Recent evolution of *Rhynchosporium secalis* populations in response to selection by triazoles

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Declaration

I confirm that this is my own work and the use of all material from
other sources has been properly and fully acknowledged.

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Abstract

Rhynchosporium secalis is a major fungal pathogen of barley. Fungicides, including triazoles and QoIs, play an important part in *R. secalis* control programmes, but can select for resistance. Reduced triazole sensitivity had been reported in *R. secalis*, but the mechanism was not known. QoI resistance had not been reported in *R. secalis* until 2008, when the G143A substitution in cytochrome *b* was reported in two *R. secalis* isolates from France.

A high-throughput fungicide sensitivity assay was developed for *R. secalis*, and isolates were screened for Quinone outside Inhibitor (QoI) and triazole sensitivity. QoI sensitivity was reduced by over 100-fold in the isolates with G143A. This was not found in any UK isolates, but smaller sensitivity shifts were detected. These sensitivity shifts were mostly reversed by alternative oxidase (AOX) inhibitors, and there is preliminary evidence of *AOX* upregulation following exposure to azoxystrobin.

Shifts in triazole sensitivity were not correlated with point mutations or constitutive over-expression of the target site encoding gene, *CYP51*, or reversed by putative efflux inhibitors. However, a second *CYP51* paralogue, *CYP51A*, was sequenced from less-sensitive isolates but absent from sensitive isolates. *CYP51A* was upregulated more than *CYP51B* following exposure to tebuconazole. Pyrosequencing analysis of the Hoosfield archive showed that levels of *CYP51A* were low until 1998, then rapidly increased. Phylogenetic analysis suggests a *CYP51* gene duplication event basal to the filamentous ascomycetes, followed by multiple losses of *CYP51A*. Therefore it appears that *CYP51A* was almost lost from the *R. secalis* population, but re-emerged due to selection by triazoles.

This project has identified the mechanism responsible for an initial shift in *R. secalis* sensitivity that compromised the effectiveness of some older triazoles, and reported further variation in sensitivity to newer triazoles that currently provide control in the field. Furthermore, the G143A cytochrome *b* substitution can confer QoI resistance in *R. secalis*. Therefore resistance management is important for sustainable *R. secalis* control.

Acknowledgements

Abbreviations

ABA	Abscisic acid
ABC	ATP-binding cassette
AOX	Alternative oxidase
CDM	Czapek Dox medium with Mycological peptone
CYP	Cytochrome P450
DMI	(Sterol) Demethylation inhibitor
EC_{50}	Fifty per cent effective concentration
MBC	Methyl Benzimidazole Carbamate
MDR	Multi-Drug Resistance
MFS	Major Facilitator Superfamily
MIC	Minimum Inhibitory Concentration
PC	Principal Component
PCA	Principal Components Analysis
PDA	Potato dextrose agar
PDR	Pleiotropic Drug Resistance
QiI	Quinone inside Inhibitor
Q_o	Quinone outside (redox site)
QoI	Quinone outside Inhibitor
RFLP	Restriction Fragment Length Polymorphism
SBI	Sterol Biosynthesis Inhibitor
SDHI	Succinate Dehydrogenase Inhibitor
SHAM	Salicylhyroxamic acid
s.l.	*sensu lato*
YPD	Yeast peptone dextrose

Contents

Chapter 1

Introduction

1.1 *Rhynchosporium secalis*

Rhynchosporium secalis (Oud.) Davis *sensu lato* (*s.l.*) is a phytopathogenic fungus, causing barley leaf blotch or scald. This foliar disease can cause serious reductions in grain yield. If untreated, yield losses within affected crops may be as high as 30% (Mayfield and Clare 1991). *Rhynchosporium secalis* infection can also reduce grain quality characters such as specific weight and protein content (Khan and Crosbie 1988).

Rhynchosporium secalis is found in barley-growing areas worldwide. When the last Commonwealth Mycological Institute Disease Distribution Map for *R. secalis* was published in 1986, it had been reported in over 50 countries, including across Europe, the Middle East and North America, Northern and Eastern coastal parts of Africa, North-western and South-eastern areas of South America, New Zealand and Southern coastal regions of Australia, the West coast of India and across South East Asia (CMI 1986).

In the U.K., *R. secalis* is especially problematic on winter barley. The Defra 2003 Cereal Disease Database Report estimated that of £4.95 million in yield losses caused by foliar pathogens, £2.57 million was due to *R. secalis*, making it the most economically-damaging foliar pathogen of barley. This represents a total yield loss of 1.39% nationally, even with £24.8 million, an average of £55/ha, spent on fungicide use, and 96.4% of crops receiving at least one spray (DEFRA 2003). Data after 2003 are more limited, but 2005 figures estimate that with increased grain prices, yield losses amounted to £4.8 million (Blake *et al.* 2011).

Rhynchosporium secalis s.l. also affects rye and various wild grass species (Caldwell 1937). While some early studies claimed isolates were able to cross-infect the full range of hosts (Bartels 1928), subsequent work has found isolates to be predominantly host-specialised. Caldwell (1937) described six different host groups, comprising barley (*Hordeum vulgare*), rye (*Secale cereale*), *Agropyron repens*, *Bromus inermis*, *Elymus canadensis* and *Hordeum jubatum*. Several later studies have found barley and rye isolates to be unable to cross-infect (Müller 1953; Owen 1958; Dodov, 1963; Kajiwara 1968; Lebedeva and Tvaruzek 2006). Müller (1953) and Owen (1958) both proposed designating the barley-infecting and rye-

infecting races as *formae specialis*, *R. secalis* f.sp. *hordeum* and *R. secalis* f.sp. *secalis*, but these names have not entered common usage. Kajiwara and Iwata (1963) also found host-specialised races infecting the grass species *Agropyron semicostatum* and *Phalaris arundinacea* (now under investigation as a potential energy crop), which they named *R. secalis* f.sp. *agropyri* and *R. secalis* f.sp. *phalaridis*. Zaffarano *et al.* (2008) found host-specialised races infecting rye, barley or *Agropyron* spp. and constructed a molecular phylogeny based on ITS sequences, which showed these three host-specialised lineages to be monophyletic, and it was concluded that they are three separate species. However, for the purpose of this thesis, *R. secalis* refers to *R. secalis s.l.*, including the barley-infecting lineage to which this study predominantly relates.

The genus *Rhynchosporium* also contains the morphologically distinct *R. orthosporum* Caldwell which infects the grass *Dactylis glomerata* (Caldwell 1937) and may cause reduced yield (Isawa 1983; Welty 1991) and some reduction in nutritional value (Isawa 1983) in this popular forage grass. A species sometimes referred to as *R. alismatis* was excluded from the genus *Rhynchosporium* by Caldwell (1937) on morphological grounds, and reclassified as *Plectosporium alismatis* (Oudem.) W. M. Pitt, W. Gams and U. Braun, by Pitt *et al.* (2004) based on molecular analysis. Molecular phylogenetic analyses have placed *R. secalis* and *R. orthosporum* close to *Oculimacula* in the Leotiomycetes (Goodwin 2002), whereas *P. alismatis* was placed close to *Verticillium* in the Sordariomycetes (Pitt *et al.* 2004). A species initially described as *R. oryzae*, causing a scald-like disease of rice, placed in *Rhynchosporium* based on a superficial similarity in the conidia which are sometimes two-celled and curved, has since been reclassified as *Gerlachia oryzae* (Hashioka & Yokogi) Gams (Gams and Muller 1980), and then *Microdochium oryzae* (Hashioka & Yokogi) Samuels & I.C. Hallet (Samuels and Hallett 1983), following more detailed morphological studies.

1.1.1 Infection process

Rhynchosporium secalis is a foliar pathogen, mainly producing symptoms on the leaf blades and sheaths. Spores landing on barley leaves germinate in response to nutrients or other substances exuded at the leaf surface (Ayres and Owen 1969). An early study by Bartels (1928) claimed that the fungus enters the leaf through stomata, but subsequent studies have repeatedly shown direct entry through the epidermis by forming appressoria (Caldwell 1937; Ayesu-Offei and Clare 1970; Jones and Ayres 1974).

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There is an initial symptomless phase in which the fungus produces subcuticular hyphae, growing through the pectic layer between the cuticle and the epidermal cell walls (Ryan and Grivell 1974). The fungus penetrates the epidermal cells, causing epidermal cell collapse. At this stage bluish-grey, 'lenticular' (lens-shaped) lesions, with a water-soaked appearance, become visible (Caldwell 1937). Fungal hyphae then grow down into the mesophyll, causing mesophyll cell collapse. The centre of the lesion then dries, leaving a light brown or grey necrotic lesion with a characteristic dark margin (Bartels 1928) (Figure 1.1). Lesions may spread outwards to form concentric rings, and in heavily-infected leaves they may coalesce to cover a large proportion of the leaf area. The two-celled conidia, with a beaked shape that gives the genus its name, form directly from hyphae, on one side of the leaf only, mostly in the centre of the lesion where hyphal growth is most dense, dislodging the cuticle so the spores are exposed (Caldwell 1937).

Subsequent investigations using electron microscopy (Jones and Ayres 1974; Hosemans and Branchard 1985), GFP-transformed strains (Newton *et al.* 2010) and qPCR detection (Fountaine *et al.* 2007) of *R. secalis* have shown the symptomless phase to be more extensive. The fungus does not produce haustoria, but during the symptomless phase it may increase the permeability of plant cell membranes, increasing the availability of sugars and amino acids from plant cells to the fungus in the subcuticular space (Jones and Ayres 1972). The symptomless phase can persist for months (Fountaine *et al.* 2010), and the fungus can produce conidia through the cuticle (Howlett and Cooke 1987).

The formation of necrotic lesions causes a reduction in photosynthetic area, reducing photosynthetic productivity and ultimately grain yield. James *et al.* (1968) found a clear correlation between the percentage of the flag leaf and leaf two visibly infected after ear emergence and the percentage yield lost, suggesting that loss of photosynthetic area during grain filling is especially damaging to yields. However, such studies have correlated yield loss with visibly-infected lead area, and so do not take account of asymptomatic infection. While there is experimental evidence that photosynthetic rate, measured by carbon dioxide uptake, only decreases from mesophyll cell collapse, the fungus is already taking up photosynthetic products from the plant during subcuticular growth (Jones and Ayres 1972). Mayfield and Clare (1991) studied plants grown in controlled environment, artificially inoculated with *R. secalis* at different growth stage. They found that reductions in yield were mainly due to fewer ears being produced per plant, and fewer grains per head, but still found

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that infection later in the growing season was most damaging to grain yields. However, the plants were grown in controlled environment chambers, with water provided directly into the pots. Therefore in the absence of rainfall to cause splash-dispersal, this experiment did not account for the importance of earlier infection for the build-up of secondary inoculum that can cause later-season infection in the field. Taggart *et al.* (1998) found that control failure of *R. secalis* resulted in reduced grain weight.
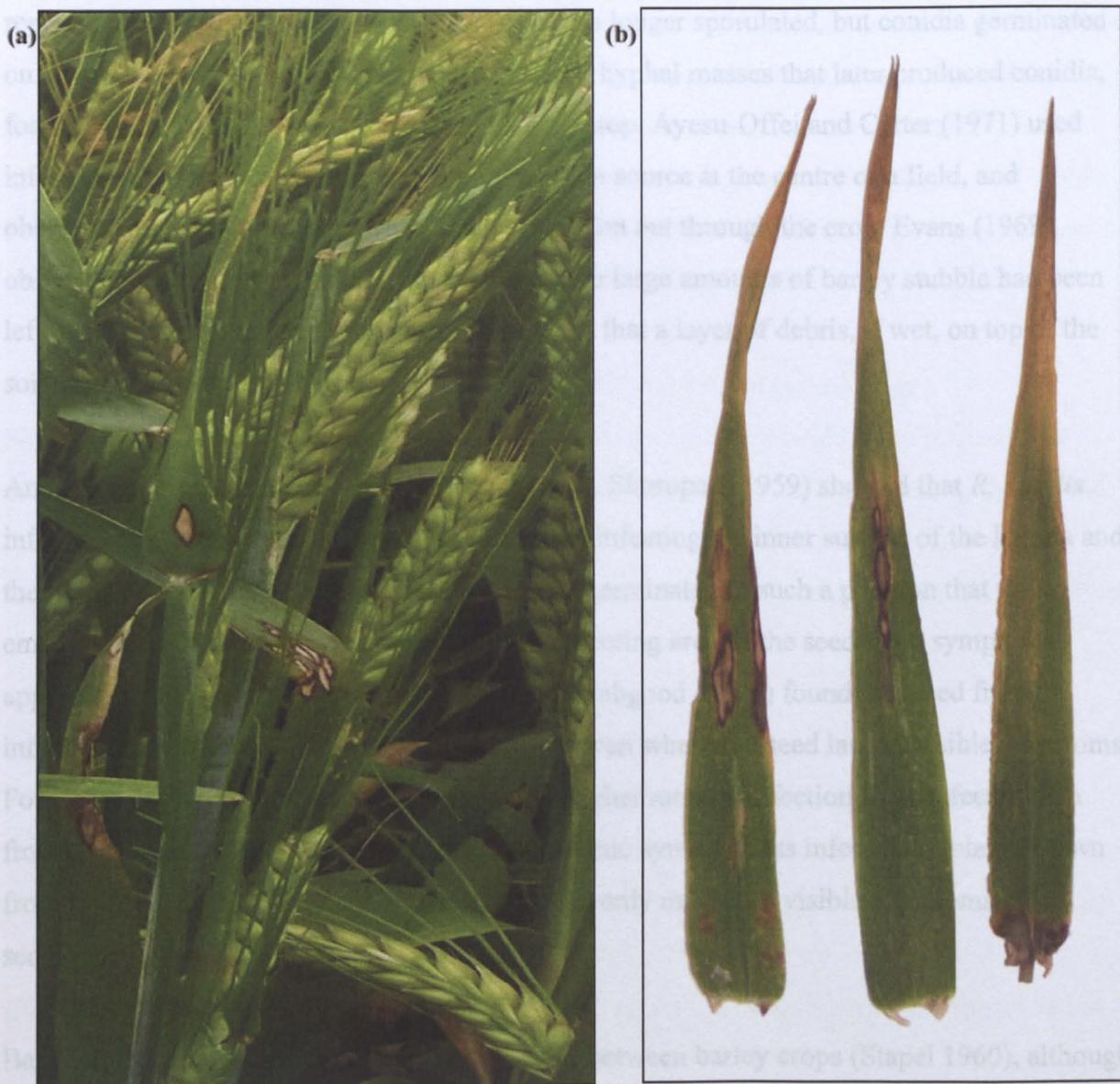
Figure 1.1. *Rhynchosporium secalis* **lesions on barley (a) in the field, (b) on leaf blades and leaf sheath.**

1.1.2 Epidemiology

Leaf scald is a polycyclic disease. Within a growing season, the primary inoculum source is infected seed or plant debris, followed by secondary spread by splash-dispersal of conidia (Habgood 1971; Shipton *et al.* 1974).

Bartels (1928) describes *R. secalis* as overwintering on crop debris and in the soil as hyphae and conidia, sometimes forming sclerotia-like bodies of tightly-packed hyphae. Polley (1971) reports similar findings: lesions on dead leaves no longer sporulated, but conidia germinated on crop debris and grew saprophytically as dense hyphal masses that later produced conidia, forming the primary inoculum to infect the next crop. Ayesu-Offei and Carter (1971) used infected barley straw as an experimental inoculum source at the centre of a field, and observed the initial spread of symptomatic infection out through the crop. Evans (1969) observed higher disease incidence on farms where large amounts of barley stubble had been left in fields, and confirmed with glasshouse tests that a layer of debris, if wet, on top of the soil can infect growing plants.

Another major primary inoculum is infected seed. Skoropad (1959) showed that *R. secalis* infection could spread from the leaves to the ear, infecting the inner surface of the lemma and then the pericarp of the grain itself. When seeds germinated in such a position that the emerging seedling came into contact with the infecting area of the seed-coat, symptoms appeared on the emerging shoot within a week. Habgood (1971) found that seed from an infected crop could produce infected seedlings, even where the seed lacked visible symptoms. Fountaine *et al.* (2010) measured significantly higher rates of infection from infected than from clean seed in field trials, and detected systemic symptomless infection in plants grown from infected seed, whereas previous studies had only measured visible symptoms in seedlings.

Barley volunteers may act as a disease reservoir between barley crops (Stapel 1960), although this is less important than for obligate biotrophic pathogens (Yarham and Gladders 1993), as *R. secalis* can also survive saprophytically, and removal of volunteer plants may only reduce disease levels if crop debris is also removed (Evans 1969). It has also been suggested that wild grasses at field margins may act as reservoirs for the disease, but current knowledge of host-specialisation in *R. secalis* suggests this only applies to a limited range of grass species, such as wall barley, *Hordeum murinum* (Zaffarano *et al.* 2008).

Secondary dispersal by rain-splashed conidia allows the fungus to infect higher leaves, reaching epidemic levels in suitable conditions. In glasshouse tests, Polley (1971) found that epidemic levels of *R. secalis* infection occurred after periods of at least twelve hours with relative humidity over 90% and rainfall at least nine hours before the end. In the field, Ayesu-Offei and Carter (1971) detected conidia in spore-traps only after rainfall or spray-irrigation. Fitt *et al.* (1986) used simulated rainfall to show that more spores were collected in spore traps following heavier rain, with a minimum rainfall rate of 0.2mm/hour required for conidia to be detected. Occasionally spores were detected in windy conditions immediately following rainfall, but none were detected from dry leaves in wind-tunnel experiments. This corresponds with historical (Priestley and Bayles 1979) and geographical (Khan *et al.* 1968; Priestley and Bayles 1979) studies, which show that *R. secalis* epidemics are more frequent in seasons with high rainfall in May to July, and in regions with higher annual rainfall.

Rhynchosporium secalis is an imperfect fungus, with no known sexual stage. Phylogenetically, however, it is a member of the Ascomycota, thought to be related to discomycete fungi in the Leotiales (Goodwin 2002; Crous *et al.* 2003), including the related species *Oculimacula yallundae* and *Pyrenopeziza brassicae*, which produce airborne ascospores from apothecia on crop debris (Wallwork 1987; McCartney and Lacey 1989; Dyer *et al.* 2001).

There is some evidence of genetic recombination in *R. secalis* populations (Burdon *et al.* 1994), but it is not yet clear whether this is due to an as yet undiscovered sexual stage, perhaps occurring on different host species (Zaffarano *et al.* 2006), or somatic recombination (Bo *et al.* 1994; Salamati *et al.* 2000). Spore trapping has repeatedly found only conidia of *R. secalis* (e.g. Skoropad 1959; Ayesu-Offei and Carter 1971), and very low levels of DNA (Fountaine *et al.* 2010), consistent with occasional capture of splash-dispersed spores rather than common wind-dispersed ascospores.

Bartels (1928) described the formation in culture of gemmae, vegetative fragments that subsequently germinate, but there have been no reports of such propagules being produced *in planta* or dispersed in the field. Jones and Ayres (1974) found that when plants were inoculated with a mixture of hyphal fragments and spores, some hyphal fragments grew over the leaf surface, but were not able to penetrate the cuticle and infect the leaf. Microconidia

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have also been reported in culture (Skoropad and Grinchenko 1957), but they did not germinate and have not been found in the field.

1.1.3 Control

Effective control relies on the combined use of more resistant barley varieties and fungicide applications (Zhan *et al.* 2008; Blake *et al.* 2011). Crop rotation, tillage to reduce surface crop debris and removal of volunteer plants have a limited effect in reducing those inoculum sources (Oxley 2010), but no effect on seed-borne infection. Work is underway to evaluate seed treatments for *R. secalis* control, but so far, effective control has not been achieved (Oxley and Burnett 2010). Later sowing date reduces infection levels, but also causes direct yield reduction (Cooke and Oxley 2000; Kavak 2004).

Cultivar resistance includes major-gene-mediated and partial (or polygenic) resistance. In major-gene-mediated resistance, a single host gene enables recognition of pathogen strains with the corresponding avirulence gene, eliciting a defence response that prevents lesion-forming infection (Zhan *et al.* 2008). For example, the *Rrs1* resistance allele in barley gives resistance to *R. secalis* isolates with the corresponding avirulence allele, *AvrRrs1*. The resistance elicitor for *AvrRrs1* has now been identified as the protein NIP1, a necrosis-inducing protein produced by *R. secalis* at the mesophyll-collapse, lesion-forming stage of infection (Rohe *et al.* 1995). So far, 17 resistance genes have been described (Wagner *et al.* 2008), although some of these map to the same chromosome location and may represent multiple alleles rather than separate loci (Genger *et al.* 2005).

The effect of major-gene-mediated resistance on asymptomatic infection has proven less tractable. Jones and Ayres (1972) found some subcuticular growth in a susceptible and a resistant variety, but in the resistant variety the levels of nutrients from the plants cells available to the fungus in the intercellular space were lower, and mycelial growth was less than in the susceptible variety. Lehnackers and Knogge (1990) observed some differences in spore germination rates and subcuticular hyphal growth levels, and in some cases host cell wall alterations blocking epidermal penetration, but these were not correlated with the presence of *Rrs1/NIP1*. Subcuticular hyphal growth was less dense in the resistant interaction, but some sporulation still took place. Steiner-Lange *et al.* (2003) found that defence responses in resistant plants were triggered within 24 hours post inoculation, just after the fungus penetrated the cuticle. One of the *Rrs1-AvrRrs1*-specific pathogenesis-related

proteins was expressed specifically in the epidermis, but three were expressed specifically or most highly in the mesophyll. Microscopy studies with a GFP-labelled *R. secalis* transformant on one susceptible barley cultivar and a near-isogenic resistant cultivar found that spore germination and mycelial growth rate were reduced on the resistant cultivar, with the hyphal network mostly limited to subcuticular growth parallel to the leaf surface rather than growing deeper into the leaf tissue. However, some sporulation did take place (Thirugnanasambandam *et al.* 2010).

Major-gene-mediated resistance is vulnerable to resistance-breaking pathogen strains. Schurch *et al.* (2004) tested *R. secalis* isolates from a range of locations and found the *NIP1* gene had been deleted in 45% of isolates, resulting in virulence on *Rrs1* barley varieties. Of the remaining isolates, 10% of those sequenced had mutations in *NIP1* associated with virulence on *Rrs1* barley varieties. Lehnackers and Knogge (1990) describe several *R. secalis* field isolates virulent on *Rrs2* cultivars. Such gene-for-gene interactions provide the basis for the 'physiologic races' of *R. secalis* on barley described in earlier literature (Shipton *et al.* 1974). *Rhynchosporium secalis* populations contain a range of pathogenicity types (Burdon *et al.* 1994; Newton *et al.* 2001), and are able to evolve quickly to different barley cultivars as virulent strains are selected (Xi *et al.* 2003).

Polygenic resistance, whereby multiple loci contribute to quantitative resistance, is thought to be more durable, but only provides partial resistance. In a cultivar with partial resistance, visible lesions will appear, but they may be smaller, fewer, develop later or have lower spore production (Habgood 1977; Kari and Griffiths 1993). Partial resistance is sometimes referred to as adult resistance, and major-gene-resistance as seedling resistance. Studies in *R. secalis* have shown that some, but not the majority, of partial resistance to *R. secalis* in barley is effective only in adult plants (Xue and Hall 1995). Recently, some components of polygenic resistance have been genetically analysed as QTLs and mapped to chromosomes (Wagner *et al.* 2008), but comparison of analyses is hampered by strong genotype x environment interactions (Kari and Griffiths 1993).

Therefore a more durable resistance strategy would involve a combination of partial resistance, and multiple major resistance genes, combined in one cultivar by 'pyramiding' (Jefferies *et al.* 2000), or in a cultivar mixture (Oxley and Burnett 2010). There has also been some interest in disease escape, whereby aspects of crop growth, such as earlier stem

elongation, lower canopy density, or more horizontal leaf surfaces, reduce secondary spread (Zhan *et al.* 2008). However, there may be associated yield penalties, such as increased shading of lower leaves. It has also been suggested that disease tolerance could be improved, so yield loss is lower for a given disease level, although it is not yet clear whether this would be possible without reducing the disease-free yield (Bingham *et al.* 2009).

Blake *et al.* (2011) suggest that net blotch, *Pyrenophora teres*, overtook *R. secalis* as the most damaging foliar pathogen of winter barley in the UK in 2005 due to the introduction of barley varieties with higher resistance to *R. secalis*, although Oxley and Burnett (2010) list *R. secalis* as the most damaging foliar disease of barley in the UK. However, barley varieties still only provide moderate levels of resistance to *R. secalis* (Wagner *et al.* 2008) and fungicides remain a key part of disease control. HGCA Recommended List Varieties in 2010 trials gave yields 8%-23% lower without fungicides than with standard treatment programmes, with a median yield difference of 17% (HGCA 2010).

1.1.4 Chemical control

Current UK recommendations for *R. secalis* control in winter barley include a T1 spray, at growth stage 31-32 for winter barley or growth stage 25-30 for spring barley, containing a triazole with a QoI (Quinone outside inhibitor) or SDHI (Succinate dehydrogenase inhibitor), or a morpholine if eradicant activity is needed; followed in wet summers by a T2 spray, at growth stage 39-49, containing a triazole with a different mixing partner such as cyprodinil or an SDHI if not used at T1. An earlier spray, before GS30, containing morpholines and cyprodinil, may be applied on winter barley if early disease levels are high (Blake *et al.* 2011). The UK Pesticide Usage Survey Report in 2008 showed that these recommendations were widely followed, with most fungicides applied in a two-way mixture and the most common mixtures containing a triazole with a QoI or morpholine (Figure 1.2) (Garthwaite *et al.* 2008). The modes of action and resistance mechanisms for these fungicide groups are discussed in Secion 1.2.

Figure 1.2. Pie chart showing fungicide use on barley in the UK in 2008 by proportion of active substance treated area. Series 1 (inner circle): proportion of total fungicides used. Series 2: mixing partners used with the fungicide groups shown in series 1. Series 3: additional mixing partners used with the fungicide combinations shown in series 2. Red: anilinopyrimidines; orange: imidazoles; yellow: benzimidazoles; light green: mildewicides; dark green: morpholines; light blue: multi-site inhibitors; dark blue: QoIs; light purple: SDHIs; dark purple: triazoles. Grey: no additional mixing partner.

1.2 Fungicides and resistance

Fungicides are a key part of control programmes for *R. secalis*. The Defra 2003 Cereal Disease Database Report estimated that £24.8 million, an average of £55/ha, was spent on fungicide use on winter barley in the UK, with 96.4% of crops receiving at least one spray (DEFRA 2003).

There are currently 408 fungicide products approved for use on barley in the U.K., comprising 379 field fungicides and 29 seed treatments (Chemicals Regulation Directorate 2010). However, the 379 field fungicides contain only 35 different active ingredients, with just 11 different modes of action (FRAC 2010a), three of which are mildew-specific. Bringing a new compound to market takes over ten years (Morton and Staub 2008) and costs up to £200 million (Case 2010). Therefore the monitoring and management of fungicide resistance is essential, to prolong the effective life of the available products.

1.2.1 Fungicide resistance

Fungicide resistance has been defined as "stable, inheritable adjustment by a fungus to a fungicide, resulting in less than normal sensitivity to that fungicide… for strains of a sensitive species that have changed, usually by mutation, to be significantly less sensitive" (European and Mediterranean Plant Protection Organization 1988). This definition specifies acquired resistance, as opposed to intrinsic differences between species. In some cases, resistance has been defined as having occurred only when the frequency of such strains in field populations results in the failure of disease control (HGCA 2000), a situation which may be referred to as "practical resistance" (European and Mediterranean Plant Protection Organization 1988).

Fungicide resistance develops when naturally-occurring genetic variation in a pathogen population includes mutations conferring reduced fungicide sensitivity, and fungicide use exerts a selective pressure that favours such mutations. Both of these stages, emergence and selection, have implications for resistance risk and management (Georgopoulos and Skylakakis 1986). Therefore detection of less-sensitive fungal isolates, before they reach sufficient frequency in the population to result in loss of control in the field, is vital in order to assess the risk of field resistance occurring and give appropriate management guidelines.

The continued efficacy of most broad-spectrum fungicides relates to their multi-site mode of action, since mutations affecting a single target site will not confer resistance. Hence only

isolated cases of resistance have been reported, which are non-target-site based (e.g. Barak and Edgington 1984). In contrast, for many fungicides with a site-specific mode of action, a single mutation can confer high levels of resistance. Consequently in many cases resistance has developed rapidly, with resistance to benzimidazoles and strobilurins reported in some pathogens within two years of their introduction (Brent and Hollomon 2007b).

When mutations conferring reduced sensitivity do arise, their persistence and spread will depend upon population genetic factors such as ploidy level and recombination frequency (Milgroom *et al.* 1989); biological factors relating to the level of selective advantage given by the mutation, a product of the resistance factor balanced against any fitness penalties resulting from reduced target-site efficiency or energy expenditure on over-expression or efflux; and agronomic factors relating to the level of selective pressure exerted by the fungicide class in question.

Therefore fungicide risk can be broken down into fungicide-related factors, pathogen-related factors and agronomic factors (Brent and Hollomon 2007b). Where fungicide and pathogen risk are moderate to high, agronomic risk must be reduced through resistance management practices. These involve limiting the selective advantage conferred by resistance to a fungicide class by reducing exposure of pathogen populations to selection by those fungicides. This can be achieved by reducing the pathogen population under selection by the fungicide by utilising other control methods including resistant varieties and cultural control (HGCA 2000). Initially, cases where resistance emerged first in high disease pressure areas were given as anecdotal evidence that reducing pathogen populations by other means could prove useful in resistance management (Staub 1991). More recently field experiments have shown a lower shift in *R. secalis* sensitivity to epoxiconazole over a growing season on less sensitive barley cultivars (Oxley *et al.* 2003). Conversely, the use of fungicides alongside resistant cultivars can reduce the pathogen population under selection for virulence, prolonging the useful life of resistance genes (Staub 1991).

Reducing the exposure of the pathogen population to a fungicide may be achieved by limiting the number of applications per season (Brent and Hollomon 2007a). This means using fungicides only when warranted by the presence or risk of disease (HGCA 2000). However, waiting for an epidemic to develop and then attempting eradicant use of fungicides results in a large pathogen population under selection by the fungicide, increasing resistance risk

(Staub 1991). Therefore disease forecasting and early detection are key. The number of applications of a single group of fungicides to a single crop during a growing season should also be limited, but here some compromise may be necessary between resistance management and disease control. The selection imposed by any one fungicide can be reduced by mixing or alternating fungicides from different cross-resistance groups (HGCA 2000), although experimental data regarding the effectiveness of using fungicide mixtures are limited and conflicting. Mixing or alternating with different fungicide groups delayed the selection of QoI and triazole resistance in *Sphaerotheca fuligenea* (Wyenandt *et al.* 2008) and QoI resistance in *Blumeria graminis* f.sp. *hordei* (Fraaije *et al.* 2006), and reduced sensitivity shifts of *R. secalis* against epoxiconazole and prothioconazole within individual sites over a single season (Oxley *et al.* 2008). However, QoI resistance in *M. graminicola* was rapidly selected even when QoI fungicides were only used once, in mixtures and alternations (Fraaije *et al.* 2006). In *Plasmopara viticola*, the benefit of a mixing partner in delaying the selection of QoI resistance depended on the mixing partner used and the dose rate of QoI and mixing partner (Genet *et al.* 2006). In *R. secalis*, the frequency of MBC resistance in populations proved too erratic between years and sites to determine the effect of fungicide mixtures on the frequency of resistant isolates (Holloman 1997).

Fungicides used in mixtures or alternations should belong to different groups which are not cross-resistant. Positive cross-resistance occurs when different fungicides share a mode of action, and a single resistance mechanism confers resistance to both, whereas multiple resistance is the occurrence of separate mechanisms conferring resistance to different fungicides in the same pathogen. Fungicides have been classified into cross-resistance groups, each sharing a mode of action and affected (or potentially affected) by a common resistance mechanism (FRAC 2010a). Groups currently authorised for use on barley in the UK are the amines (morpholines), carboxamides, anilinopyrimidines, MBCs, QoIs, DMIs (demethylation inhibitors) and multi-site inhibitors, along with some specific mildewicides (Chemicals Regulation Directorate 2010).

1.2.2 Fungicide classes used against *R. secalis*

The amines, sometimes referred to as morpholines but also including piperadines and spiroxamine (FRAC 2010a), are a class of sterol biosynthesis inhibitors (SBIs) (Leroux and Gredt 1978), inhibiting sterol Δ^8-Δ^7 isomerase (Kato *et al.* 1980) and Δ^{14}-reductase (Kerkenaar *et al.* 1981). They are in a separate cross-resistance group from the DMIs

(Hollomon 1994; Godet and Limpert 1998), which inhibit a different step in sterol biosynthesis. Their inhibition of two different enzymes is thought to make target-site resistance unlikely to evolve, since appropriate mutations in both target site encoding genes would be required (Hollomon 1994). The only reports of resistance so far are of a quantitative shift in *B. graminis* (f.sp. *tritici* and *hordei*) which was significant but smaller than that for DMIs (Godet and Limpert 1998; Napier *et al.* 2000), and laboratory mutants of *Ustilago maydis* (Markoglou and Ziogas 2001) and *Nectria haematococca*, the latter due to altered sterol metabolism, and possibly mutation or over-expression of one of the two target sites, sterol Δ^{14}-reductase (Lasseron-De Falandre *et al.* 1999). Whilst the amines are not the most effective fungicides against *R. secalis* when used alone, their use in mixtures can improve results relative to use of another fungicide alone (Phillips and Frost 1975), including use as a mixing partner for triazoles (Taggart *et al.* 1998). The eradicant activity of amine fungicides means they can be used against established *R. secalis* infections, although their use is not recommended after canopy expansion as they can cause leaf die-back (Oxley and Burnett 2010). In the UK in 2008, amines accounted for 17% of fungicide applications by active substance treatment area on barley (Garthwaite *et al.* 2008) (Figure 1.2) and nearly 10% of fungicide applications by treatment area on all cereals (FERA 2010) (Figure 1.3).

The carboxamides, or SDHIs, inhibit succinate dehydrogenase of respiratory complex II (White 1971). Complex II comprises sdhA, or sdhFp, the flavoprotein subunit; sdhB, or sdhIp, the iron-sulphur protein subunit; and the membrane anchor, in fungi consisting of two subunits, sdhC and sdhD (Ackrell 2000). The first SDHI fungicides, such as carboxin, were introduced in the late 1960s for use against rusts and other basidiomycetes (Avenot and Michailides 2010). Field resistance to carboxin and fenfuram was detected in *Ustilago nuda* in 1986 (Leroux and Berthier 1988). Studies of carboxin-resistant laboratory mutants of *U. maydis* (Broomfield and Hargreaves 1992) and *M. graminicola* (Skinner *et al.* 1998) found single-site substitutions substitutions L257K and H267 Y/L, respectively, in the third cysteine-rich cluster of the iron-sulphur redox centre in sdhB. However, studies of resistant laboratory mutants of *Coprinopsis cinerea* found a single-site substitution (N80K) in sdhC (Ito *et al.* 2004). Mutations in sdhB and sdhD have been found in phytopathogenic bacteria (Matsson *et al.* 1998; Li *et al.* 2006), and mutations in sdhB, sdhC and sdhD have been found in carboxin-resistant laboratory mutants of *Aspergillus oryzae*, with each mutation independently conferring carboxin resistance (Shima *et al.* 2009).

Figure 1.3. : Fungicide use on cereals in the UK by percentage of active substance treated area, excluding seed treatments.

Green: triazoles; Light green: imidazoles; Dark green: heterocyclic DMIs; Yellow: Morpholines; Red: Benzimidazoles; Blue: QoIs;

Dark blue: SDHIs; Orange: cyprodinil; Purple: chlorothalonil; Grey: Dithiocarbamates. (Data: FERA 2010)

Since 2002, a new generation of SDHI fungicides, effective against ascomycete pathogens, has been introduced (Ammermann *et al.* 2002). The first such compound was boscalid, released for use on cereals in 2005, followed by isopyrazam and bixafen in 2010 and 2011, with further compounds including penthiopyrad yet to be released (Avenot and Michailides 2010; Chemicals Regulation Directorate 2010; FRAC 2010a; Oxley and Burnett 2010). By 2008, boscalid accounted for 3.6% of fungicide applications on cereals in the UK by active substance treated area (FERA 2010), and whilst SDHIs accounted for only 0.28% of fungicide applications on barley in 2008 (Garthwaite *et al.* 2008), this is likely to increase as more products are introduced. In 2006, boscalid-resistant field isolates of *Botrytis cinerea* were found, with substitutions in sdhB (Stammler *et al.* 2007). Resistance to boscalid has also recently been reported in field isolates of *Alternaria alternata* carrying mutations in sdhB (Avenot *et al.* 2008), sdhC or sdhD (Avenot *et al.* 2009), with any one mutation conferring a resistance factor of over 900 against boscalid but lower resistance factors against carboxin. Resistance has also been reported in *B. cinerea* (Stammler *et al.* 2007), *Sclerotinia sclerotiorum* and some diseases of horticultural crops (Avenot and Michailides 2010), but so far there have been no reported cases of field resistance to the newer SDHIs in cereal pathogens (FRAC 2010b). Products currently registered for use on barley in the UK contain either boscalid in mixture with epoxiconazole, bixafen with prothioconazole or isopyrazam with cyprodinil (Chemicals Regulation Directorate 2010).

The anilinopyrimidines were introduced in the 1990s. The mode of action has not yet been fully elucidated, but sensitivity is reduced *in vitro* in rich media and so the mode of action is believed to be linked to extracellular secretion of hydrolytic enzymes (Miura *et al.* 1994), or to methionine biosynthesis (Masner *et al.* 1994), although cystathionine β-lyase appears not to be the target site (Fritz *et al.* 2003). Field resistance was detected in *B. cinerea* in 1993 (Leroux and Gredt 1978), and subsequently in *Venturia inaequalis* (Kung *et al.* 1999) and the post-harvest pathogen *Penicillium expansum* (Li and Xiao 2008), and shifts were seen in *Oculimacula* spp. in field experiments (Babij *et al.* 2000). The anilinopyrimidine cyprodinil is effective against *R. secalis* (Heye *et al.* 1994), including as a mixing partner for triazoles (Cooke *et al.* 2004) or isopyrazam (Blake *et al.* 2011), but it accounts for under 5% of UK fungicide treatments on barley (Figure 1.2) and less than 1% of UK fungicide treatments on cereals (Figure 1.3), and some formulations cannot be used at T2 where certain plant growth regulators are used (Oxley and Burnett 2010).

The main multi-site inhibitor currently in use against *R. secalis* is chlorothalonil, which reacts with thiols such as glutathione so various thiol-dependent enzymes cannot function (Tillman *et al.* 1973). Generally multi-site inhibitors have very low risk of resistance, although *B. cinerea* isolates with cross-resistance to chlorothalonil and five other multi-site inhibitors were reported, suggesting that a non-target-site mechanism is involved, such as thiol over-production (Barak and Edgington 1984). Chlorothalonil shows good protectant activity against *R. secalis*, but lacks systemicity and curative action (Oxley and Burnett 2010). Therefore, it is often used in mixtures with systemic but higher resistance risk fungicides, and accounted for 16% of fungicide applications on cereals in the UK by active substance treated area in 2008 (FERA 2010) (Figure 1.3).

1.2.3 Benzimidazoles

Benzimidazoles, introduced in the late 1960s, were among the first selective, site-specific fungicides (Morton and Staub 2008). Benzimidazoles bind to β-tubulin, preventing microtubule assembly (Davidse and Flach 1977), causing mitotic failure as pairs of chromatids cannot be separated (Hammerschlag and Sisler 1973). The thiophanate fungicides are converted by fungi to the benzimidazole carbendazim (Uesugi 1998), so the thiophanates and benzimidazoles are grouped together as methyl benzimidazole carbamates (MBCs) (FRAC 2010a).

Field resistance was first reported in *S. fuliginea* in 1971, followed rapidly by many other pathogens of horticultural crops, and by *B. graminis* on the grass *Poa pratensis* in 1973 (FRAC 2010b). MBCs were used on cereals from the mid-1970s. Field resistance was reported in *Oculimacula* spp. in 1982, *Microdochium nivale* in 1983 (FRAC 2010b), and *M. graminicola* in 1985 (Fisher and Griffin 1984). The Fungicide Resistance Action Committee now lists 120 plant pathogens that have developed benzimidazole resistance (FRAC 2010b). In 1990, benzimidazoles accounted for 16% of fungicide treatments on cereals in the UK by active substance treated area. By 2008, this had fallen to 0.15% (FERA 2010) (Figure 1.2).

The β-tubulin of MBC-resistant fungal strains has a greatly reduced binding affinity to MBCs (Davidse and Flach 1977), resulting from single-site mutations, predominantly at codons 198 or 200 (Koenraadt *et al.* 1992). Substitutions include E198G, E198K, E198A and F200Y in *V. inaequalis*, with the same mutations in *Monilinia fructicola*, *Sclerotinia homoeocarpa* and various *Penicllium* species, as well as E198V in *P. expansum* (Koenraadt *et al.* 1992); the

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same mutations have been found in other species in subsequent studies (e.g. Wong *et al.* 2005; Ziogas *et al.* 2009), along with E198Q and L240F in *O. yallundae* (Albertini *et al.* 1999) and F167Y in *Fusarium graminearum* (Chen *et al.* 2009). Y200 corresponds to the wild-type sequence in some intrinsically insensitive species, including humans (Davidse and Ishii 1995). Further substitutions have been found in laboratory mutants but have not in field isolates, possibly due to fitness costs resulting from impaired tubulin-tubulin binding in microtubule formation making microtubule structure less stable and more temperature-sensitive (Davidse and Ishii 1995). Alternatively, some laboratory mutants of *Oculimacula* spp. had changes at multiple residues, or amino acid substitutions resulting from a two-base-pair mutation, which may not have been found in the field because these multiple changes have not occurred at natural mutation rates (Albertini *et al.* 1999). Benzimidazoles are also used as nematicides, and the substitutions E198A, F200Y and F167Y have been found in benzimidazole-resistant nematodes (Skuce *et al.* 2010). Furthermore, some mutations only reported in resistant laboratory mutants in fungi correspond to wild-type sequences found in intrinsically insensitive organisms (Davidse and Ishii 1995). This suggests that any fitness penalty depends on the genetic background.

Substitutions E198A and E198G conferred negative cross-resistance to N-phenylcarbamate fungicides, such as diethofencarb: wild-type isolates are insensitive to these fungicides, but isolates with these substitutions are sensitive. Therefore fungicide mixtures of carbendazim and diethofencarb were introduced to control mixed populations of carbendazim sensitive and resistant isolates of *Cercospora beticola* (Kato *et al.* 1984). However, the F200Y and L240F substitutions confer resistance to the MBCs while retaining insensitivity to the N-phenylcarbamates, and E198K, E198Q and E198V confer resistance to MBCs, with negative cross-resistance to methyl N-(3, 5-dichlorophenyl)-carbamate (MDPC) but retaining insensitivity to diethofencarb (Koenraadt *et al.* 1992). Therefore attempts to exploit the negative cross-resistance for resistance management by combining carbendazim and diethofencarb resulted in the selection of genotypes resistant to both compounds (Faretra *et al.* 1989; Katan *et al.* 1989; Ziogas *et al.* 2009).

MBC fungicides were introduced for use on barley in the UK in 1975, but resistance in *R. secalis* was not reported until 1989 in Northern Ireland (Hollomon 1992) and 1990 on the UK mainland (Wheeler *et al.* 1995b). The β-tubulin of resistant isolates bound radio-labelled carbendazim less strongly than that the β-tubulin of sensitive isolates (Kendall *et al.* 1994).

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All resistant field isolates of *R. secalis* sequenced have the β-tubulin substitutions E198G (Wheeler *et al.* 1995a) or F200Y (Butters and Hollomon 1999). Substitution E198K has been found in a benzimidazole-resistant laboratory mutant of *R. secalis* (Wheeler *et al.* 1995a). This laboratory mutants had lost pathogenicity, whereas field isolates with E198G or F200Y had not (Wheeler *et al.* 1995b). However, it is not clear whether E198K itself has fitness costs *in planta*, or whether the loss of pathogenicity resulted from co-incidental mutations or growth in culture. The E198G substitution, but not the F200Y substitution, confers negative cross-resistance to the N-phenylcarbamate fungicide, diethofencarb (Wheeler *et al.* 1995a).

The spread of MBC-resistant genotypes in *R. secalis* populations has been described as "erratic". Ten years after the first report of resistance, the occurrence was still sporadic in the UK mainland, with farmers advised that carbendazim-containing mixtures still "may be useful" in some cases. In contrast, in Northern Ireland, resistance became "common and widespread" within three years (HGCA 2000). A survey in 1993 found 39% of isolates from Northern Ireland were carbendazim-resistant (Taggart *et al.* 1994), compared to 16.6% of isolates, with no resistant isolates obtained from over half of crops surveyed, in England and Wales in the same year (Locke and Phillips 1995). However, the apparent division between Northern Ireland and mainland Britain may be misleading, since resistant isolates were also found at higher frequencies in wetter regions of England and Wales, where higher rainfall results in higher disease pressure. For example, 12 out of 35 Welsh isolates were resistant to carbendazim, compared to none of 54 isolates from Suffolk and Essex (Locke and Phillips 1995). Differences between Northern Ireland and Wales were not significant (Taggart *et al.* 1999), and there was considerable variation between sites in Northern Ireland, with the proportion of resistant isolates ranging from 7.6% to 85.7%, and carbendazim still giving disease control at sites with the lowest frequency of resistance (Taggart *et al.* 1994). From 1993 to 1995, overall resistance frequency in Northern Ireland stabilised at around 40%, but remained highly variable between sites (Taggart *et al.* 1999). Differences in resistance frequency between sites were not correlated with carbendazim use within each growing season, as large differences in the initial frequency of resistant isolates prior to fungicide treatment had a greater impact on the final frequency of resistant isolates (Taggart *et al.* 1999). However, within sites the frequency of resistant isolates was higher after carbendazim treatment (Taggart *et al.* 1998). However, frequency of resistance was negatively correlated with disease control (Taggart *et al.* 1999), and by 2002, resistance was sufficiently widespread that MBCs were no longer considered useful for *R. secalis* control in the UK.

Morpholines and the newer anilinopyrimidine and QoI fungicides were recommended instead alongside DMIs (Cooke and Locke 2002).

1.3 QoI fungicides

The Quinone-outside inhibitors (QoIs) (Figure 1.4) effect reversible competitive inhibition of the quinone outside redox site (Q_o) of cytochrome b of the cytochrome bc_1 complex in respiration (Becker *et al.* 1981; Brandt *et al.* 1988) (Figure 1.5), a mitochondrially-encoded target site. This group includes the strobilurins, based on natural products of various basidiomycete fungi. The antifungal activity of strobilurins A and B, from *Strobilurus tenacellus*, was first reported in 1977, but extensive chemical modification was necessary to develop fungicides with sufficient stability and photo-stability to be useful in the field (Sauter *et al.* 1995). The first commercial strobilurin fungicides were released in 1996 and 1997 (Joshi and Sternberg 1996; Bartlett *et al.* 2002). They now account for 21.4% of fungicide applications by active substance treated area on cereals in the UK (Chemicals Regulation Directorate 2010) (Figure 1.3), and 19.5% of applications on barley (Garthwaite *et al.* 2008) (Figure 1.2).

(a)   **(b)**
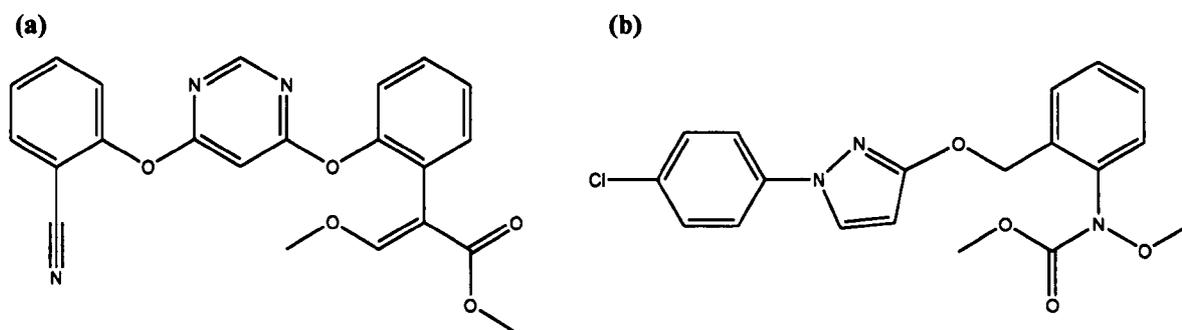
Figure 1.4. Structures of two QoI fungicides: (a) Azoxystrobin, (b) Pyraclostrobin.

Resistance was first found in *P. viticola* in a field trial in 1997 (Heaney *et al.* 2000). These isolates were cross-resistant to strobilurins (azoxystrobin, kresoxim-methyl and trifloxystrobin were tested), fenamidone and famoxadone. These fungicides, therefore, were placed in the QoI-STAR (Strobilurin Type Action and Resistance) cross-resistance group (Heaney *et al.* 2000), which now includes a further eight strobilurins and the benzylcarbamate pyribencarb (FRAC 2010a). Kataoka *et al.* (2010) suggested that pyribencarb may interact with different Qo site domains from other QoIs due to its higher activity against *B. cinerea* strains with target-site resistance to other QoIs, but the resistant

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isolates still had a resistance factor of 65, and higher EC_{50} values than most non-target organisms. The quinone inside inhibitors (QiIs), such as cyazofamid and amisulbrom (FRAC 2010a), inhibit a different region of cytochrome *b*, and are in a different cross-resistance group from the QoIs (di Rago *et al.* 1989). The QiIs are used for the control of oomycetes, and at present in the UK, they are only registered for use on potatoes (Chemicals Regulation Directorate 2010).
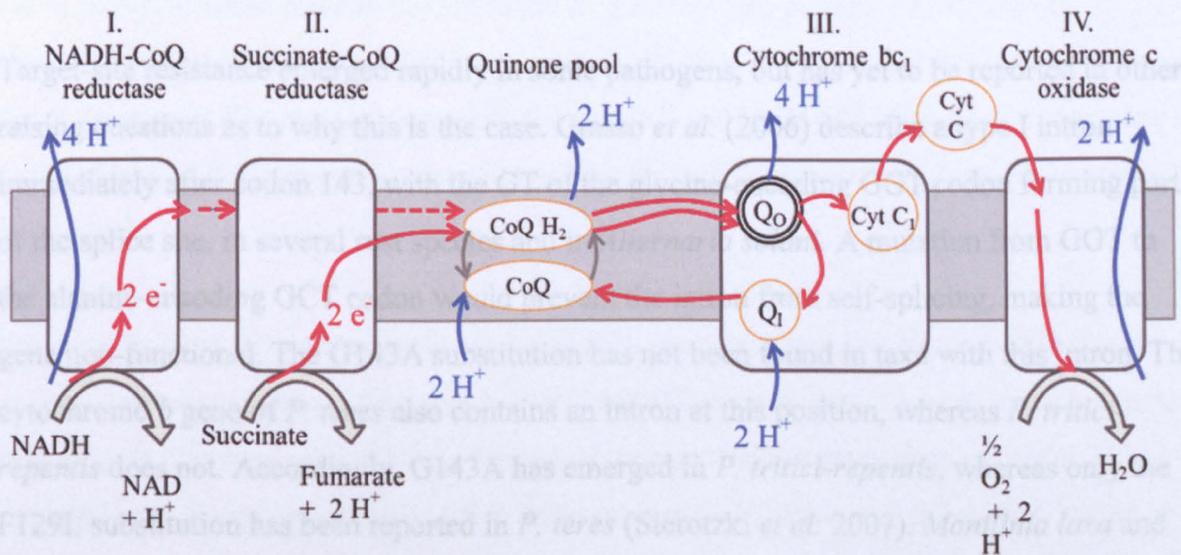
Figure 1.5. Schematic diagram of the mitochondrial electron transfer chain, including the Quinone outside (Qo) site, target site of the QoI fungicides.

1.3.1 Target-site resistance

The ability of target-site mutations to confer QoI resistance was shown by di Rago *et al.* (1989), who generated laboratory mutants of *Saccharomyces cerevisiae* with amino acid substitutions including F129L and G137R, as well as N256Y and three different substitutions at residue 275. Studies of naturally-resistant strobilurin-producing basidiomycetes revealed further amino acid changes associated with resistance, including A143 (Kraiczy *et al.* 1996).

The G143A substitution, with a change from glycine to alanine encoded by a single nucleotide mutation from GGN to GCN at codon 143, was found in a field isolate of *B. graminis* f.sp. *tritici* collected in 1998, with around a 200-fold reduction in sensitivity to strobilurins (Sierotzki *et al.* 2000b). A study of field isolates of *Magnaporthe oryzae* found some with the G143A substitution, and some with F129L, with G143A conferring around ten times the level of resistance conferred by F129L (Farman 2001). In field populations of *Pyrenophora tritici-repentis*, G143A, F129L and G137R have all been found, with G143A

conferring the highest level of resistance, and G137R similar to F129L (Sierotzki *et al.* 2007). In *P. teres* only F129L has been found so far, and control is still possible using QoIs with higher activity, such as pyraclostrobin and picoxystrobin (Oxley and Burnett 2010). As of January 2010, resistance to QoIs had been reported in 39 phytopathogenic fungi and oomycetes, including *Mycosphaerella graminicola*, *B. graminis* f. spp. *hordei* and *Ramularia collo-cygni* (FRAC 2010b).

Target-site resistance emerged rapidly in some pathogens, but has yet to be reported in others, raising questions as to why this is the case. Grasso *et al.* (2006) describe a type I intron immediately after codon 143, with the GT of the glycine-encoding GGT codon forming part of the splice site, in several rust species and in *Alternaria solani*. A mutation from GGT to the alanine-encoding GCT codon would prevent the intron from self-splicing, making the gene non-functional. The G143A substitution has not been found in taxa with this intron. The cytochrome *b* gene of *P. teres* also contains an intron at this position, whereas *P. tritici-repentis* does not. Accordingly, G143A has emerged in *P. tritici-repentis*, whereas only the F129L substitution has been reported in *P. teres* (Sierotzki *et al.* 2007). *Monilinia laxa* and *M. fructicola* also possess this intron, whereas *M. fructigena* does not (Miessner and Stammler 2010). In *B. cinerea*, some isolates have an intron after codon 143, but the G143A substitution is found only in isolates lacking this intron (Banno *et al.* 2009). Possible compensatory mutations restoring cytochrome *b* function in the presence of G143A and an intron at codon 143 have recently been reported in laboratory mutants of *S. cerevisiae* (Vallières *et al.* 2011). A mutation to restore correct base pairing for intron formation would have to take place simultaneously with G143A as either mutation alone would prevent splicing, and overexpression of mitochondrial metal ion carriers resulted in other fitness penalties, so these mutations are unlikely to emerge in the field, but loss of the intron may be possible in the field.

In species without an intron after codon 143, there may be fitness penalties associated with cytochrome *b* substitutions, due to effects on the structure or function of cytochrome *b*, such as steric hindrance during protein-folding, or impaired quinone-binding or electron transfer (Fisher *et al.* 2004). Di Rago *et al.* (1989) reported QoI resistant laboratory mutants of *S. cerevisiae* with impaired growth and reduced oxygen consumption. Avila-Adame and Köller (2003a) obtained two QoI-resistant strains of *Magnaporthe oryzae* by *in vitro* forced selection, one with the G143A substitution and another with G143S. The G143A isolate had

no apparent fitness costs or reduction in pathogenicity, whereas the G143S isolate had reduced sporulation *in vitro* and *in planta*. G143S has been reported in laboratory mutants of *C. beticola*, but this has not been found in the field (Malandrakis *et al.* 2011). Fisher *et al.* (2004) compared the effects of G143A and F129L in yeast cytochrome *b* with the Qo-site altered to resemble that found in a range of species, and fitness penalties were found to be species-dependent. For example, G143A was found to be deleterious to protein function in the *V. inaequalis*-like protein, but not in the *B. graminis*-like protein. F129L did not drastically affect the function of either protein. In G143A-F129L double mutants, function of the *V. inaequalis*-like protein was markedly reduced, and as G143A alone confers resistance to QoIs beyond field rates, it is unsurprising that no such double mutants have been reported in the field.

Two studies have shown that the G143A mutation has arisen multiple times within species, with at least two independent origins in *P. viticola* (Chen *et al.* 2007), and at least four in *M. graminicola* (Torriani *et al.* 2009b). Among *M. oryzae* isolates from ryegrass turf in the Eastern USA, G143A was found in five different genetic backgrounds (Kim *et al.* 2003), and in European *P. teres* isolates carrying F129L, three different codons for L129 were found in field isolates (Sierotzki *et al.* 2007).

The mitochondrially-encoded nature of the QoI target site raises the question of how a mutation in a single mitochondrion may increase in frequency in the mitochondrial population within a cell. Indeed, it was initially suggested that this would be unlikely to happen and therefore resistance risk for QoIs was low (Koller *et al.* 2004). Therefore the rapid emergence of QoI resistance in many pathogen species demonstrates that mitochondrial mutations can be selected more readily than predicted. Barr *et al.* (2005) give some examples of differential replication rates within intracellular mitochondrial populations, but these relate to mutations directly increasing replication rates of the mitochondria themselves, not those that benefit the cell as a whole. In addition, there are variations in patterns of mitochondrial inheritance between fungal species. In anisogamous species, ascogonium-producing strains always act as the maternal parent, contributing organelle DNA to all progeny. However, in isogamous, hermaphroditic species such as *B. graminis*, either isolate can act as maternal parent (Robinson *et al.* 2002). Studies of the spread of mitochondrial mutations mostly focus on the accumulation or removal of mutations deleterious to the organism, rather than possible selection of beneficial mutations. Nevertheless, such studies do provide evidence that in large

populations and under high selective pressure at the organism level, selection at the inter-organism level becomes the dominant evolutionary force (Taylor *et al.* 2002).

In the apple powdery mildew fungus *Podosphaera leucotricha*, Lesemann *et al.* (2006) reported widespread G143A heteroplasmy in the isolates tested. Sensitive isolates contained nearly all G143 mitochondria, whereas less-sensitive isolates had varying proportions of G143A correlated with resistance. The least sensitive isolates, which were able to infect leaves treated with 10 times the recommended field concentration of trifloxystrobin, had around 50% G143A mitochondria. In *V. inaequalis*, QoI-resistant laboratory mutants with predominantly GCC, the resistant genotype, at codon 143 reverted to predominantly GGC, the sensitive genotype, when two generations of selection on fungicide-amended medium were followed by growth on fungicide-free medium, showing that some wild-type mitochondria had persisted in the intra-cellular population. After eight generations on fungicide-amended medium, however, the resistant genotype appeared to have reached fixation (Zheng *et al.* 2000). Furthermore, QoI-resistant field isolates of *V. inaequalis* reported the following year appeared to be homoplasmic for G143A (Koller *et al.* 2004). Sirven *et al.* (2002) reported stable resistance to QoIs in *P. viticola* with a frequency of resistant *cytochrome b* alleles of just 2%, which has been cited as an example of heteroplasmy. However, sporidial populations were studied, not single-spore isolates, so it is not clear whether allele frequencies represent heteroplasmy within isolates or polymorphism between them. G143A heteroplasmy has since been reported in several pathogens (Fraaije *et al.* 2002; Ishii *et al.* 2007; Ishii *et al.* 2009), with frequency of resistant alleles often decreasing after several generations without fungicide, whereas in other species, such as *M. graminicola* (Fraaije *et al.* 2005), only A143 was detected in resistant isolates.

1.3.2 Alternative oxidase

Another possible resistance mechanism to strobilurins is target-site circumvention by an alternative respiratory pathway, using the alternative oxidase (AOX) as a penultimate electron acceptor directly from ubiquinone, which is oxidised at the Q_o site in the core respiratory pathway (Joseph-Horne and Hollomon 2000) (Figure 1.6).

I.	II.		III.	IV.
NADH-CoQ reductase	Succinate-CoQ reductase	Quinone pool	Cytochrome bc_1	Cytochrome c oxidase
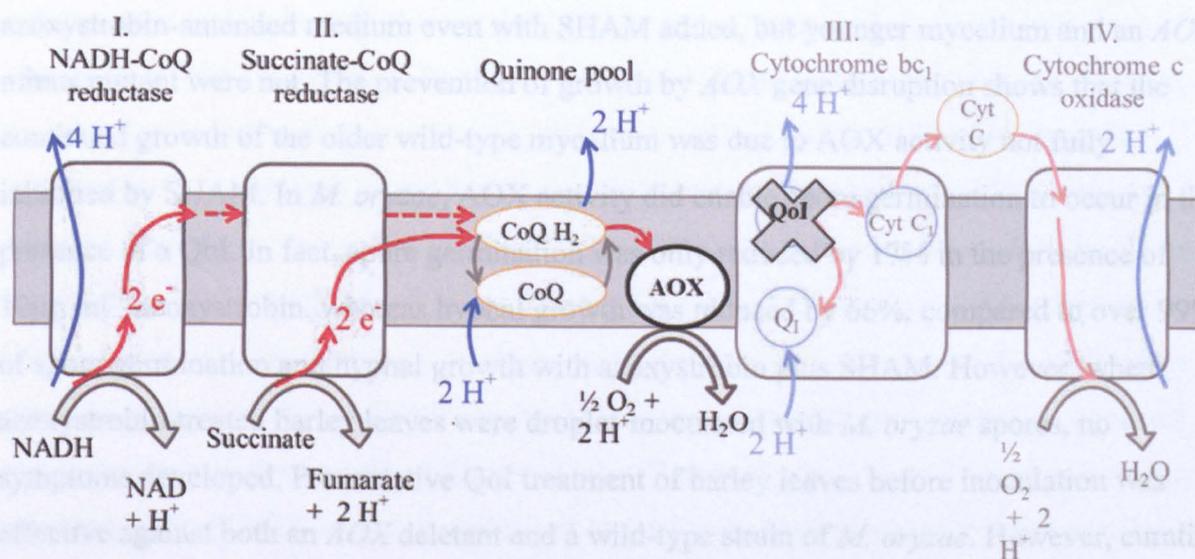
Figure 1.6. Schematic diagram of the mitochondrial respiratory electron transfer chain, with the Quinone outside pocket of Complex III inhibited by a QoI fungicide, and alternative oxidase acting as penultimate electron acceptor.

Ziogas *et al.* (1997) describe a laboratory mutant of *M. graminicola* with around a ten-fold decrease in azoxystrobin sensitivity that was negated by the addition of salicylhydroxamic acid (SHAM), an AOX inibitor. However, respiratory rate was lower for the alternative pathway and there was no loss of control *in planta*. In *V. inaequalis*, AOX activity gave a 60-fold reduction in kresoxim-methyl sensitivity, but again, this was not seen *in planta* (Olaya *et al.* 1998). The lack of *in planta* effect may be because *AOX* is induced by the generation of reactive oxygen species (Yukioka *et al.* 1998), which are inactivated by antioxidant compounds such as flavonoids in plant tissue (Mizutani *et al.* 1996; Tamura *et al.* 1999). Zheng *et al.* (2000) used flavones to inhibit *AOX* expression in *V. inaequalis*, achieving a similar synergistic effect to AOX inhibitors such as SHAM. However, laboratory mutants with reduced QoI sensitivity but no *cytochrome b* mutations, consistent with increased AOX activity, were also less sensitive to the effects of flavones on QoI sensitivity, demonstrating the potential for isolates to evolve which could express *AOX in planta*.

Alternatively, the observed ineffectiveness of AOX *in planta* may be because its effects are dependent upon fungal growth stage. AOX-terminated oxidative phosphorylation bypasses 60% of the proton-pumping activity of the core pathway, so ATP production from carbohydrates and oxygen is less efficient, and may be insufficient for more energy-intensive or food-limited stages (Wood and Hollomon 2003). Avila-Adame and Köller (2003a) found that older (melanised) mycelium of a wild-type *M. oryzae* isolate was able to grow slowly on

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azoxystrobin-amended medium even with SHAM added, but younger mycelium and an *AOX*-minus mutant were not. The prevention of growth by *AOX* gene disruption shows that the continued growth of the older wild-type mycelium was due to AOX activity not fully inhibited by SHAM. In *M. oryzae*, AOX activity did enable spore germination to occur in the presence of a QoI. In fact, spore germination was only reduced by 17% in the presence of $10\mu g\ ml^{-1}$ azoxystrobin, whereas hyphal growth was reduced by 66%, compared to over 99% of spore germination and hyphal growth with azoxystrobin plus SHAM. However, when azoxystrobin-treated barley leaves were droplet-inoculated with *M. oryzae* spores, no symptoms developed. Preventative QoI treatment of barley leaves before inoculation was effective against both an *AOX* deletant and a wild-type strain of *M. oryzae*. However, curative treatment of leaves colonised with established mycelium was only effective against the *AOX* deletant, and the wild-type isolate continued to grow (Avila-Adame and Köller 2003b). This suggests that in wild-type *M. oryzae*, either AOX activity is insufficient for the energetic demands of early mycelial growth post-germination, or *AOX* expression is prevented during the early stages of host colonisation. In *M. graminicola*, a laboratory mutant with increased AOX activity had reduced *in vitro* sensitivity but no loss of *in planta* control (Ziogas *et al.* 1997).

Lower intrinsic QoI sensitivity in *F. graminearum* than *M. nivale* is correlated with increased oxygen uptake and transcriptional upregulation of the *AOX* gene in response to azoxystrobin in *F. graminearum* and not in *M. nivale*. The increased oxygen uptake in *F. graminearum* was prevented by the addition of n-propyl gallate, an AOX inhibitor (Kaneko and Ishii 2009). Yukioka *et al.* (1998) reported that *AOX* transcript levels increased following the addition of a QoI fungicide, but transcript formation appeared constant, suggesting that reduced transcript degradation was responsible. However, Mizutani *et al.* (1996) detected a 40kDa protein when alternative respiration was induced by a QoI fungicide, but when *AOX* induction was suppressed by a reactive oxygen scavenger, a 41.3 kDa polypeptide was detected, which was converted to the 40kDa protein when the oxygen scavenger was removed, suggesting that the induction of AOX activity in that species was due to post-translation modification from a precursor to the active form. Analysis of *AOX* expression in *Neurospora crassa* identified at least four loci required, suggesting a complex regulatory pathway is involved (Descheneau *et al.* 2005).

Avila-Adame and Köller (2003a) obtained QoI-resistant isolates of *M. oryzae* only from older mycelium of wild-type isolates, in which AOX enabled growth to continue in the presence of azoxystrobin before mutations arose. In younger mycelium and an *AOX*-minus mutant, growth was fully inhibited on the first azoxystrobin-amended plate, and so serial transfers were not possible. In contrast, Zheng *et al.* (2000) only obtained *cytochrome b* mutants when UV mutagenesis was carried out on media with SHAM and kresoxim-methyl combined. When carried out with the fungicide alone, only mutants with increased AOX activity were obtained, suggesting that the ability to use AOX removed the selective advantage of target site mutations which may carry a fitness penalty. However, just $0.1 \mu g \, ml^{-1}$ kresoxim-methyl was used in the SHAM-amended media, but $1 \mu g \, ml^{-1}$ was used alone, so it is not possible to say whether AOX activity would have been sufficient to remove the selection pressure for target-site resistance at the higher fungicide dose. It has also been suggested that AOX may facilitate the evolution of QoI resistance in the field, as pathogens may continue to grow in the presence of a QoI, effectively giving a higher pathogen population under prolonged selection by the fungicide (Avila-Adame and Köller 2003a; Miguez *et al.* 2004). Furthermore, it has been suggested that since inhibition of cytochrome bc_1 results in increased levels of reactive oxygen species (Mizutani *et al.* 1996), growth under these conditions may result in an elevated mutation rate of mitochondrial DNA (Avila-Adame and Köller 2003a).

There have also been reports of reduced QoI sensitivity linked to active efflux of the fungicide out of fungal cells by membrane transported proteins. Azoxystrobin-adapted isolates of *P. tritici-repentis* generated by serial transfer on fungicide amended medium showed increased efflux of a fluorescent substrate, and returned to QoI-sensitivity on the addition of a transporter inhibitor (Reimann and Deising 2005). *Aspergillus nidulans* strains transformed with an overexpression construct of ABC transporter *atrB* had decreased sensitivity to a range of fungicides including kresoxim-methyl (Andrade *et al.* 2000). Roohparvar *et al.* (2008) report field strains of *M. graminicola* with increased expression of major facilitator *MgMFS1* and a further decrease in QoI sensitivity in addition to that conferred by G143A target-site resistance. There are also some cases of QoI resistance where the mechanism is not yet known, such as isolates of *Podosphaera fusca* resistant to QoIs even in the presence of SHAM, but with no mutations in the Qo site encoding region of the *cytochrome b* gene correlated with QoI resistance (Fernandez-Ortuno *et al.* 2008).

Up until 2008, extensive monitoring of *R. secalis* did not find any target-site resistance (Torriani *et al.* 2009a). *Rhynchosporium secalis* field isolates resistant to QoIs and carrying the G143A mutation were found at one site in France in 2008 (FRAC QoI Working Group 2008). However, G143A has not yet been reported in *R. secalis* from any other location, and was not found again in France in 2009 or 2010 (FRAC QoI Working Group 2009; FRAC QoI Working Group 2010). McCartney (2006) identified *R. secalis* isolates in which *in vitro* azoxystrobin EC_{50} was increased around ten-fold. There were no target-site mutations, and the decrease in sensitivity was negated by the addition of AOX inhibitors SHAM and propyl-gallate, suggesting that AOX was involved, but this was not investigated at the molecular level.

1.4 Triazoles

The triazoles (Figure 1.7) are demethylation inhibitor (DMI) fungicides, along with the imidazoles and heterocyclic DMIs (pyrimidines, pyridines and piperazines) (FRAC 2010a). They inhibit the14α-demethylation step in sterol biosynthesis (Sherald and Sisler 1975; Buchenauer 1977). As such, DMIs fall into the wider fungicide class of sterol biosynthesis inhibitors (SBIs), along with the morpholines and hydroxyanilides, which inhibit different stages in sterol biosynthesis and are therefore in different cross-resistance groups (FRAC 2010a).

The first DMI fungicide, the piperazine triforine, was introduced in 1969 (Schicke and Veen 1969). The first imidazole, imazilil, was introduced in 1972, and the first triazole, triadimefon, was introduced in 1973 (Schultz and Scheinpflug 1988). In 2008, triazoles represented 47% of UK cereal crop fungicide treatments by active substance treated area (Chemicals Regulation Directorate 2010) (Figure 1.3), and 41.6% of active substance treated area on UK barley (Garthwaite *et al.* 2008) (Figure 1.2). Furthermore, on UK barley in 2008, 60.9% of QoI applications, 62.1% of morpholine applications, 57.6% of MBC applications and 100% of SDHI applications by active substance treatment area were in a mixture containing a triazole (Garthwaite *et al.* 2008) (Figure 1.2).

Azoles (triazoles and imidazoles) are also used as clinical anti-fungal drugs. Initially, azoles were developed for topical treatments of superficial infections, and no resistance was reported in over fifteen years (Plempel and Berg 1988). However, since the introduction of

azoles for long-term systemic treatment of opportunistic pathogens such as *Candida* spp. and *Aspergillus* spp. in immunocompromised patients, there have been many reports of resistance by various mechanisms (Edlind 2008), some of which are common to pathogens of humans and plants.

Some triazoles, including tebuconazole and propiconazole, are also used as plant growth regulators, due to their inhibition of ABA 8'-hydroxylase, a cytochrome P450 involved in abscisic acid catabolism in plants (Kitahata *et al.* 2005).
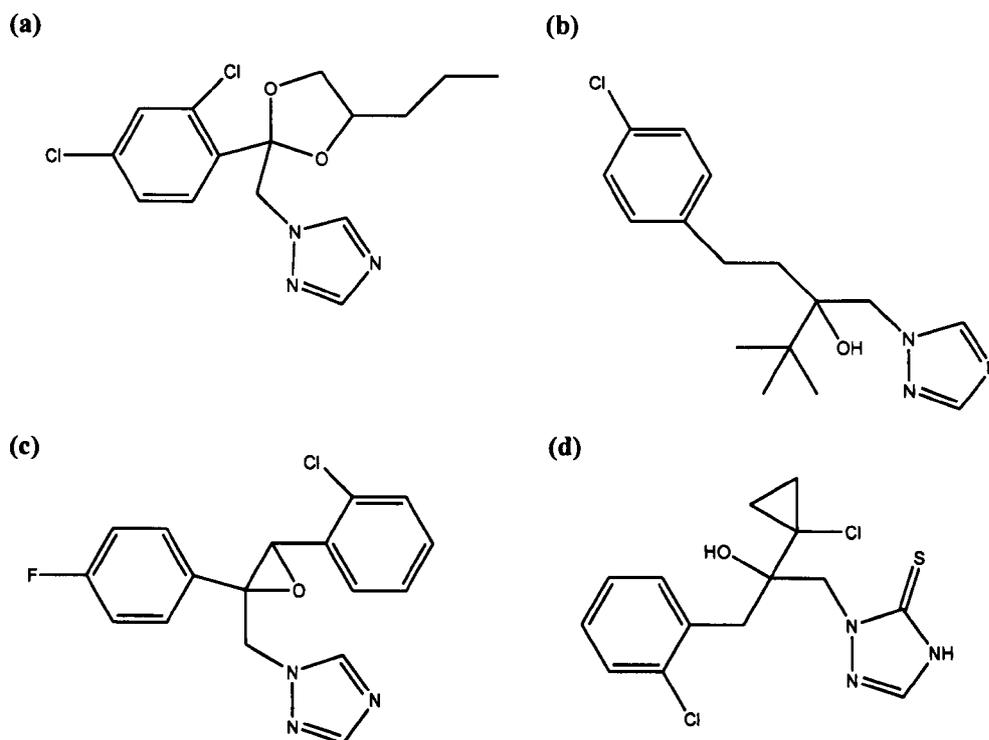
Figure 1.7. Structures of four triazole fungicides: (a) propiconazole, (b) tebuconazole, (c) epoxiconazole, (d) prothioconazole.

1.4.1 Mode of action

Triazoles and other DMIs inhibit the 14α-demethylation of lanosterol or eburicol (2-methylene-dihydrolanosterol) in sterol biosynthesis (Sherald and Sisler 1975; Buchenauer 1977) (Figure 1.7). Sterol 14α-demethylase is encoded by the gene *CYP51* (also known as *Erg11* due to its role in ergosterol biosynthesis), and is a member of the cytochrome P450 family of proteins (Lepesheva and Waterman 2007).

The main sterol in most fungi is ergosterol, a key component of cell membranes, which is synthesised *de novo* in fungi from acetyl CoA (Paltauf *et al.* 1992). Treatment with DMIs results in a decrease in ergosterol levels, and an accumulation of sterol intermediates including 2-methylene-dihydrolanosterol and obtusifoliol, and the aberrant sterol 14α-methyl-$\Delta^{8,24(28)}$-ergostadienol (14-methyl-3,6-diol), formed by the metabolism of 14-methylfecosterol by sterol 5,6 desaturase (Ragsdale and Sisler 1973; Kato *et al.* 1980) (Figure 1.8). The sterol 14-methyl-3,6-diol is toxic and cannot properly fulfil the function of ergosterol in cell membranes (Kelly *et al.* 1995).

The effects of CYP51 inhibition on sterol composition varies between species. In *R. secalis*, the proportion of ergosterol was not reduced by the addition of azole fungicides, but sterol intermediates downstream of 14α-demetylase were reduced, suggesting that later steps may have been upregulated, although total growth was reduced so the absolute level of ergosterol was lower (Kwok and Loeffler 1993). In *R. secalis*, addition of triadimenol, propiconazole or prochloraz resulted in an increase in [U-^{14}C] acetate incorporation into total sterols and in particular lanosterol, the substrate of CYP51, confirming 14α-demethylase as the site of action (Kendall and Hollomon 1990).

Although all DMIs share the same mode of action, cross-resistance is incomplete and complex (Kendall 1986), so as efficacy of older products has been eroded, new products have provided effective control (Klix *et al.* 2007). In some cases, as sensitivity to older products has declined, no significant cross-resistance has been found with newer products (Robbertse *et al.* 2001). In other cases, resistance has been correlated, but is quantitatively far lower for new products (Girling *et al.* 1988; Kendall *et al.* 1993). Furthermore, cross-resistance may depend on products used. Robbertse *et al.* (2001) found that the use of triadimenol as a seed-treatment selected for decreased sensitivity to triadimenol in particular, but when triadimenol and tebuconazole were used together as foliar treatments, isolates less sensitive to both triadimenol and tebuconazole were found, suggesting that different mutations or different mechanisms were responsible.

In some cases, triazoles have been found to show no or negative cross-resistance with some imidazoles (Kendall *et al.* 1993; Mavroeidi and Shaw 2005). For example, the I381V CYP51 substitution in *M. graminicola* results in reduced sensitivity to tebuconazole but increased sensitivity to prochloraz (Fraaije *et al.* 2007). However, the S524T substitution confers reduced sensitivity to all tested triazoles and to prochloraz (Cools *et al.* 2011). Conflicting results have been obtained for other pathogens. For example, following studies of *C. beticola*, Karaoglanidis and Thanassoulopoulos (2003) place DMIs into different cross-

LANOSTEROL

14α-demethylase → 4,4-DIMETHYL-8,14, 24-CHOLESTATRIENOL

14α-demethylase inhibited by azole fungicide → (LANOSTEROL) (No C14 reductase substrate)

C14 reductase → 4,4-DIMETHYL-8,24-CHOLESTADIENOL

(LANOSTEROL)

C4 methyl oxidase plus second C4 demethylation → ZYMOSTEROL

C4 methyl oxidase → 14-METHYL ZYMOSTEROL

C24 methyltransferase → FECOSTEROL

C24 methyltransferase → 14-METHYL FECOSTEROL

C8 isomerase → EPISTEROL

(No C8 isomerase activity) (14-METHYL FECOSTEROL)

C5 desaturase → ERGOSTA-5,7,24(24¹)-TRIENOL

C5 desaturase (incorrect activity when 14-methyl group present) → 14-METHYL-3,6-DIOL

C22 desaturase, C24 reductase → ERGOSTEROL

Figure 1.8. Ergosterol biosynthetic pathway from lanosterol onwards, showing the effect of azole fungicides (Paltauf *et al.* 1992; Kelly *et al.* 1995).

resistance subgroups, some containing a mixture of chemical classes (triazoles, imidazoles and pyrimidines). This is because the chemical groups are delineated by the nature of the heterocyclic ring (Uesugi 1998), whereas side chain substituents, which vary within each group, also affect target-site binding because the chemical groups are delineated by the nature of the heterocyclic ring (Uesugi 1998), whereas side chain substituents, which vary within each group, also affect target-site binding (Ito *et al.* 2005). Patterns of cross-resistance also depend on the resistance mechanisms involved. Reported resistance mechanisms against triazoles are target site mutations, target site over-expression, active efflux and altered sterol metabolism.

1.4.2 Target-site mutations

In contrast to the single-site, qualitative resistance to benzimidazoles and QoIs, target-site resistance to triazoles involves multiple mutations with varying and interacting effects. For example, amino acid substitutions S405F, G464S, and R467K, found in clinical isolates of *Candida albicans*, each reduced fluconazole and ketoconazole sensitivity by a factor of four but itraconazole only by a factor of two, whilst substitution Y132H decreased itraconazole, fluconazole and ketoconazole sensitivity by factors of two, four and 16, respectively (Edlind 2008). Over 140 predicted CYP51 amino acid substitutions have been reported in clinical isolates of *C. albicans*, although not all have been directly linked to quantified resistance factors and there is extensive polymorphism even within sensitive isolates (Morio *et al.* 2010).

In the plant pathogen *M. graminicola*, 19 target-site mutations have so far been identified (Cools *et al.* 2010), most of which have small but additive effects on triazole sensitivity (Leroux *et al.* 2007; Cools and Fraaije 2008). However, the I381V substitution in *M. graminicola* (Fraaije *et al.* 2007), as well as Y136F in *Uncinula necator* (Délye *et al.* 1997), and K147Q in *B. graminis* f.sp. *hordei* (Wyand and Brown 2005), are associated with higher levels of resistance to particular compounds (tebuconazole; triadimenol; and triadimenol and propiconazole, respectively). Other mutations in *M. graminicola* include Y137F, found as the sole change in isolates with specific resistance to triadimenol; substitutions at at codons 459-461, or the deletion of codons 459-460; V136A, found in combination with changes at codons 459-461 but not with I381V; and A379G, found in combination with I381V in the least sensitive isolates (Leroux *et al.* 2007; Cools and Fraaije 2008). The specific combinations of changes may reflect the evolutionary origins of the different mutations

(Brunner *et al.* 2008) or structural constraints to maintain enzyme activity (Bean *et al.* 2009; Cools *et al.* 2010). Substitutions equivalent to Y136F, codon 459 or 461 substitutions and A311G have been found in the related plant pathogen *Mycosphaerella fijiensis*, in isolates with a 4- to 20-fold reduction in propiconazole sensitivity. However, the involvement of non-target-site mechanisms in addition to these substitutions prevented the calculation of the contribution of each mutation to reduced sensitivity (Canas-Gutierrez *et al.* 2009).

In the cereal eyespot fungi *Oculimacula acuformis* and *O. yallundae*, phytopathogenic fungi closely related to *R. secalis* (Goodwin 2002; Crous *et al.* 2003), *O. acuformis* is naturally less sensitive to triazoles than *O. yallundae*, but sensitivity has decreased in both species, and *CYP51* is highly polymorphic (Albertini *et al.* 2003). With 14 species-specific amino acid substitutions, and 15 other amino acid substitutions in 27 isolates between the two species, individual substitutions were not definitively linked to differences in sensitivity, but comparisons with any mutations found in *R. secalis* could prove informative.

Some filamentous ascomycetes carry two paralogous *CYP51* genes. This was first observed in *Aspergillus fumigatus*, in which the two genes were designated *CYP51A* and *CYP51B* (Mellado *et al.* 2001). Subsequently, clinical isolates with reduced azole sensitivity were found to possess mutations (Diaz-Guerra *et al.* 2003; Mellado *et al.* 2004), or a different mutation accompanied by tandem repeats in the promoter region leading to over-expression (Mellado *et al.* 2007), in the *CYP51A* paralogue. Furthermore, knocking out *CYP51A* by gene disruption resulted in decreased azole sensitivity in sensitive as well as less-sensitive isolates, suggesting that the presence of the second copy may confer reduced intrinsic sensitivity (Mellado *et al.* 2005).

1.4.3 Target-site over-expression

Target site over-expression may result in reduced sensitivity to azoles, by reducing the ratio of fungicide to target site molecules (Hamamoto *et al.* 2000). Field isolates of *V. inaequalis* with reduced triazole sensitivity but no associated target-site changes had a 533-bp insertion upstream of the *CYP51* gene corresponding to increased *CYP51* expression (Schnabel and Jones 2001). Hamamoto *et al.* (2000) reported field isolates of *Penicillium digitatum* with reduced triazole sensitivity, but no mutations to the *CYP51* coding region, containing a 126-bp section of the promoter region that was tandemly repeated five times, resulting in constitutive *CYP51* expression around 100 times higher than sensitive isolates. Ghosoph *et al.*

(2007) reported another *CYP51* promoter aleration in *P. digitatum*, comprising a 199-bp insert within the single copy of the 126-bp promoter region, with around a tenfold increase in constitutive *CYP51* expression measured for either modification. In recent clinical isolates of *A. fumigatus*, over-expression was linked to a single tandem repeat in the *CYP51* promoter region, but this only conferred a marked decline in sensitivity in combination with an L98H substitution (Mellado *et al.* 2007). Field isolates of *Blumeriella japii* with reduced sensitivity to fenbuconazole overexpressed *CYP51* five- to 12-fold compared to sensitive isolates, and the promoter regions of these isolates contained inserts of varying lengths but all with a common 2120-bp section (Ma *et al.* 2006). Constitutive *CYP51* overexpression correlated with reduced azole sensitivity has also been reported in *C. beticola* (Nikou *et al.* 2009) and *Puccinia triticina* (Stammler *et al.* 2009), but the promoter regions have not been studied. In field isolates of *M. fructicola*, a repetitive insertion 117 base pairs upstream of the start of the *CYP51* coding region has been linked to a ten-fold increase in *CYP51* expression (Luo and Schnabel 2008).

In some clinical isolates of *C. albicans* in which reduced azole sensitivity was correlated with increased *CYP51* expression, mutations were found in the transcription factor encoding gene *Upc2p*. This transcription factor is involved in the upregulation of *CYP51* and other genes in the sterol biosynthesis pathway in response to ergosterol depletion, whereas gain of function mutations, resulting in substitutions G648D (Dunkel *et al.* 2008) and A643T (Heilmann *et al.* 2010), lead to constitutive upregulation of these genes.

There have also been reports in *Candida* spp. of chromosome or chromosome arm duplications resulting in increased expression of *CYP51*. Whole-chromosome duplication has been reported in *C. glabrata*, where it was associated with increased *CYP51* transcript levels and reduced intracellular levels of fluconazole (Marichal *et al.* 1997). In *C. albicans*, (Selmecki *et al.* 2006) report clinical isolates in which reduced fluconazole sensitivity is associated with a partial duplication of chromosome 5 to form an isochrome comprising two copies of the left arm, chr5L, rather than a left arm and a right arm. Genes on chr5L include *CYP51*, as well as two efflux pump encoding genes and one ABC transporter transcription factor, *TAC1*, and expression of these genes was higher in strains with isochromosome 5L. Successive deletions of individual copies of each of the four genes were carried out. Copy number of the two efflux transporter encoding genes did not affect fluconazole sensitivity, whereas *CYP51* and *TAC1* had independent, additive effects, with each extra copy of either

gene leading to a doubling of fluconazole MIC (minimum inhibitory concentration) (Selmecki *et al.* 2008).

In *Cryptococcus neoformans*, reduced azole sensitivity has been associated with disomy, a full extra copy in the haploid genome, of chromosome 1, containing *CYP51* and ABC transporter encoding gene *AFR1*. A proportion of isolates with disomic chromosome 1 were found in the progeny of sensitive isolates and selected in the presence of azoles, but the disomic chromosomes were subsequently lost from the population during serial transfers on fungicide-free medium. The effect of chromosome 1 disomy was partly mimicked by the insertion of a second copy of *CYP51* on a different chromosome, but also reduced by the deletion of *AFR1*, showing that both genes contributed to reduced azole sensitivity (Sionov *et al.* 2010).

1.4.4 Enhanced efflux

In other cases, reduced fungicide sensitivity has been linked to ATP-dependent reduction in the intracellular fungicide concentration (de Waard and van Nistelrooy 1980). This has been attributed to active efflux by ATP-binding cassette (ABC) transporter proteins (Balzi *et al.* 1994). Resistance due to ABC transporters is also be referred to as multidrug resistance (MDR) or pleiotropic drug resistance (PDR), sometimes according to the topology of the ABC transporter involved, with the transmembrane domain or the ATP-binding domain at the N-terminal of MDR and PDR proteins, respectively (de Waard *et al.* 2006). Another group of proteins involved in active efflux of fungicides is the major facilitator superfamily (MFS) (Tenreiro *et al.* 2000), which do not bind ATP but depend on protein-motive force (Del Sorbo *et al.* 2000).

In clinical *C. albicans* isolates, ABC transporters confer cross-resistance to multiple triazoles, whilst the MF transporter MDR1 confers specific resistance to fluconazole (Sanglard and Odds 2002). In *A. fumigatus*, 278 MF and 49 ABC transporters have been identified. So far, genes *atrF*, *AfumDMR3* and *AfumDMR4* have been linked to reduced triazole activity, with increased constitutive expression, or induced expression on exposure to itraconazole, in isolates with a high level of itraconazole resistance (Da Silva Ferreira *et al.* 2005).

The role of efflux transporter gene expression in drug resistance has prompted work on the regulatory pathways involved (Hiller *et al.* 2006). In the model organism *S. cerevisiae*,

mutations in the transcriptional regulator locus *PDR1* result in over-expression of three ABC-transporter proteins, leading to multi-drug resistance (Carvajal *et al.* 1997). In *C. albicans*, the transcription factor *TAC1* regulates the ABC transporters *CDR1* and *CDR2*, and some less sensitive isolates have a gain-of-function mutation (Coste *et al.* 2006) or duplication of the chromosome arm carrying this gene (Selmecki *et al.* 2006), whereas gain of function mutations in transcription factor *MRR1* resulted in constitutive overexpression of major facilitator gene *MDR1* (Morschhauser *et al.* 2007), although *TAC1* and *MRR1* also regulate other genes that may be involved in cellular stress responses (Cowen and Steinbach 2008).

In some clinical *C. albicans*, constitutive overexpression of the ABC transporter *CDR1* in isolates with reduced azole sensitivity was partly due to increased transcription, but also to increased transcript stability (Manoharlal *et al.* 2008). This was subsequently found to be due to hyperadenylation of *CDR1* transcripts. *CDR1* hyperadenylation correlated with poly(A)-polymerase 1 (*PAP1*) genotypes, comprising *PAP1a-PAP1α* in sensitive isolates and *PAP1a-PAP1α* in resistant isolates. Deletion of *PAP1a* from sensitive isolates results in reduced azole sensitivity, suggesting that *PAP1a* has a negative regulatory effect on poly(A)-polymerase 1, and the loss of this allele in less sensitive isolates results in hyperadenylation of *CDR1* transcripts (Manoharlal *et al.* 2010).

In plant pathogens, over-expression of an ABC transporter has been identified in field isolates of *P. digitatum* with reduced triazole sensitivity (Nakaune *et al.* 1998). In laboratory mutants of *B. cinerea* showing cross-resistance to oxpoconazole, tebuconazole and prochloraz, constitutive and induced expression of the ABC transporter encoding gene *BcatrD* was negatively correlated with azole sensitivity (Hayashi *et al.* 2001). *BcatrD* deletion increased azole sensitivity, and overexpression resulted in further reductions in sensitivity (Hayashi *et al.* 2002). In other cases, identifying specific ABC transporters with a clear link to changes in sensitivity has proven more elusive. Zwiers *et al.* (2002) reported one laboratory isolate of *M. graminicola* with reduced azole sensitivity in which ABC transporter *MgAtr1* was constitutively overexpressed, and deletion of this gene restored wild-type sensitivity. However, in other isolates, reduced azole accumulation was not linked to expression changes in ABC transporter encoding genes *MgAtr1-MgAtr5*. In *M. graminicola* field isolates with a range of azole sensitivities, constitutive *MgAtr* expression also varied, and *MgAtr4* expression was induced by cyproconazole, but expression of these genes was not correlated with azole sensitivity (Stergiopoulos *et al.* 2003). Isolates of *P. tritici-repentis* described as

showing reduced sensitivity to QoI fungicides due to efflux transporters were also cross-resistant to azoles, with sensitivity increased by the addition of efflux inhibitors (Reimann and Deising 2005), but the transporters responsible were not identified.

In addition to increased efflux, Mansfield *et al.* (2010) recently reported clinical isolates of *C. albicans* with reduced azole uptake. They present evidence of azole uptake taking place by facilitated diffusion, rather than active transport or passive diffusion, since uptake is unaffected in de-energised cells or efflux pump knockout strains, but does not take place in heat-killed cells, and uptake can reach saturation. Similar results were obtained for *Candida krusei, S. cerevisiae* and *C. neoformans*, but no filamentous ascomycetes and no plant pathogens were investigated. All triazole and imidazole fungicides tested appeared to use the same uptake mechanism, whereas other unrelated fungicides did not. They suggest that mutations leading to loss or loss-of-function of the protein responsible for facilitated diffusion of azoles may be found in *C. albicans* isolates with reduced uptake, but the protein involved has not yet been identified.

1.4.5 Altered sterol biosynthesis

Another possible means of reduced triazole sensitivity involves alterations in enzyme activity downstream of sterol 14 α-demethylase in the sterol biosynthetic pathway, producing alternative sterols that are non-toxic and partially functional in cell membranes. In particular, the presence of the 14-methyl group impairs the function of sterol 5,6-desaturase, resulting in the addition of a 4-methyl group (Kelly *et al.* 1995). It has been shown that a 14α-methyl group does not directly destroy membrane function, but a 4-methyl group does (Debieu *et al.* 1998).

Two fluconazole-resistant laboratory mutants of *S. cerevisiae* were found to have reduced sterol C5-6 desaturase (Erg3) activity, preventing accumulation of toxic 14-methyl-3,6-diol, and accumulation instead of 14α-methyl fecosterol (Watson *et al.* 1989) (Figure 1.8). A similar mutation restored aerobic growth in *CYP51* deletants (under anaerobic conditions, yeast is able to take up exogenous ergosterol), again resulting in accumulation of 14α-methyl fecosterol instead of 14-methyl-3,6-diol (Kelly *et al.* 1995). This mechanism was subsequently reported in clinical *C. albicans* isolates (Chau *et al.* 2005). These isolates had azole MICs over 100-fold greater than sensitive isolates, correlated with internal stop codons in *Erg3*, and accumulated 14α-methylfecosterol instead of 14-methyl-3,6-diol. The changes

were reversed by complementation with a wild-type *Erg3*. There was some evidence of reduced virulence, as mice infected with *erg3* mutant isolates survived longer than those infected with wild-type isolates, although tissue colonisation levels after 24 hours were similar and the isolates had been obtained from a patient following failure of azole treatment.

In *C. glabrata*, Geber *et al.* (1995) found that *Erg11* deletants were aerobically inviable whereas *Erg3-Erg11* double deletants were viable. However, they also obtained spontaneous mutants of *Erg11* deletants which were aerobically viable, but *Erg3* was expressed and contained no mutations. These mutants primarily accumulated lanosterol and obtusifoliol, suggesting loss of function or downregulation of genes involved in a different step in the conversion of lanosterol to ergosterol.

Azole tolerance in *Erg3* loss-of-function mutants depends on calcineurin-Hsp90 signalling, a pathway involved in responses to cell wall and membrane stress, and full sensitivity is restored in double mutants lacking both *Erg3* and components of this pathway (Cowen *et al.* 2006). Subsequent studies have identified further components of the signalling pathway, including protein kinases Pkc1 and MAPK (LaFayette *et al.* 2010). So far, these mechanisms have only been reported in laboratory and clinical isolates of *Candida* spp. and laboratory mutants of *S. cerevisiae*.

1.4.6 Resistance in *R. secalis*

Triadimenol, the first triazole to be recommended for winter barley in the UK, was introduced in the mid-1970s (Morris *et al.* 1977). Field strains of *R. secalis* with a 16-fold range in sensitivity to triadimenol were isolated in 1975-1981, but no loss of control was reported, and no differences in sensitivity were observed between isolates from azole-treated and untreated crops (Hollomon 1984). The potential for resistance development in *R. secalis* populations against the triazoles triadimenol and propiconazole, and the imidazole prochloraz, was demonstrated in glasshouse tests reported in 1986 (Hunter *et al.* 1986). Spores were transferred four times onto successive plants treated with the same fungicide, after which isolates showed over a thirty-fold shift in sensitivity to that fungicide (cross-resistance between fungicides was not tested). At that point, however, there were still no reports of loss of control in the field. This is in contrast to the barley pathogen *B. graminis* f.sp. *hordei*, in which reduced triazole sensitivity was reported in 1981, with a survey of

England, Wales and Scotland finding 53% of isolates were insensitive (Fletcher and Wolfe 1981).

A survey in England and Wales in 1987 found that triadimenol sensitivity in *R. secalis* populations, as determined by the *in vitro* sensitivity of 464 isolates, had declined from levels recorded in 1981(Jones 1990). A subsequent survey in south-west England in 1989 found that triadimenol sensitivity had declined further, with an increase in both the highest MIC recorded and the proportion of isolates with higher MIC values, and propiconazole sensitivity had also declined. Triadimenol sensitivity followed a bimodal distribution, with cross-resistance against propiconazole, but propiconazole was still effective at lower concentrations than triadimenol, and propiconazole or prochloraz gave good field control (Jones 1990). More detailed studies of eight isolates confirmed positive cross-resistance between triadimenol and propiconazole, but no clear cross-resistance to prochloraz (Kendall and Hollomon 1990). Studies with [U-^{14}C] found lanosterol accumulation after azole treatment in all isolates, although a higher fungicide dose was required in less sensitive isolates. There was no evidence of metabolism or decreased uptake of the fungicides in less sensitive isolates, with [^{14}C] triadimenol uptake similar in all isolates and all subsequently recovered as unmetabolised triadimenol, so it was suggested that target-site mutations may be responsible.

A four-year UK-wide survey of field isolates from 1987-1990 reported similar results: triadimenol sensitivity followed a bimodal distribution, with mean MIC 40-fold higher in 1990 than in 1975-1981, and propiconazole sensitivity followed a unimodal distribution, with an eightfold shift from 1987-1990 (Kendall *et al.* 1993). In 1990, tebuconazole sensitivity was also measured, and found to be positively correlated with triadimenol and propiconazole sensitivity. There was no significant correlation with sensitivity to the imidazole prochloraz. By 1990, triadimenol no longer provided field control , and control by propiconazole alone was reduced, but a propiconazole-carbendazim mixture or tebuconazole were effective (Kendall *et al.* 1993). In 1993-1994, MBC resistance in *R. secalis* was threatening the performance of DMI-carbendazim mixtures, but propiconazole, alone or with carbendazim, gave good disease control in field trials in Northern Ireland, even where control failed with carbendazim alone (Taggart *et al.* 1998).

In 1996-1998, new fungicides showed some promise against *R. secalis*, with the new triazole epoxiconazole and new mixing partners in the form of QoIs and cyprodinil introduced to the

market (Jones *et al.* 2000). However, in field trials in Northern Ireland and south west England in 1998-2000, epoxiconazole use resulted in sensitivity shifts, both between pre- and post-treatment isolates and between years, although epoxiconazole continued to provide yield benefits and some visible disease control in the field, especially in combination with azoxystrobin or cyprodinil (Cooke *et al.* 2004). By 2002, sensitivity shifts to epoxiconazole were reported in field isolates of *R. secalis*. Sensitivity of the population appeared to be moving towards a bimodal distribution in 2000-2002, with an increase in isolates with higher MICs (Oxley *et al.* 2003). By 2005-2007, epoxiconazole sensitivity had declined, especially in northern Scotland, but it was generally still moderately effective (Oxley *et al.* 2008). Prothioconazole had been introduced and was proving highly effective against *R. secalis*. Sensitivity shifts were observed when prothioconazole was used alone, with some evidence of cross-resistance with epoxiconazole (Oxley *et al.* 2008), but in general prothioconazole sensitivity appeared stable from 2005-2008 (Oxley and Hunter 2009).

Studies in other countries have also found successive declines in sensitivity to various triazole fungicides. In New Zealand, Sheridan and Nendick (1989) obtained *R. secalis* isolates with reduced triadimenol sensitivity from a field trial in 1988-1989, and disease control by triadimenol seed treatment or foliar spray was reduced, but cyproconazole seed treatment and propiconazole sprays were effective. In South Africa, Robbertse *et al.* (2001) reported isolates collected in 1993-1995 with significantly reduced sensitivity to triadimenol, propiconazole, flusilazole and tebuconazole compared to 'wild-type' isolates collected from an untreated field in a predominantly wheat-growing region. Triadimenol sensitivity was further reduced in 1995 compared to 1993-1994, a shift which the authors attributed to the heavy use of triadimenol as a seed treatment. Also, tebuconazole sensitivity was lower in isolates collected from two sites where barley had been grown and treated with tebuconazole every year for five and six years, but otherwise triazole sensitivity fluctuated between years.

Current HGCA monitoring in the UK has reported reduced azole sensitivity in *R. secalis*, with higher doses required for control of less sensitive strains, although sensitivity is higher in Northern Ireland (Blake *et al.* 2011). Prothioconazole and higher doses of epoxiconazole still provide disease control (Blake *et al.* 2011), although curative activity of epoxiconazole is reduced so this azole may now be more useful as a protectant (Oxley and Burnett 2010), and shifts in sensitivity to prothioconazole when used alone (Oxley *et al.* 2008) mean chemical diversity is important and there is a need for reistance monitoring and management.

In summary, QoI and triazole fungicides are important in the control of *R. secalis*. For the QoIs, a single case of target site resistance has been reported, so continued monitoring is needed. Furthermore,the role of AOX in reduced QoI sensitivity merits further investigation. In the case of the triazoles, previous studies have reported sensitivity shifts to different compounds, but the resistance mechanism is not yet known. Further sensitivity testing is needed to clarify the sensitivity shifts that may have occurred over time against different triazoles, using a consistent sensitivity assay method in order to obtain comparable results. Identifying the molecular mechanism or mechanisms underlying triazole sensitivity shifts in *R. secalis* would enable molecular diagnostics to be developed.

1.5 Aims of current work

This project aims to optimise culture conditions and develop a high-throughput fungicide sensitivity assay for *R. secalis*, in order to assess the QoI and triazole sensitivity of a range of older and recent isolates.

In the case of the QoIs, isolates will be screened for sensitivity shifts due to target-site mutations or AOX. The *cytochrome b* gene will be sequenced from a range of isolates to look for target-site mutations. The effect of AOX will be assessed through the use of AOX inhibitors, and analysis of *AOX* gene sequences and expression levels.

Isolates will also be screened for sensitivity shifts to a range of azole fungicides. The *CYP51* genes will be sequenced from a range of isolates to determine whether sensitivity shifts correlate with target-site mutations. A possible role of efflux transporters in azole sensitivity will also be tested through the use of efflux inhibitors.

The occurrence of multiple *CYP51* paralogues in fungal genomes will be surveyed through bioinformatic and phylogenetic methods. The role of multiple *CYP51* paralogues in azole sensitivity in *R. secalis* will be investigated. Assays will be developed to detect *CYP51* paralogues in *R. secalis* isolates, expression analysis of the *CYP51* genes will be carried out, and a pyrosequencing assay will be used to investigate the occurrence of *CYP51* paralogues in *R. secalis* populations from the Hoosfield spring barley experiment.

Elucidation of the methods responsible for altered fungicide sensitivity in *R. secalis* and investigation of their occurrence will help to inform future fungicide use and resistance management, as well as providing fundamental insights into the evolution of fungicide resistance and the adaptive potential of *R. secalis* populations.

Chapter 2

Method development: *R. secalis* culture conditions and fungicide sensitivity assay

2.1 Introduction

Assessing the occurrence of fungal strains with reduced sensitivity and investigating the molecular mechanisms responsible requires accurate measurements of the fungicide sensitivity of individual isolates. An *in vitro* fungicide sensitivity bioassay can be used to determine the fungicide concentrations required to inhibit growth of fungal isolates.

 Most previous studies of fungicide sensitivity in *R. secalis* have used agar media amended with a range of fungicide concentrations. Some methods have used agar slants in test tubes, which are inoculated with spores and the Minimum Inhibitory Concentration (MIC) assessed as the lowest fungicide concentration at which no growth occurs (Hollomon 1984). Other methods use agar in petri dishes or 24-well plates, on which a mycelial disc is placed. MIC may be assessed as the lowest concentration at which no growth occurs, as for agar slants, Kendall *et al.* (1993), or the Effective Concentration at which growth is reduced by 50% (the EC_{50}) may be assessed based on measurements of colony diameter (Sierotzki and Morchoisne 2006).

In other species, fungal growth has been assessed by optical density, measuring turbidity by passing light through a liquid culture. Georgopoulos and Sisler (1970) compared *U. maydis* growth in liquid culture in microtubes with and without fungicide. They subsequently measured growth at a range of fungicide concentrations and generated a dose response curve, but each fungicide concentration had to be set up and read in a separate tube, limiting the number of isolates that could be tested (Georgopoulos *et al.* 1972). In 1978, the first 96-well microplate reader was introduced, initially for ELISA (Enzyme-linked immunosorbant assay) plates (Thermo Scientific 2008). In 1982, Genta *et al.* (1982) reported using a microplate reader for a microtitre sensitivity assay for bacteria, and in 1986, Drouhet *et al.* (1986) described the use of this assay for a range of clinical pathogenic fungal species, including *C. albicans*, *C. neoformans* and *A. fumigatus*. The assay used is based on serial dilutions of fungicide in liquid growth media. Fungal isolates are then grown in 96-well microtitre plates

with a concentration series of fungicide-amended medium. Fungal growth is assessed by optical density readings, or with fluorescent metabolic indicators, using an automated plate reader. Dose-response curves are fitted, and MICs calculated. This method has also been developed for *M. graminicola*, for which EC_{50} values were calculated from the dose-response curves (Pijls *et al.* 1994; Fraaije and Cools 2006). This assay is preferable to those previously used for *R. secalis*. The 96-well format is suitable for high-throughput screening of large numbers of isolates, EC_{50} values reflect the point at which the majority of fungal growth is inhibited whereas MICs may be elevated when low levels of residual growth occur, and the automated reading of plates removes the need for subjective judgements in assessing fungal growth by eye.

The liquid-media based sensitivity bioassay requires spores as inoculum. Therefore it was necessary to optimise growth conditions of *R. secalis* in culture for conidia production. Previous authors have noted that *R. secalis* grows slowly in culture (Brooks 1928; Caldwell 1937; Schein and Kerelo 1956). A range of different growth media have been used. Caldwell (1937) tried agar media containing barley leaf decoction, potato or corn meal with and without dextrose, lima bean, oatmeal and malt extract, of which the fastest growth was observed on potato dextrose agar. Lebedeva (2005) reported the fastest growth in colony diameter on yeast potato sucrose agar, followed by potato sucrose agar, oatmeal agar, carrot dextrose, Czapek dox and finally water agar. Cooke *et al.* (2004) used malt yeast agar to isolate *R. secalis*, Czapek dox agar with mycological peptone for subculturing and yeast glucose medium for fungicide sensitivity testing.

Skoropad (1966) noted that when *R. secalis* grows saprophytically on barely debris, conditions conducive to sporulation did not result in greater hyphal growth. Similar observations had been made in culture by Schein and Kerelo (1956), who noted that only slow hyphal growth and small colonies were obtained on the media producing the highest spore yields, whereas potato dextrose agar, which previous studies reported as giving good growth, produced very low spore yields. Therefore conditions must be optimised for spore production without excessive hyphal growth, to produce suitable inoculum for an optical density-based sensitivity assay.

Rhynchosporium secalis growth is also temperature-dependent. Bartels (1928) found the optimal growth temperature to be 19-21°C, with a minimum temperature of 2-3°C and a

maximum of 30-31°C. Caldwell (1937) recorded maximum growth at temperatures of 18-21°C. The minimum temperature for germination and growth was 2-4°C. The maximum temperature was 28-30°C, with spores in water rupturing above 30°C, but growth rates dropped rapidly as temperature increased above 21°C. Subsequent studies have reported similar results for growth in culture (Schein and Kerelo 1956; Owen 1958; Lebedeva 2005) and infection and sporulation *in planta* (Skoropad 1959).

It was then necessary to optimise growth conditions for the bioassay itself. For sensitivity testing, sporulation is not required and maximising total growth rate would allow measurements to be taken after a shorter incubation time. Therefore different growth media and temperatures were tested again, and the inoculum density was also optimised for the sensitivity assay. However, a major problem with the use of optical density readings for *R. secalis* is the heterogeneous growth, or "clumping", of *R. secalis* in liquid culture. This covers two different growth states. Initially, *R. secalis* grows in discrete colonies: different growth levels result in smaller colonies with larger gaps, or larger colonies with smaller gaps, rather than homogeneous growth at lower or higher density as is seen with the yeast-like growth of *M. graminicola*. Later, fungal material forms thick clumps in the middle of each well. Neither state is conducive to the accurate assessment of growth by turbidity measurements. Different approaches have been used to deal with this, including gelatin-amended growth medium (Havis 2006), or the use of fluorescent metabolic indicators such as resazurin so fluorescence rather than optical density is measured (McCartney 2006). Earlier plate readers scanned plates with a light beam covering approximately a 3mm diameter in the centre of each well (Pijls *et al.* 1994). The plate reader used in the current study (Optima Fluostar, BMG Labtech, Germany) measures a single point at the centre of each well.

Finally, for DNA and RNA extraction, rapid growth in liquid culture was required, since agar contamination or secondary metabolites from older cultures may inhibit subsequent reactions such as PCR. Furthermore, for analysis of induced gene expression in response to fungicides, fungicide addition and RNA extraction should take place during the linear phase of growth, after spore germination but while the fungus is still actively growing. Therefore growth curves were produced for *R. secalis* in suitable growth conditions.

This chapter describes experiments carried out to optimise growth conditions for *R. secalis* in culture and to develop a protocol for a high-throughput *in vitro* fungicide sensitivity bioassay.

2.2 Materials and Methods

2.2.1 Culture conditions

2.2.1.1 Medium

Isolates used in initial tests were OSB 28-2-2, LARS 12-4-2 and LARS 12-4-3 and SAC 0003 1-4-2 30 (Table 2.1), collected by J. Fountaine from field trials in 2001-2002 and stored as conidia in silica gel at -80°C. All were grown on Czapek Dox agar with mycological peptone at 15°C for 12 days, and spores harvested into sterile distilled water.

Media tested for spore production were malt yeast glucose agar 1, as described in Cooke *et al.* (2004) (yeast extract 10 g l^{-1}, malt extract 10 g l^{-1}, agar 20 g l^{-1}); CDM agar (Czapek Dox broth mix 33.25 g l^{-1}, mycological peptone 5 g l^{-1}, agar 20 g l^{-1}); V8 agar (V8 juice 165 ml l^{-1}, calcium chloride 2 g l^{-1}, agar 16 g l^{-1}; pH-adjusted to pH7 with sodium hydroxide); Sabouraud agar (Sabouraud broth mix 30 g l^{-1}, agar 20 g l^{-1}); malt yeast glucose agar 2, with added glucose (yeast extract 10 g l^{-1}, malt extract 10 g l^{-1}, glucose 20gl^{-1}, agar 20 g l^{-1}); PDA (potato dextrose agar) and YPD (yeast peptone dextrose) agar (according to pack instructions). Media were poured into 90mm diameter Petri dishes, inoculated with 50µl spore suspension, sealed with parafilm and incubated in darkness at 15°C for seven days.

Subsequently, when a link between inoculum density and spore yield was established (Section 2.3.1.3), the experiment was repeated with a fixed inoculum density of 2.5 x 10^6 spores ml^{-1}, with 50µl spore suspension spread evenly over the plate surface. Isolates OSB 28-2-2 and LARS 12-4-2 were used, with two replicates of each.

Spores were harvested by adding 2ml sterile distilled water to the plate, scraping the surface with a sterile disposable spreader for two minutes, and pouring off the spore suspension. Spores were counted in an Improved Neubauer haemocytometer (Weber Scientific International, Middlesex, UK), with 5/25 squares chosen by random number.

Table 2.1. List of _R. secalis_ isolates used in this project

Isolate	Year of collection	Location
K1124	Pre-1994	Long Ashton Research Station, UK
SK7	1984	Long Ashton Research Station, UK
NKT12	1996	Norway
FI12-63	1996	Finland
R157 (1)	1997	USA
R157 (2)	1997	USA
788	1997	France
SAC 0003 1-4-2 30	2000	Scottish Agricultural College, UK
SAC 1-4-8 (0003)	2000	Scottish Agricultural College, UK
SAC 8-3-8 (0003)	2000	Scottish Agricultural College, UK
SAC 0004 1.15 0030	2000	Scottish Agricultural College, UK
SAC 8.83 00 (0.04)	2000	Scottish Agricultural College, UK
SAC 0003 1.2.4 00 (3.33)	2000	Scottish Agricultural College, UK
SAC 0003 1.2.5 00 1.11	2000	Scottish Agricultural College, UK
SAC 0003 1.4.8 00 (10)	2000	Scottish Agricultural College, UK
SAC 0003 8.1.8 00 (0.123)	2000	Scottish Agricultural College, UK
SAC 0004 8.1.2 2000 10.07	2000	Scottish Agricultural College, UK
SAC 0004 1.2.4 00	2000	Scottish Agricultural College, UK
SAC 0003 16/20	2000	Scottish Agricultural College, UK
QUB 9-10	2001	Northern Ireland
QUB 30-10	2001	Northern Ireland
QUB 18-2	2001	Northern Ireland
QUB 30-13	2001	Northern Ireland
QUB 12-3	2001	Northern Ireland
QUB 18-9	2001	Northern Ireland
R 9517.1	2001	ARINI, Northern Ireland
R 9524.2	2001	ARINI, Northern Ireland
R 9528.4	2001	ARINI, Northern Ireland
R 9516.1	2001	ARINI, Northern Ireland
R 9511.4	2001	ARINI, Northern Ireland
R 9522.3	2001	ARINI, Northern Ireland
R 9519.2	2001	ARINI, Northern Ireland
RS01ch2.126	2001	Switzerland
RS01ch2.126.5.5	2001	Switzerland
RS01ch2.306.5.5	2001	Switzerland
RS01ch01A12a5.5	2001	Switzerland
3.1 2/7	2001	Rothamsted Research, UK
3.1 4/7	2001	Rothamsted Research, UK
3.1 5/7	2001	Rothamsted Research, UK
5.2 2/7	2001	Rothamsted Research, UK
T.I. 3.1 11/7	2001	Trenthome Farm, Nottingham, UK
M.S. 1.1 13/7	2001	Trenthome Farm, Nottingham, UK
M.S. 5.1 13/7	2001	Trenthome Farm, Nottingham, UK

Table 2.1 continued

Isolate	Year of collection	Location
GKII 18-2-1	2002	Rothamsted Research, UK
GKII 18-2-3	2002	Rothamsted Research, UK
GKII 18-3-1	2002	Rothamsted Research, UK
GKII 18-3-2	2002	Rothamsted Research, UK
GKII 18-3-3	2002	Rothamsted Research, UK
GKII 20-3-1	2002	Rothamsted Research, UK
OSA 28-2-2	2002	Rothamsted Research, UK
OSA 10-4-1	2002	Rothamsted Research, UK
OSA 10-4-28	2002	Rothamsted Research, UK
OSB 28-2-2	2002	Rothamsted Research, UK
OSB 24-4-1	2002	Rothamsted Research, UK
OSB 24-4-21	2002	Rothamsted Research, UK
OSB 24-4-47	2002	Rothamsted Research, UK
LARS 12-4-2	2002	Long Ashton Research Station, UK
LARS 12-4-3	2002	Long Ashton Research Station, UK
LARS 8-4-2.5	2002	Long Ashton Research Station, UK
Sheringham 1	2002	Sheringham, Norfolk, UK
Sheringham 2	2002	Sheringham, Norfolk, UK
XNC 2000 3-2 T1 A	2002	Northern Ireland
XNC 2000 3-2 T1 B	2002	Northern Ireland
XNC 2000 4-2-4 T1	2002	Northern Ireland
SAC 09/943/186	2007	Scottish Agricultural College, UK
SAC 09/943/62	2007	Scottish Agricultural College, UK
SAC 09/943/73	2007	Scottish Agricultural College, UK
SAC 09/943/13	2007	Scottish Agricultural College, UK
SAC 09/943/115	2007	Scottish Agricultural College, UK
SAC 09/943/131	2007	Scottish Agricultural College, UK
SAC 09/943/14	2007	Scottish Agricultural College, UK
SAC 09/943/132	2007	Scottish Agricultural College, UK
SAC 09/943/178	2007	Scottish Agricultural College, UK
RS 219	2004	UK (Syngenta sensitive reference isolate)
RS 683	2004	UK (Syngenta intermediate reference)
RS 783	2004	UK (Syngenta less sensitive reference)
R.s. 2310 4.2	2008	France (BASF QoI monitoring)
R.s. 2313 4.2	2008	France (BASF QoI monitoring)
R.s. 2314 4.2	2008	France (BASF QoI monitoring)
R.s. 2318 4.2	2008	France (BASF QoI monitoring)
SCRI 13-13		Scottish Crop Research Institute, UK (genome sequencing)

2.2.1.2 Temperature

Silica gel stocks of the four isolates used in media tests were used to inoculate CDM Agar. These were incubated at 15°C and 21°C. Spores were harvested and counted after ten days.

2.2.1.3 Inoculum density

The four isolates used in the media tests were sub-cultured onto CDM Agar. Spore suspensions were diluted to concentrations of 2.5×10^4, 2.5×10^5, 2.5×10^6 and 2.5×10^7 spores ml^{-1}, with 50µl spore suspension used per 90mm diameter Petri dish. There were two replicates at each concentration, except LARS 12-4-2, LARS 12-4-3 and SAC 0003 1-4-2 30 at 2.5×10^7, for which insufficient inoculum was available. Plates were incubated in darkness at 15°C. Spores were harvested and counted after ten days.

2.2.2 Assay method

2.2.2.1 Medium

The preliminary assay used the same four isolates as culture condition tests. Spore suspensions were harvested from 10-day-old cultures on CDM agar. Spore counts were carried out, and spore concentration was adjusted to 2.5×10^5 spores ml^{-1} by dilution in sterile distilled water. Double-strength media were amended with epoxiconazole. A 10 mg ml^{-1} solution of epoxiconazole in acetone was made, and added to the media to a final concentration of 100 µg ml^{-1}. Ten serial dilutions were carried out with a dilution factor of 2/5. Media used were Czapek Dox (McCartney, 2006), Sabouraud (Pijls *et al.* 1994), alkyl ester (Sierotzki and Morchoisne 2006) and yeast glucose (Cooke *et al.* 2004).

The assay was carried out in 96-well microtitre plates (TPP, Switzerland). 100µl of fungicide-amended medium and 100µl of concentration-adjusted spore suspension were added to each well. Columns 1-12 contained increasing concentrations of epoxiconazole, with a final concentration of 50 µg ml^{-1} in column 12, ten serial dilutions (dilution factor 2/5) in columns 11-2 and 0 µg ml^{-1} in column 1. Rows A-H comprised two replicate rows for each of the four isolates. Plates were sealed with parafilm and incubated in darkness at 15°C.

After 7, 9, 11 and 13 days, fungal growth was measured by optical absorbance at 620nm using an Optima Fluostar plate reader (BMG Labtech, Germany), with the accompanying software used to fit a dose-response curve (4-parameter fit) and calculate EC_{50} values.

2.2.2.2 Incubation Temperature and Time

Epoxiconazole sensitivity assays were carried out as described above, but with Sabouraud medium used throughout and one plate incubated at 15°C, one at 18°C and one at 21°C. Growth was measured after 7, 9 and 12 days.

2.2.2.3 Inoculum density

Epoxiconazole sensitivity assays were set up as described above, using Sabouraud medium throughout, and isolates OSB 28-2-2 and LARS 12-4-2. Inoculum concentrations of 2.5 x 10^4, 1 x 10^5, 2.5 x 10^5, 1 x 10^6 and 2.5 x 10^6 spores ml^{-1} were used. Plates were incubated at 18°C for seven days.

2.2.2.4 Growth Heterogeneity: Gelatin

Double-strength Sabouraud medium was amended with 180-bloom gelatin to final concentrations of 0, 0.4, 1, 2 and 4% w/v. Epoxiconazole sensitivity assays were set up as described above, for those four isolates, with plates incubated at 18°C and read after seven days. This was then repeated for final gelatin concentrations of 0 and 1%.

2.2.2.5 Growth Heterogeneity: Shaking

Epoxiconazole sensitivity assays were set up as described above, using Sabouraud medium, spore suspension at 2.5 x 10^5 spores ml^{-1}, incubating at 15°C for 12 days. In addition to isolates OSB 28-2-2 and LARS 12-4-2, isolates QUB 9.10 and QUB 30.10, previously found to have differing levels of sensitivity to epoxiconazole (L Black, unpublished data), were used. Plates were read without shaking, then after 30 seconds shaking at medium speed (c. 1125 rpm), 2 minutes at medium speed, 2 minutes at top speed (1300 rpm) then 5 minutes at top speed, on an Orbis Plate Shaker (Mikura, West Sussex). Further assays were carried out taking readings before and after shaking for 30 seconds at medium speed, with a wider range of isolates and the four triazole fungicides described in Chapter Four.

2.2.2.6 Growth Heterogeneity: Fluorescence-Based Assays

Epoxiconazole sensitivity assays were set up as described. After seven days, optical density readings were taken and 20 μl of 80 μg ml^{-1} resazurin solution was added to each well. Plates were incubated overnight (16 hours) in darkness at 18°C, then fluorescence at 530 nm was measured using the Optima Fluostar plate reader (BMG Labtech, Germany).

An Epoxiconazole sensitivity assay was set up using Sabouraud medium, spore suspension at 2.5 x 10^5 spores ml^{-1}, and incubating at 18°C for seven days. Isolates SAC 09/943/186, SAC 09/943/73, SAC 09/943/115 and RS 219 were used. The assay plate was read with the following settings (well inside diameter = 6.7 mm): endpoint mode, single reading per well; endpoint mode, orbital averaging, 20 flashes around a 4mm-diameter orbit; well-scanning mode, 3x3 matrix, 6mm diameter; well-scanning mode, 6x6 matrix, 6mm diameter; well-scanning mode, 6x6 matrix, 4mm diameter; well-scanning mode, 4x4 matrix, 3mm diameter. The well-scanning points are arranged in a square grid with sides equal to the specified diameter; points in the matrix that fall wholly outside a circle of the specified diameter are excluded. Hence for a 3x3, 4x4 and 6x6 matrix, readings are taken at 9, 12 and 32 points, respectively. Further assays were carried out taking readings in end-point mode and in well-scanning mode with a 4x4 matrix of 3mm diameter before and after shaking for 30 seconds at medium speed, with a wider range of isolates and the four triazole fungicides described in Chapter 4.

2.2.3 Growth curve

Isolates 788 and K1124 were each grown in 50 ml tubes containing 10 ml Sabouraud medium inoculated with 2.5 x 10^4 spores ml^{-1}, in an orbital shaker at 150 rpm at 20 °C. At day 0 and subsequent 2-day intervals, 2 tubes of each isolate were selected at random, removed, and frozen at 20 °C. Subsequently all isolates were harvested by vacuum filtration onto a nylon filter disc, placed into individual plastic bags, re-frozen and freeze-dried. The dry mass of fungus on the filter discs was measured on a fine balance.

2.3 Results

2.3.1 Culture conditions

2.3.1.1 Medium

CDM agar yielded the highest spore numbers, V8 agar produced good hyphal growth but lower spore counts, and the malt yeast glucose agar and PDA gave no growth after seven days for all four isolates tested (Table 2.2).

Table 2.2. Spore yields obtained by subculturing _R. secalis_ isolates on different agar media

Isolate	Spore counts (10^5 spores ml^{-1})			
	Agar Medium			
	Czapek Dox with mycological peptone	V8	Malt Yeast Glucose 1	Potato Dextrose
OSB 28-2-2	95	46.5	N[a]	N
LARS 12-4-2	26.5	5	N	N
LARS 12-4-3	22.5	5.5	N	N
SAC 0003 1-4-2 30	51	6.5	N	N

[a] N indicates no growth.

In the second experiment, spore counts for both isolates were highest on CDM agar. V8 agar still produced good hyphal growth but lower spore counts, as did the Sabouraud agar. The addition of extra glucose to the malt yeast glucose agar and yeast extract to the potato dextrose agar resulted in some growth and sporulation, but less than was obtained with the CDM agar (Table 2.3).

Table 2.3. Spore yields obtained by subculturing _R. secalis_ isolates on five different agar media.

Isolate	Spore counts (10^5 spores ml^{-1})				
			Agar Medium		
	Czapek Dox + Mycological Peptone	V8	Malt Yeast Glucose 2	Yeast Potato Dextrose	Sabouraud
OSB 28-2-2	339	11	21.8	40.5	19.8
LARS 12-4-2	159.5	17.5	24.8	45	85.5

2.3.1.2 Temperature and Time

All four isolates grew well at 15°C, sporulating within ten days. There was little or no germination and growth at 21°C.

2.3.1.3 Inoculum density

Spore yields for all isolates increased with inoculum density for inoculum concentrations up to 2.5×10^6 spores ml^{-1} (Figure 2.1). However, increasing inoculum concentration to 2.5×10^7 spores ml^{-1} resulted in a far lower increase in spore yield for isolate LARS 12-4-3, and decreased spore yields for the other isolates. An inoculum density of 2.5×10^6 spores ml^{-1}

was selected for all future work, as it produced consistently high spore yields for both replicates of all four isolates.
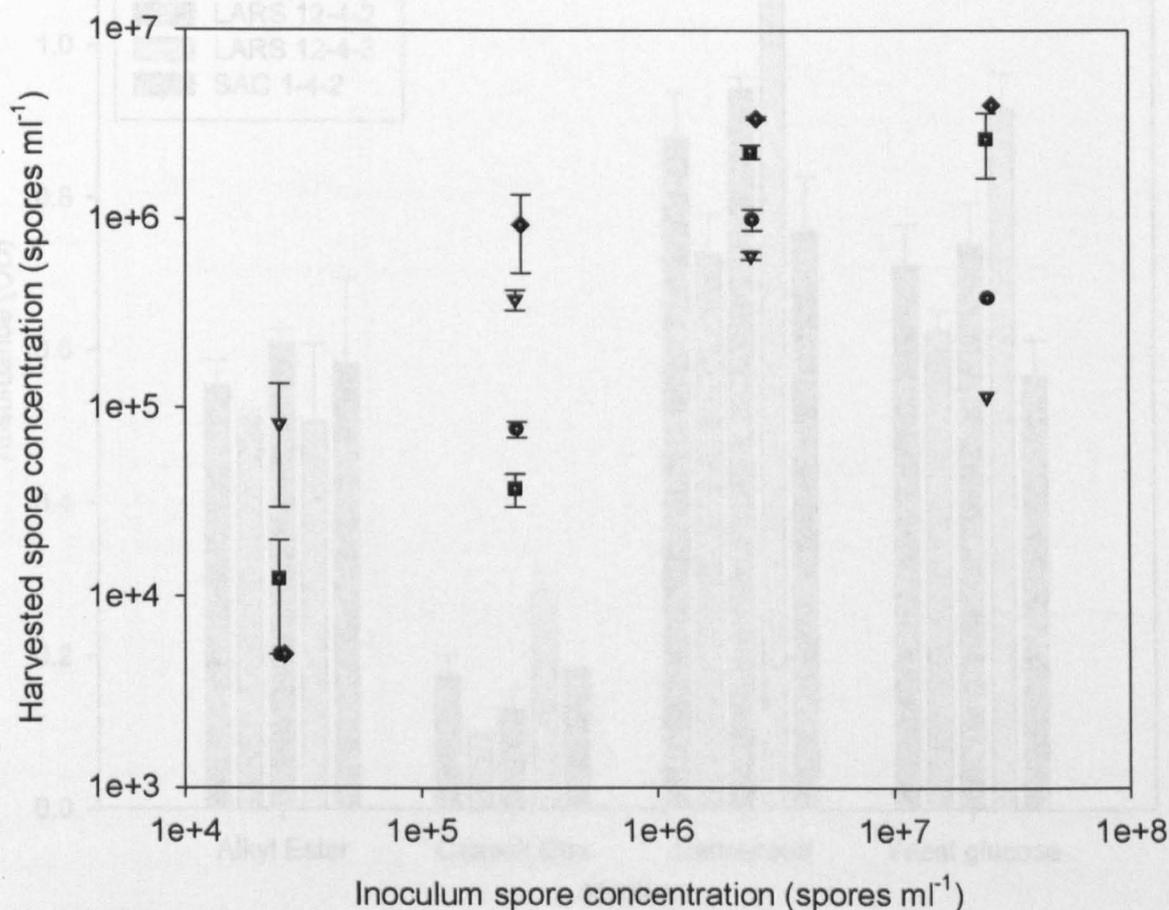
Figure 2.1. Spore yield against inoculum level for four _R. secalis_ isolates. Spore counts of zero are shown as 5 x 10³ spores ml⁻¹, the lowest detectable level, due to the log scale. Circle, isolate OSB 28-2-2; square, LARS 12-4-2; diamond, LARS 12-4-3; triangle, SAC 0003 1-4-2.

2.3.2 Assay method

<u>2.3.2.1 Medium</u>

The control growth, i.e. 0 µg ml⁻¹ epoxiconazole, was greatest in Sabouraud medium, followed by yeast glucose, alkyl ester then Czapek Dox, for all four isolates at all four measurement times. Data shown are measurements at 13 days (Figure 2.2).
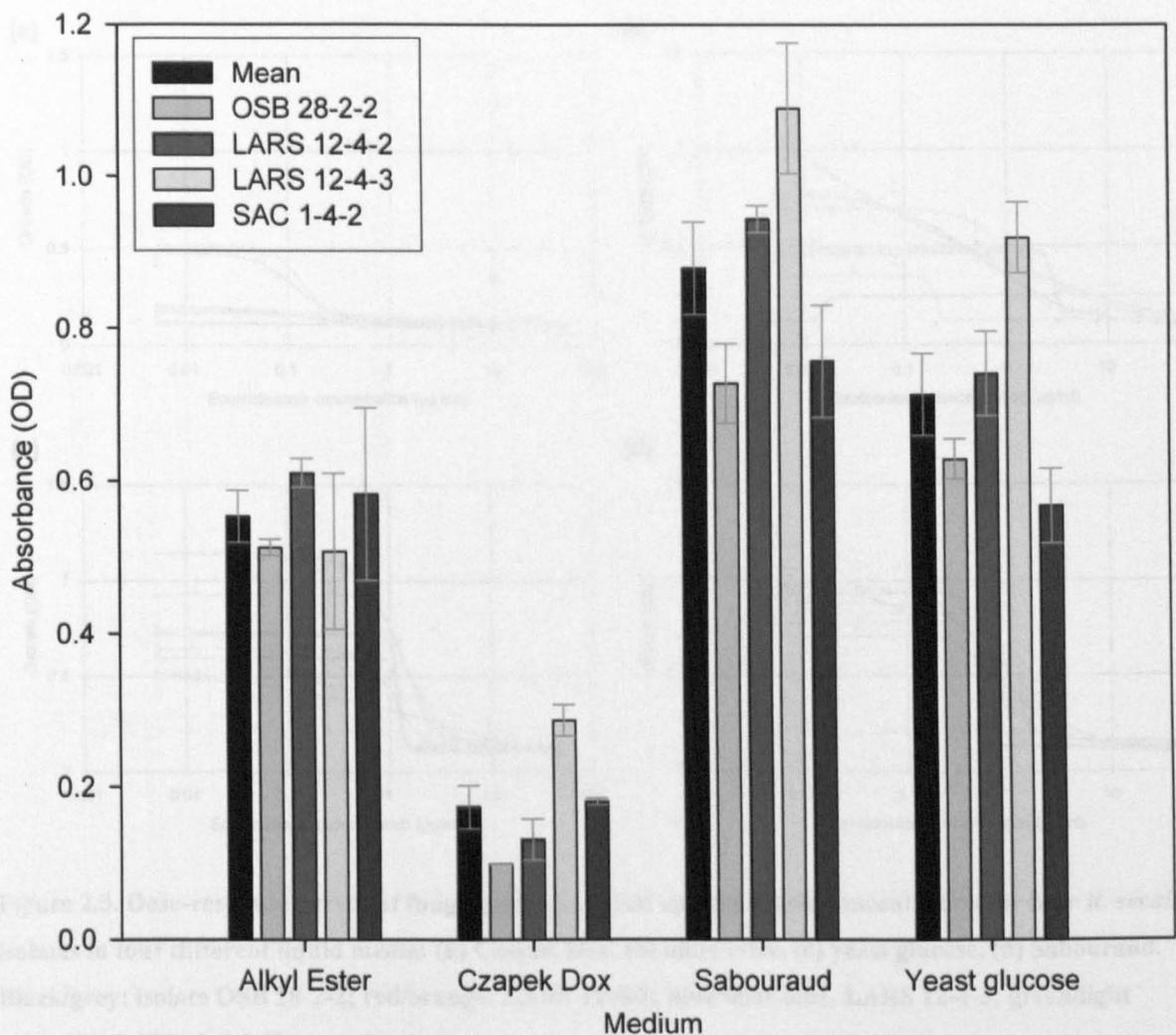
Figure 2.2. Growth of four *R. secalis* isolates after 13 days, from an initial inoculum concentration of 2.5 x 10-5 spores ml⁻¹, in four different liquid media, measured by optical absorbance.

The growth rate affected the quality of dose-response curves obtained (Figure 2.3). In Czapek Dox and alkyl ester media, some isolates did not grow sufficiently to obtain an EC_{50} value even after 13 days, but better data are obtained with yeast glucose medium, and better still with Sabouraud. This is reflected in the consistency of EC_{50} values between replicates, with Sabouraud producing the most consistent results (Figure 2.4). Therefore, Sabouraud medium was selected as the first choice of assay medium for future assays, with the caveat that a very rich medium may not be suitable for all fungicides.
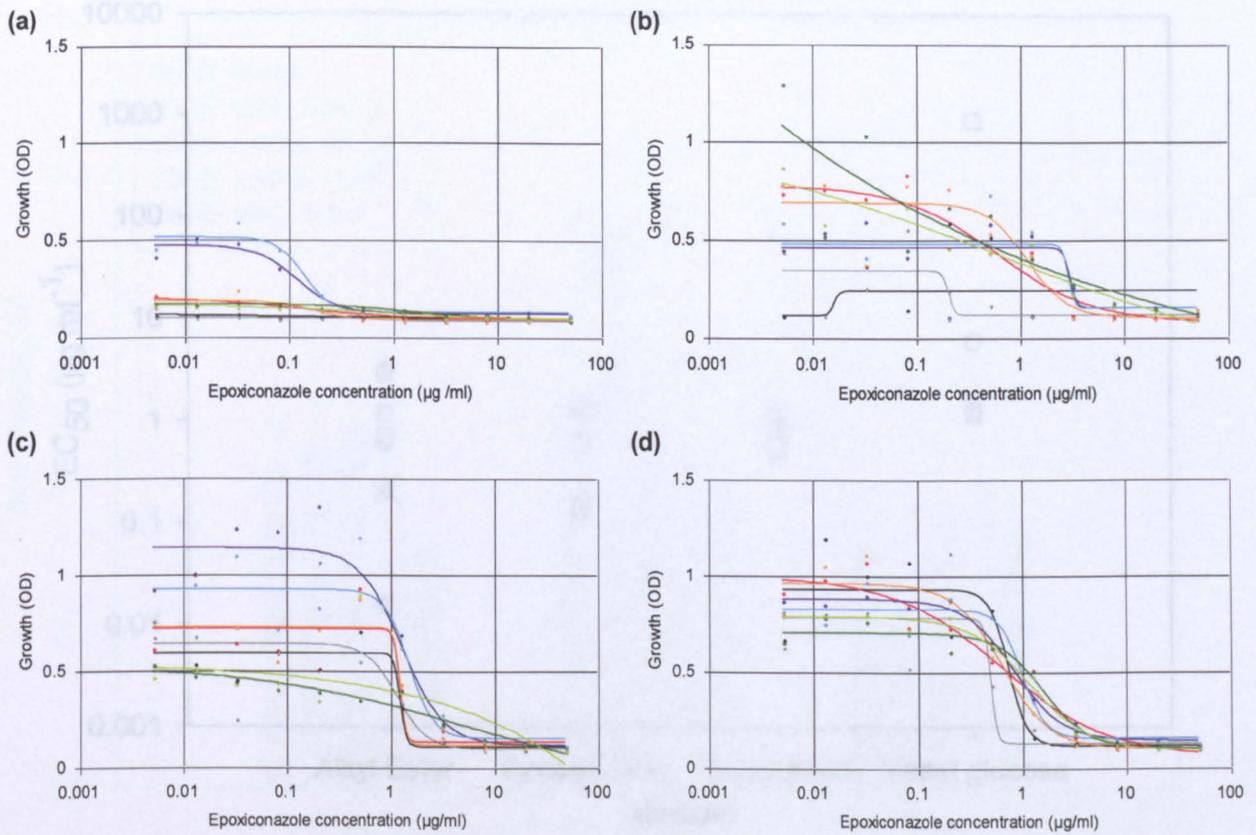
Figure 2.3. Dose-response curves of fungal growth against epoxiconazole concentration for four *R. secalis* isolates in four different liquid media: (a) Czapek Dox, (b) alkyl ester, (c) yeast glucose, (d) Sabouraud. Black/grey: isolate OSB 28-2-2; red/orange: LARS 12-4-2; blue/light blue, LARS 12-4-3; green/light green, SAC 0003 1-4-2 30.
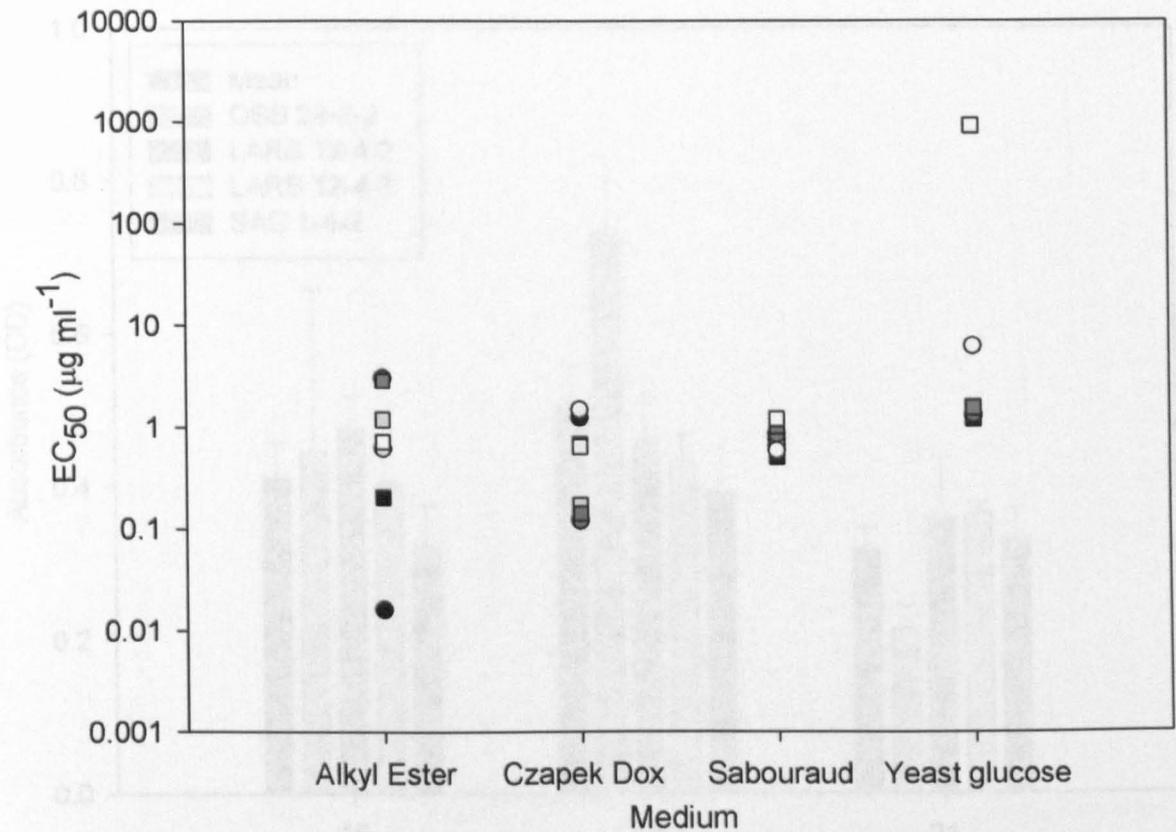
Figure 2.4. Epoxiconazole EC_{50} values obtained for four *R. secalis* isolates in four different growth media, with two replicates per isolate: Black, isolate OSB 28-2-2; light grey, LARS 12-4-2; dark grey, LARS 12-4-3; white, SAC 0003 1-4-2. Circle, replicate 1; square, replicate 2.

2.3.2.2 Temperature and Time

Growth after seven days was greatest at 18°C for three of the four isolates, slightly lower at 15°C and markedly lower at 21°C (Figure 2.5). Readings at seven days produced the most consistent epoxiconazole EC_{50} values between replicates at15°C and 18°C incubation temperatures, with greater differences at 9 and 12 days (Figure 2.6). The opposite pattern was seen at 21°C, as the slower growth rate meant the optimal measuring time had not been reached at the earlier readings.
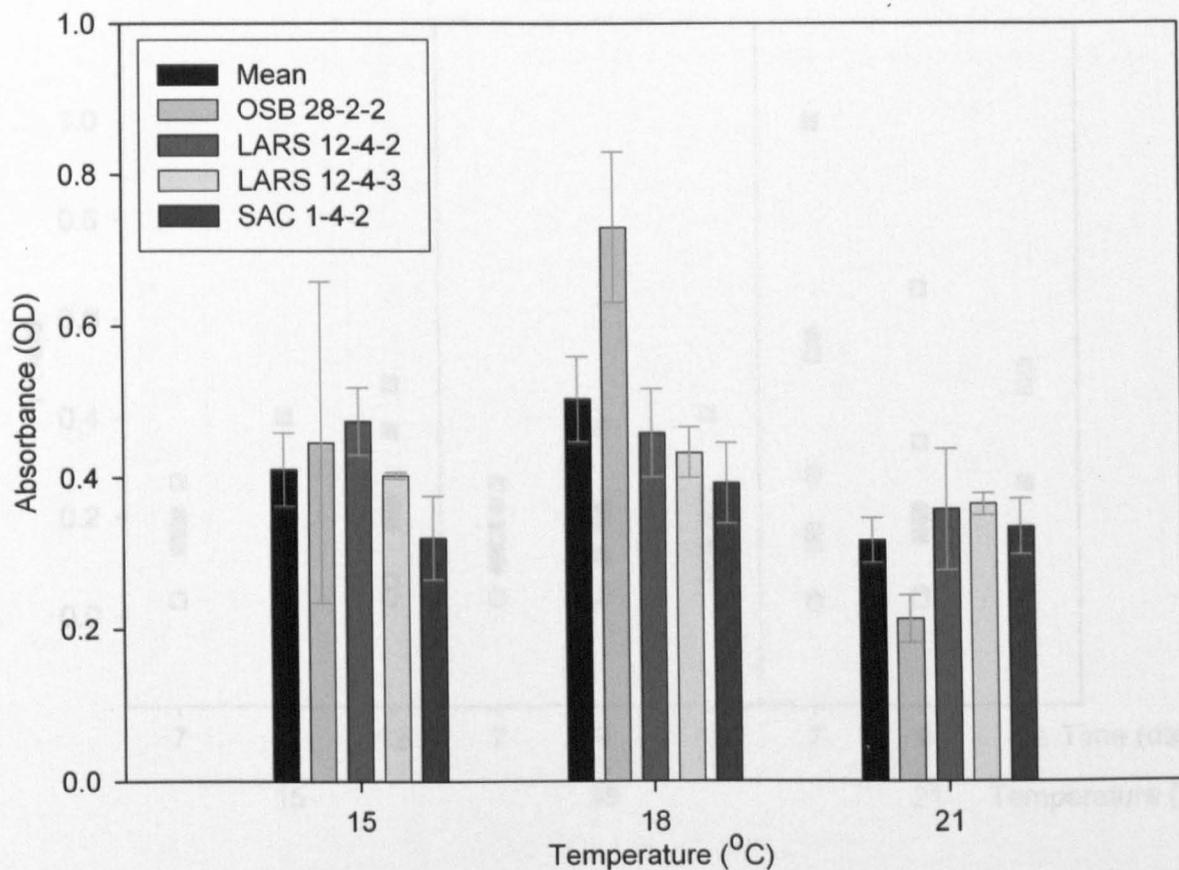
Figure 2.5. Growth of four *R. secalis* isolates at three different temperatures after 7 days, measured by optical absorbance. Black, isolate OSB 28-2-2; mid-grey, LARS 12-4-2; dark grey, LARS 12-4-3; light grey, SAC 0003 1-4-2; white, all isolates.

2.3.2.3 Inoculum density

Spore concentrations of 10^3 to 10^5 spores ml⁻¹ produced more consistent growth curves with more consistent EC₅₀ values (Figure 2.7). At lower concentrations, growth was too sparse, whereas higher concentrations resulted in excess mycelial mass before sporulation, giving elevated optical absorbance readings.

2.3.2.4 Growth characterisation, Gelatin

Initially, it appeared that a final gelatin concentration of 0.5% produced the most consistent EC₅₀ values (Figure 2.8). However, this analysis was made on only 0.5% and 1% data points for that data set, with no general pattern of response apparent across the entire gelatin concentration. When the experiment was repeated, the medium with no gelatin and the medium with 0.5% gelatin produced most consistent results.

Figure 2.6. Epoxiconazole EC_{50} values obtained for four *R. secalis* isolates at three different temperatures with readings taken at three times, with two replicates per isolate: Black, isolate OSB 28-2-2; light grey, LARS 12-4-2; dark grey, LARS 12-4-3; white, SAC 0003 1-4-2.

2.3.2.3 Inoculum density

Spore concentrations of 10^5 to 10^6 spores ml^{-1} produced better dose-response curves with more consistent EC_{50} values (Figure 2.7). At lower concentrations, growth was too sparse, whereas higher concentrations resulted in excess turbidity from inoculum material, giving elevated optical absorbance readings in the absence of any further growth.

2.3.2.4 Growth Heterogeneity: Gelatin

Initially, it appeared that a final gelatin concentration of 1% produced the most consistent EC_{50} values (Figure 2.8). However, this is solely due to the absence of outlying EC_{50} data points for that data set, with no general pattern of improved data quality around an optimum gelatin concentration. When the experiment was repeated with 0 and 1% gelatin, the assay with 0% gelatin produced more consistent results.
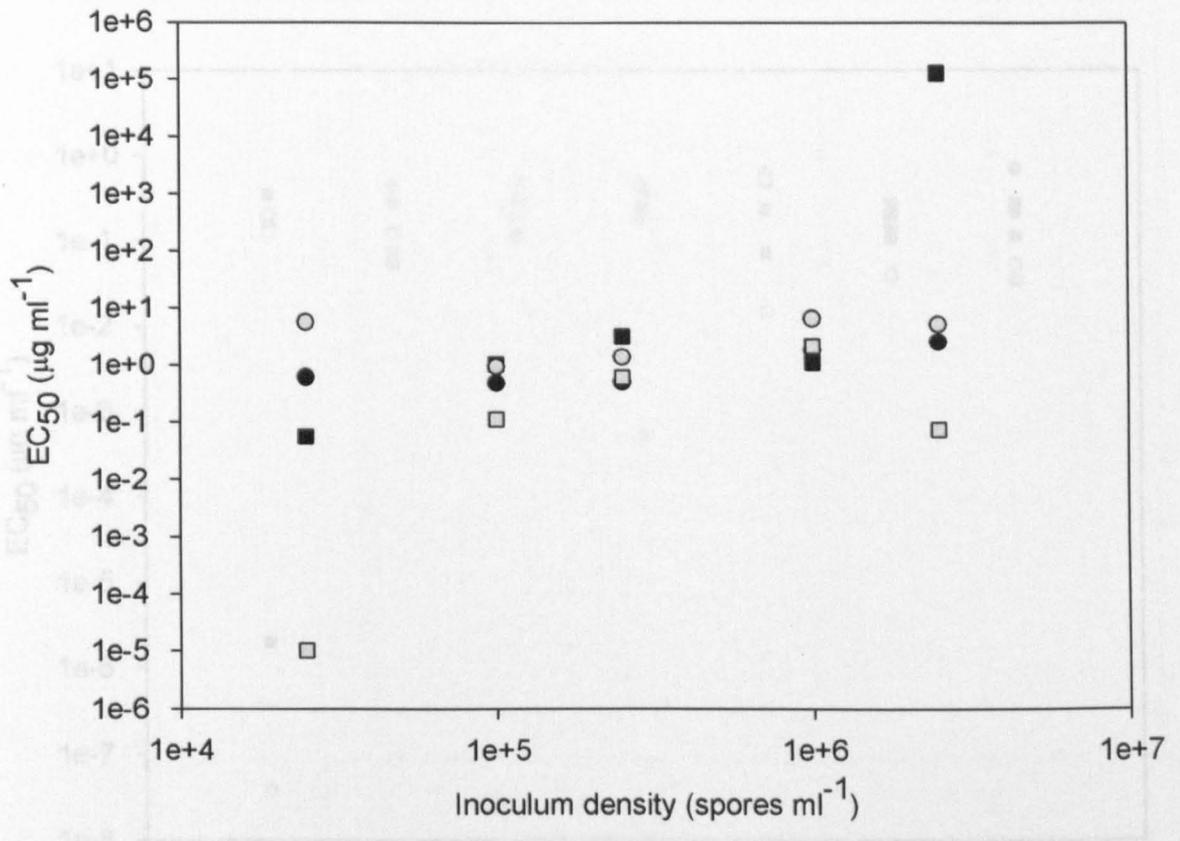
Figure 2.7. Epoxiconazole EC$_{50}$ values obtained for two *R. secalis* isolates with five different inoculum concentrations, with two replicates per isolate: Black, isolate OSB 28-2-2; grey, LARS 12-4-2.

Figure 2.8. Epoxiconazole EC_{50} values obtained for four *R. secalis* isolates in Sabouraud medium amended with different concentrations of gelatin, with two replicates per isolate: Black, isolate OSB 28-2-2; light grey, LARS 12-4-2; dark grey, LARS 12-4-3; white, SAC 0003 1-4-2. [a] Assays with 0% and 1% gelatin were carried out twice.

2.3.2.5 Growth Heterogeneity: Shaking

Shaking for 30 seconds appeared to give a slight improvement in dose-response curves and EC_{50} value consistency by breaking up some clumps of fungal growth. However, further shaking did not yield any further improvements (Figure 2.9). In subsequent assays, shaking was generally beneficial to data quality (Figure 2.10). However, in some cases, especially in prothioconazole sensitivity assays, the opposite effect was seen (Figure 2.11).
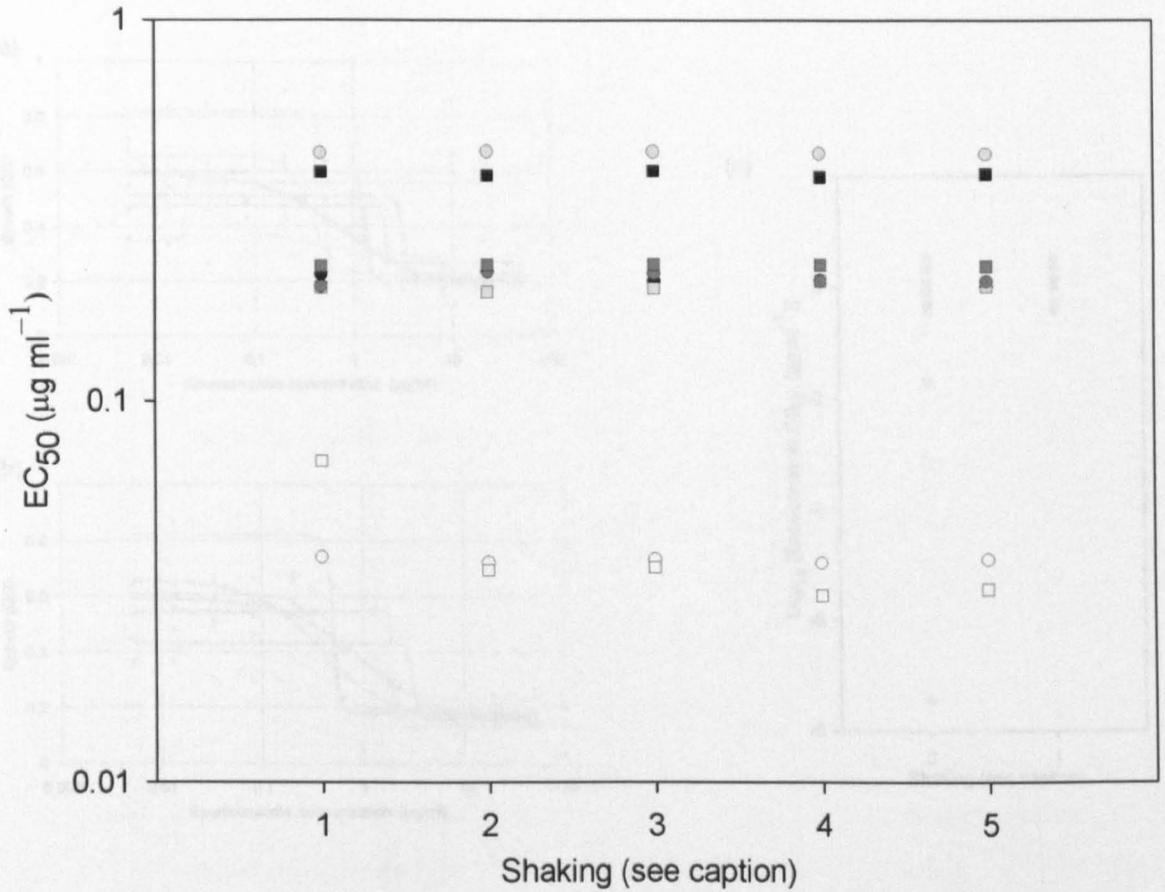
Figure 2.9. Epoxiconazole EC$_{50}$ values obtained for four *R. secalis* isolates with five different levels of shaking to disperse clumps of fungal material, with two replicates per isolate. Black, isolate OSB 28-2-2; red, LARS 12-4-2; purple, QUB 9.10; orange, QUB 30.10. Shaking used: 1, no shaking; 2, 30 seconds medium speed; 3, 2 minutes medium speed; 4, 2 minutes top speed; 5, five minutes top speed.
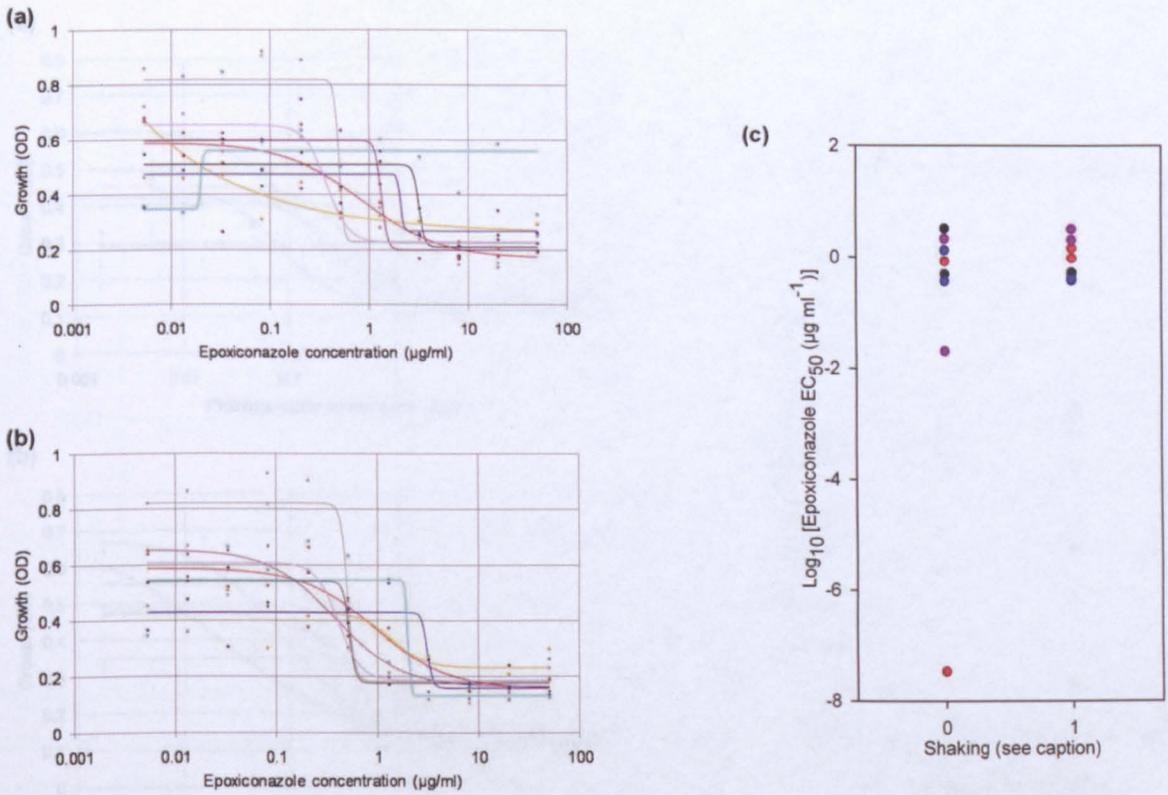
Figure 2.10. Dose-response curves of fungal growth against epoxiconazole concentration, and epoxiconazole EC$_{50}$ values, for four *R. secalis* isolates, with and without shaking the assay plates: (a) Dose-response curves obtained before shaking; (b) Dose-response curves obtained after shaking the plates for 30 seconds; (c) Comparison of EC$_{50}$ values obtained before shaking (0) and after shaking for 30 seconds (1). Black/grey: isolate OSB 28-2-2; red/orange: LARS 12-4-2; blue/light blue, LARS 12-4-3; purple/light purple, QUB 9-10.

Figure 2.11. Dose-response curves of fungal growth against prothioconazole concentration, and prothioconazole EC$_{50}$ values, for four *R. secalis* isolates, with and without shaking the assay plates: (a) Dose-response curves obtained before shaking; (b) Dose-response curves obtained after shaking; (c) Comparison of EC$_{50}$ values obtained before (0) and after shaking (1). Black/grey: isolate OSA 10-4-1; red/orange: OSA 10-4-28; blue/light blue, LARS 8-4-2.5; green/light green, OSB 24-4-47.

2.3.2.6 Fluorescence-Based Assays

Most assay plates failed to give the expected dose-response curves from fluorescence measurements. In many cases, the highest fluorescence readings were obtained for mid-range or high fungicide concentrations (Figure 2.12), whereas for slower-growing isolates, growth was insufficient for EC$_{50}$ to be determined accurately by this method (Figure 2.13).
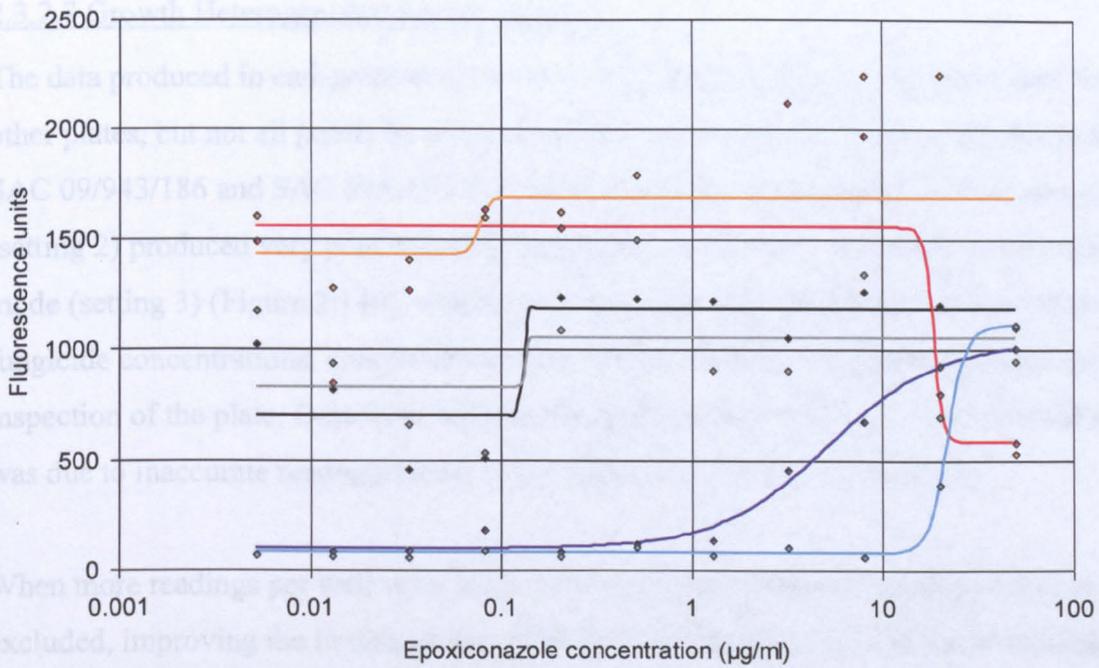
Figure 2.12. Dose-response curves of fungal growth, measured as fluorescence produced by the ingrowth indicator resazurin, against epoxiconazole concentration, for three *R. secalis* isolates. Black/grey: Isolate LARS 12-4-2; red/orange: LARS 12-4-3; blue/light blue: QUB 9-10. Fluorescence is highest for intermediate growth due to the reduction of resazurin to resorufin, and lower where growth is greatest due to the reduction of resorufin to non-fluorescent hydroresorufin.
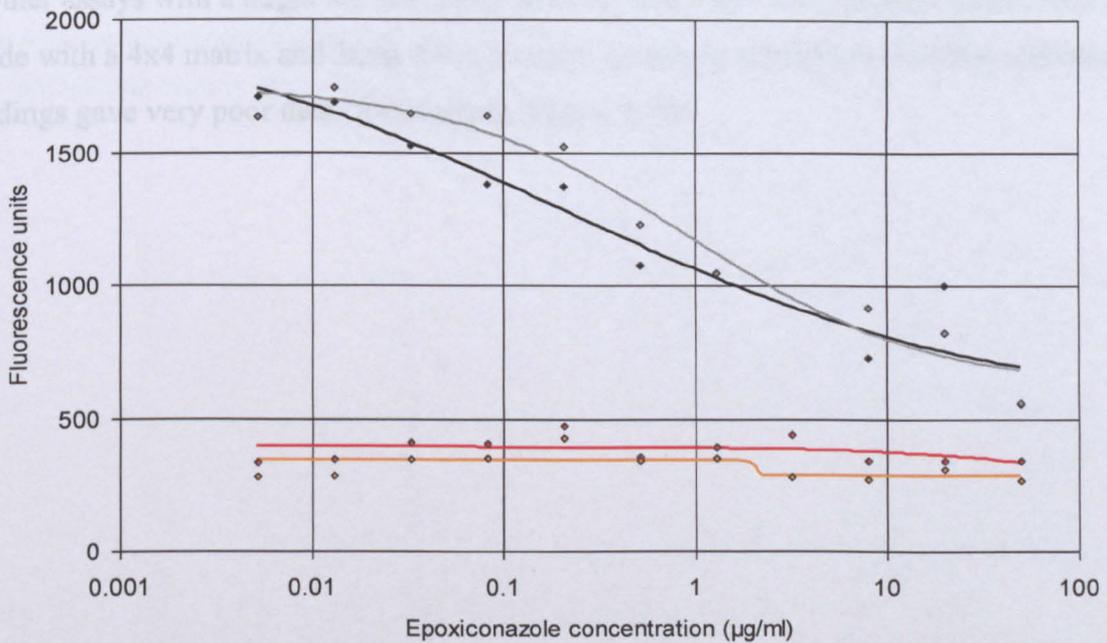
Figure 2.13. Dose-response curves of fungal growth, measured as fluorescence produced by the indicator substance resazurin, against epoxiconazole concentration, for two *R. secalis* isolates. Black/grey: Isolate R157; Red/orange: isolate RS01ch2.126. Isolate RS01ch2.126 shows insufficient growth to give a reliable EC$_{50}$ value.

2.3.2.7 Growth Heterogeneity: Reader Settings

The data produced in end-point mode when testing reader settings were better than for some other plates, but not all points fit on a smooth dose-response curve, especially for isolates SAC 09/943/186 and SAC 09/943/73 (Figure 2.14a). The reading with orbital averaging (setting 2) produced very poor data (Figure 2.14b), as did the first reading in well-scanning mode (setting 3) (Figure 2.14c), with several optical density readings increasing at the highest fungicide concentrations, contradicting what was shown by the endpoint readings and visual inspection of the plate. Data from individual reading points within the wells showed that this was due to inaccurate readings closer to the edges of the wells (Figure 2.15).

When more readings per well were taken (setting 4), the outermost readings could be excluded, improving the in data quality (Figure 2.16, Figure 2.17). Taking all readings within a smaller diameter (setting 5) produced better data still (Figure 2.18b-c, Figure 2.19). Reducing the matrix from 6x6 to 4x4 data points cut the time taken to read each plate from an hour to twenty minutes, with relatively little reduction in data quality (Figure 2.18d, Figure 2.19).

Further assays with a larger set of isolates gave consistently better readings in well-scanning mode with a 4x4 matrix and 3mm diameter than in end-point mode, even when end-point readings gave very poor data (for example, Figure 2.20).
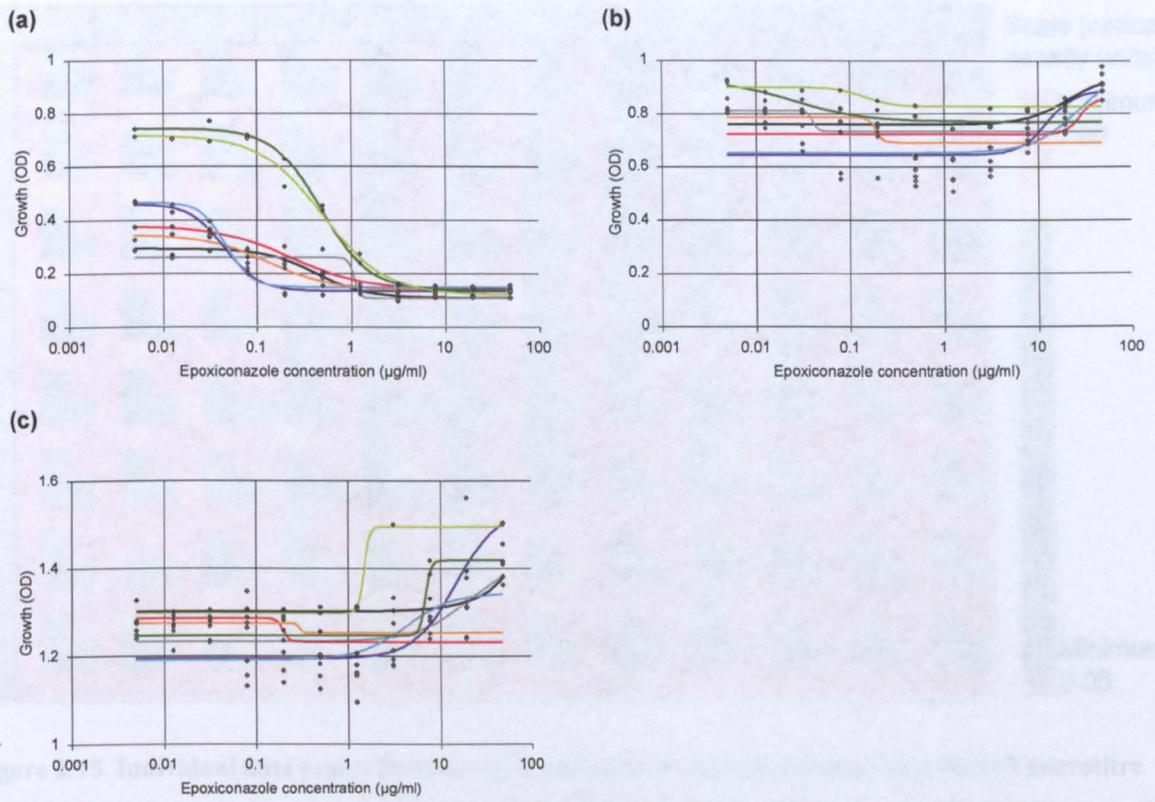
Figure 2.14. Dose-response curves of fungal growth against epoxiconazole concentration for four *R. secalis* isolates. (a) Readings obtained from a single optical density measurement per well; (b) readings obtained from 20 optical density measurments, taken in a 6mm diameter circle, per well; (c) obtained from a 3x3 matrix of optical density measurments, within a 6mm diameter, per well. Black/grey: isolate SAC 09/943/186; red/orange: SAC 09/943/73; blue/light blue, SAC 09/943/115; green/light green, RS 219.
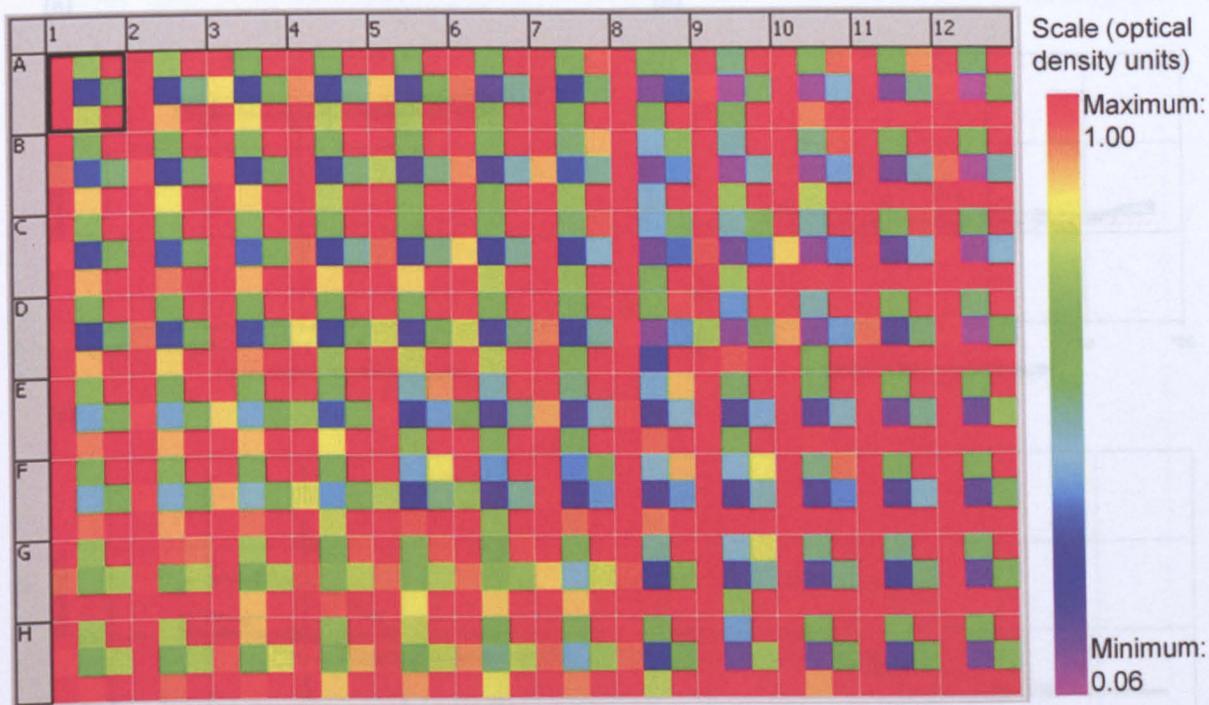
Figure 2.15. Individual data points from an epoxinconazole sensitivity bioassay in a 96-well microtitre plate, with 9 data points per well, read in well-scanning mode with a 3x3 matrix of 6mm diameter (well diameter = 6.7 mm). Columns 1-12 contain increasing epoxiconazole concentrations. Rows A-B contain *R. secalis* isolate SAC 09/943/186; C-D, isolate SAC 09/943/73; E-F, isolate SAC 09/943/115 and G-H, isolate RS 219.
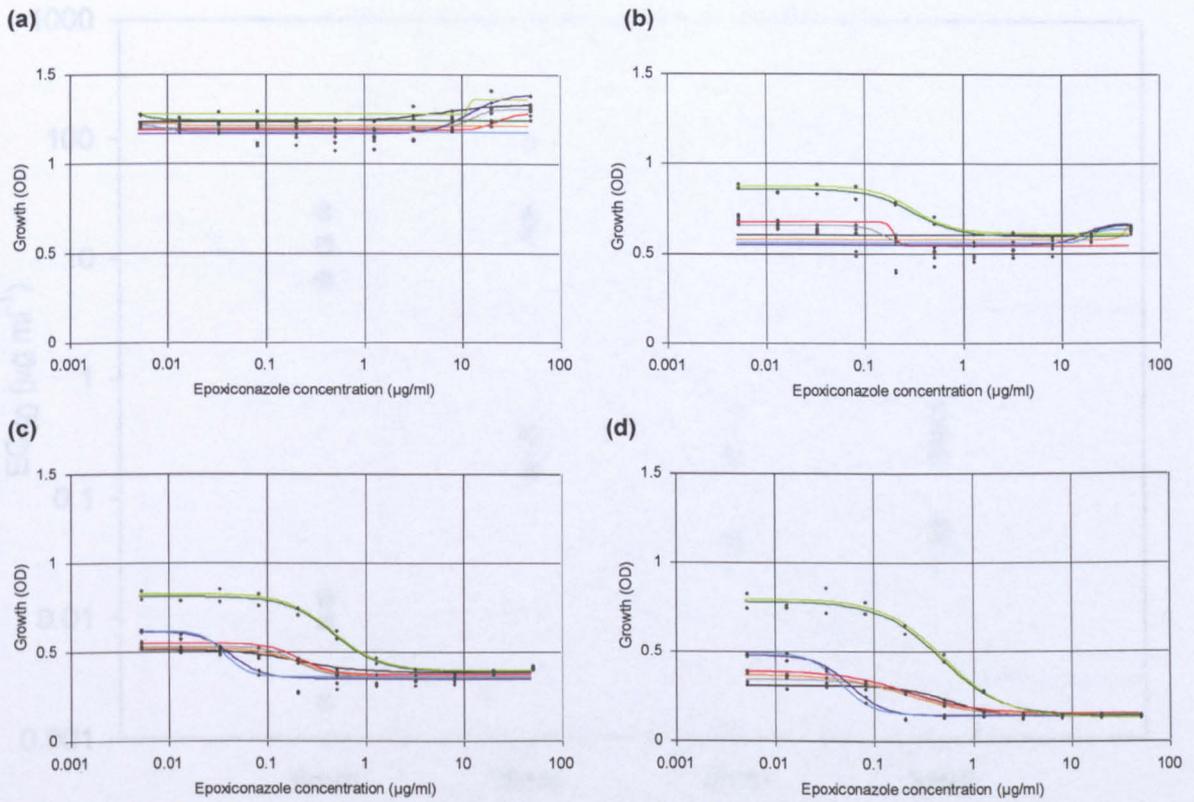
Figure 2.16. Dose-response curves of fungal growth against epoxiconazole concentration for four *R. secalis* isolates, obtained from a 6x6 matrix of optical density measurments, within a 6mm diameter, per well. (a) All readings included; (b) only readings within a 5mm diameter included; (c) only readings within a 4mm diameter included; (d) only readings within a 3mm diameter included. Black/grey: isolate SAC 09/943/186; red/orange: SAC 09/943/73; blue/light blue, SAC 09/943/115; green/light green, RS 219.

Figure 2.17. Epoxiconazole EC_{50} values for four *R. secalis* isolates with two replicates per isolate, obtained from a 6x6 matrix of optical density measurements, within a 6mm diameter, per well. (a) All readings included; (b) only readings within a 5mm diameter included; (c) only readings within a 4mm diameter included; (d) only readings within a 3mm diameter included. Black: isolate SAC 09/943/186; light grey: SAC 09/943/73; dark grey, SAC 09/943/115; white, RS 219.
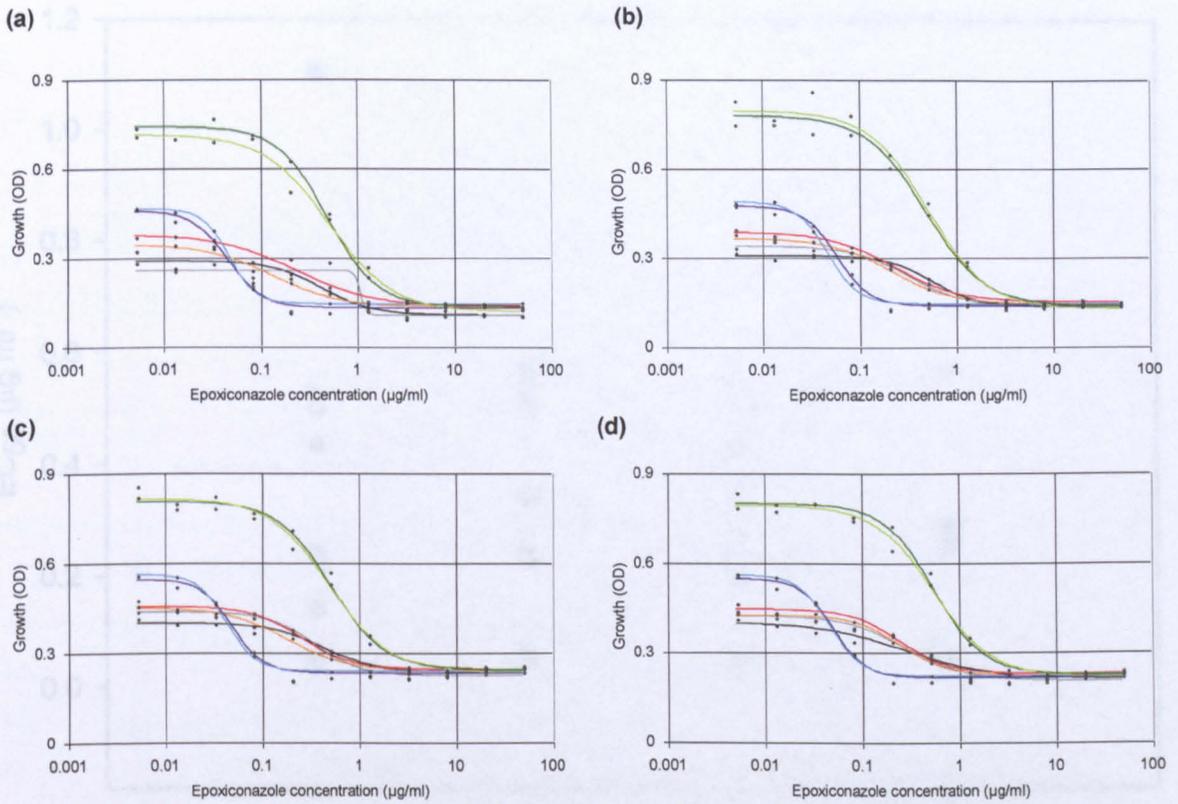
Figure 2.18. Dose-response curves of fungal growth against epoxiconazole concentration for four *R. secalis* isolates. (a) Obtained from one reading per well; (b) Obtained from a 6x6 matrix of measurements, taken within a 6mm square, per well, with only the 4 readings falling within a 3mm diameter included; (c) Obtained from a 6x6 matrix of measurements, taken within a 4mm square, per well, with only the 16 readings falling within a 3mm diameter included; (d) Obtained from a 4x4 matrix of measurements, taken within a 3mm square, per well, with only the 12 readings falling within a 3mm diameter included. Black/grey: isolate SAC 09/943/186; red/orange: SAC 09/943/73; blue/light blue, SAC 09/943/115; green/light green, RS 219.
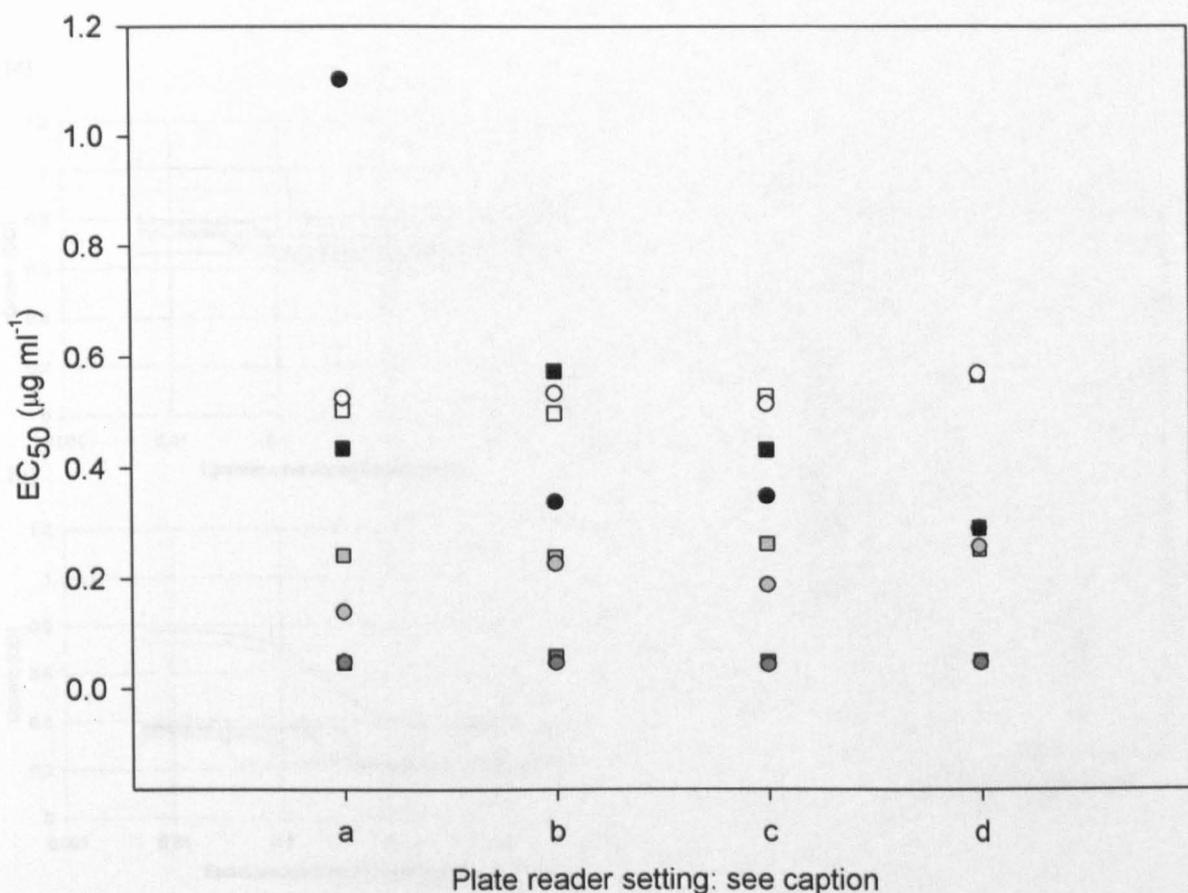
Figure 2.19. Epoxiconazole EC$_{50}$ values obtained for four *R. secalis* isolates with two replicates per isolate. (a) Obtained from one reading per well; (b) Obtained from a 6x6 matrix of measurements, taken within a 6mm, diameter per well, with only the 4 readings falling within a 3mm diameter included; (c) Obtained from a 6x6 matrix of measurements, taken within a 4mm diameter, per well, with only the 16 readings falling within a 3mm diameter included; (d) Obtained from a 4x4 matrix of measurements, taken within a 3mm diameter, per well, with only the 12 readings falling within a 3mm diameter included. Black/grey: isolate SAC 09/943/186; red/orange: SAC 09/943/73; blue/light blue, SAC 09/943/115; green/light green, RS 219.

Figure 2.20. Dose-response curves of fungal growth against epoxiconazole concentration, and epoxiconazole EC$_{50}$ values, for four *R. secalis* isolates, with optical absorbance readings taken at single and multiple points per well: (a) Dose-response curves obtained in end-point mode (one reading per well); (b) Dose-response curves obtained in well-scanning mode with 4x4 matrix and 3mm diameter (12 readings per well); (c) Comparison of EC$_{50}$ values obtained with each setting. Black/grey: isolate OSB 24-4-1; red/orange: OSA 10-4-1; blue/light blue, OSB 24-4-21; green/light green, LARS 8-4-2.5.

2.3.3 Growth curve

Fungal mass was plotted against time for the two isolates (Figure 2.21). Both isolates show a lag phase up to day 6, followed by linear growth. Some reduction in growth rate is apparent for isolate K1124 from days 12-14, whereas isolate 788 reaches stationary or declining mass after 16 days.
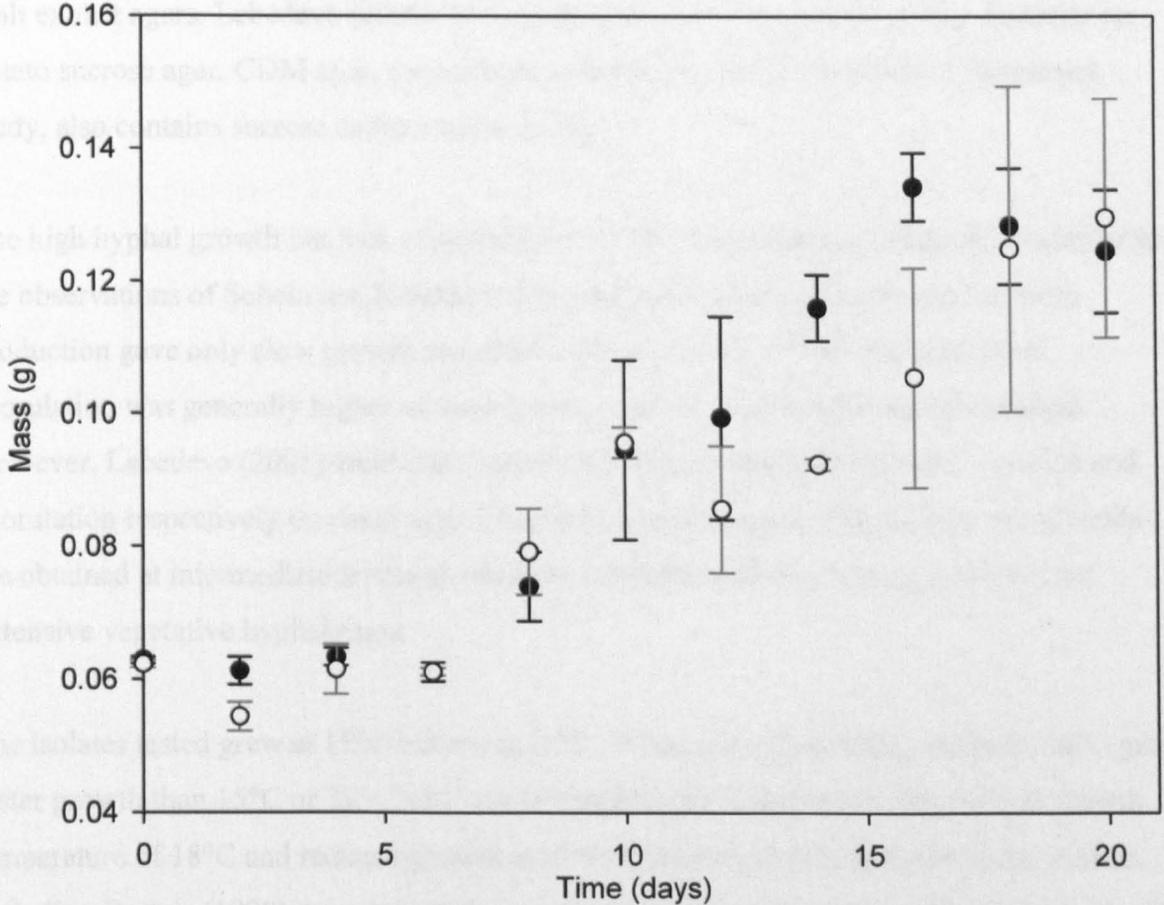
Figure 2.21. Dry mass obtained from fungal cultures at two-day intervals, for isolates 788 (black) and K1124 (white).

2.4 Discussion

2.4.1 Culture conditions

Of the media tested, CDM agar gave the most consistently high spore yields, and was selected as the growth medium. The malt yeast glucose agars, PDA and YDP agar resulted in lower total growth, whereas Sabouraud medium, and for some isolates V8 agar, resulted in extensive hyphal growth but lower sporulation. The lack of growth on PDA is in contrast with previous studies that have reported good growth on PDA, such as Caldwell (1937) who reported the fastest growth on PDA of all media tested. This may be because in the present

study the PDA was inoculated with spores that had been frozen on silica gel, resulting in additional germination requirements following prolonged dormancy. Schein and Kerelo (1956) also obtained no detectable spores on PDA, but did not mention the level of vegetative growth on this medium. Sporulation was greater on potato sucrose agar, and also on lima bean agar, with intermediate levels of sporulation on various other media including V8 and malt extract agars. Lebedeva (2005) also reported the fastest growth in colony diameter on potato sucrose agar. CDM agar, the medium with the greatest spore yields in the present study, also contains sucrose as the carbon source.

The high hyphal growth but lack of sporulation on the Sabouraud and V8 media is similar to the observations of Schein and Kerelo (1956), who noted that the best media for spore production gave only slow growth and small colonies. Owen (1958) also noted that sporulation was generally higher on media with relatively low soluble nutrient content. However, Lebedeva (2005) and Schein and Kerelo (1956) observed the lowest growth and sporulation respectively on water agar. Therefore it would appear that the best spore yields are obtained at intermediate levels of available nutrients, enabling some growth but not extensive vegetative hyphal mass.

The isolates tested grew at 15°C but not at 21°C. When comparing assay methods, 18°C gave faster growth than 15°C or 21°C, and was selected for all further work. An optimal growth temperature of 18°C and reduced growth at 21°C is consistent with many previous studies, including Bartels (1928), who reported an optimal growth temperature of 19-21°C, Caldwell (1937), who reported an optimum temperature for germination and germ-tube elongation of 18-21°C and a rapid reduction in growth as temperature increased above 21°C, and Lebedeva (2005), who reported maximum *in vitro* growth in colony diameter of *R. secalis* isolates from barley at 15-20°C. Cooke *et al.* (2004) and Oxley *et al.* (2006) also used an incubation temperature of 18°C. Skoropad (1959) reported an optimum temperature of 16°C for seedling infection from seed, with a rapid reduction above 20°C. The lower optimum temperature in this case may reflect faster growth of the seedling at higher temperatures, allowing early disease escape.

The four isolates used in initial tests sporulated within seven days of inoculation, but slower-growing isolates required 10 days. A 10-day incubation time was selected to allow a consistent protocol to be used for all isolates.

Spore yields for some isolates fluctuated greatly with subculturing, with low yields sometimes obtained from cultures inoculated with a high concentration of spores. Therefore, the effect of inoculum concentration on spore density was investigated.

Spore yields for all isolates increased with inoculum density for concentrations up to 2.5 x 10^6 spores ml^{-1}, but plateaued or decreased at higher inoculum densities. This was consistent with the appearance of the cultures: at lower inoculum concentrations, growth was limited to separate colonies with unutilised space between them; at 2.5 x 10^6 spores ml^{-1}, the surface was mostly covered but colonies remained separate; and at the highest inoculum concentration, the agar surface was covered with continuous dense hyphal growth but with lower sporulation levels. Furthermore, higher inoculum levels produced more hyphal material, which increased the turbidity of the resulting concentration-adjusted spore suspension for use in sensitivity bioassays, giving elevated optical absorbance readings in the absence of any further growth. Therefore an inoculum density equivalent to 50μl per 90mm plate at 2.5 x 10^6 spores ml^{-1} was selected for all future work, as it produced consistently high spore yields for both replicates of all four isolates. Consequently, isolates stored in silica gel at -80°C are first grown from those stocks, and then subcultured at the optimal inoculum density.

Previous publications have not addressed the impact of inoculum density on spore yields, although Schein and Kerelo (1956) mentioned that high spore yields were obtained using 'a zigzag smear technique', which would reduce overcrowding and ensure most growth took place close to the edge of the streak of fungal mass. Ayres and Owen (1969) suggested that spores may produce a 'self-inhibitor of germination', preventing germination when spores are present at high density, such as within a sporulating lesion on crop debris. However, in the present study, germination did not appear to be reduced. In fact, dense hyphal growth was produced, but with lower spore yields, similar to growth on some richer media. It is not clear whether this is due to later allelopathic effects or resource depletion by the crowded mycelium, or a response to conditions resembling *in planta* conditions during pre-sporulation stages in the infection process.

2.4.2 Assay method

To measure fungal growth in a sensitivity assay, total growth, rather than spore production, should be maximised. Growth rate was greatest in Sabouraud medium, resulting in the most consistent EC_{50} values between replicates. Therefore, Sabouraud medium was selected as the

first choice of assay medium for future assays, with the caveat that a very rich medium may be unsuitable for some fungicides. For example, anilinopyrimidines may be less effective when the medium contains a rich source of methionine (Masner *et al.* 1994), and SDHI effectiveness *in vitro* may depend on the carbon source (Shima *et al.* 2009).

Growth after seven days was greatest at 18°C for three of the four isolates, and readings taken after seven days produced the most consistent EC_{50} values at 18°C. Therefore, incubation at 18°C for seven days is optimal, which is consistent with previous studies, as previously discussed. Inoculum concentrations within the range of 10^5 to 10^6 spores ml^{-1} gave the most consistent EC_{50} values. Therefore an inoculum concentration of 2.5×10^5 was selected for future assays.

Despite the optimisation of growth medium, temperature, time and inoculum level, data quality was still excessively variable, due to the heterogeneous growth of *R. secalis* in liquid culture. Gelatin-amended growth medium, as described by Havis (2006), did not result in a consistent improvement in data quality. Furthermore, when plates were shaken, gelatin-amended medium formed large air bubbles, resulting in inaccurate optical absorbance readings. Therefore, gelatin was not used in any further tests. Shaking for 30 seconds slightly improved the data quality by breaking up some clumps of fungal growth, but further shaking gave no further improvements. Therefore shaking for 30 seconds at medium speed was selected for further assays. When further assays were carried out, in many cases shaking was beneficial to data quality, visibly dispersing clumps of fungal material and reducing corresponding anomalous data points. However, in other cases, the opposite effect was seen, with fungal material being loosened from the well bottom. This was most common in prothioconazole sensitivity assays, as fungal growth tended to form a thin film across the bottom of the well, which loosened and crumpled when the plates were shaken.

A fluorescence-based method was also tried, using the metabolic indicator resazurin. Whilst a colour gradient was observed on many plates after incubation with resazurin, most failed to give the expected dose-response curves from fluorescence measurements. In many cases, the highest fluorescence readings were obtained at mid-range or higher fungicide concentrations. This is because resazurin undergoes two reduction reactions. It is reduced first to resorufin, the fluorescent product, but then to hydroresorufin, which is non-fluorescent (O'Brien *et al.* 2000). Therefore, to use resazurin to obtain EC_{50} values, it would

be necessary to find an incubation time at which the first reaction, but not the second, will take place in fungicide concentrations where growth has been inhibited by less than 50% for all isolates. However, preliminary tests found that for the range of isolates being tested here, variation in growth rates is such that no one timing would be universally acceptable: either the slower-growing isolates will have insufficient growth for EC_{50} to be determined accurately (Figure 2.12), or the faster-growing isolates will reduce the resazurin to hydroresorufin at the lowest fungicide concentrations (Figure 2.13).

In all assays discussed so far, the plate reader (Optima Fluostar, BMG Labtech, Germany) had been used in end-point mode, taking a single reading per well. This produces good results for *M. graminicola* (for example, Bean *et al.* 2009), which gives homogeneous yeast-like growth in suitable culture conditions, but for the more heterogeneous growth of *R. secalis*, multiple readings per well can be made.

Taking multiple readings per plate, using orbital-averaging or well-scanning settings on the plate reader, initially produced very poor data due to inaccurate readings close to the edge of each well. However, reducing the diameter within which readings were taken to 3mm clearly improved the data quality, with readings lying closer to a smooth yield-density curve, resulting in more consistent EC_{50} values. This may be due to optical effects from the well walls or differences in fungal growth at the edge of the well. The 3mm diameter is similar to that scanned by the more diffuse light sources of some other plates readers (Pijls *et al.* 1994).

Within a 3mm diameter, taking multiple readings per well improved data quality, as a more representative sample of the fungal growth was measured and chance effects of whether a single reading point fell within or between fungal colonies was reduced. Increasing the matrix of measurement points per well from a single point, to a 2x2, 3x3 then 4x4 matrix, gave successive improvements in data quality, with smoother yield-density curves and more consistent readings between replicates. However, increasing the matrix from 4x4 to 6x6 data points increased the time taken to read each plate from twenty minutes to an hour, with relatively little further improvement in data quality.

Therefore, well-scanning mode was selected for use in all further assays. A 4x4 matrix gave a suitable balance between data quality and read time, and reading within a 3mm diameter gave sufficient coverage to allow for growth heterogeneity while avoiding inaccurate readings

from around the well edges. When well-scanning was used, shaking plates was no longer necessary. The assay plate used to test these settings produced relatively good data even in end-point mode, but further assays with a wider range of isolates and fungicides gave consistently better readings in well-scanning mode (with a 4x4 matrix and 3mm diameter) than in end-point mode, particularly when end-point readings gave very poor data due to patchy growth. The final assay protocol is described in Section 3.2.1.

2.4.3 Growth curve

When testing liquid media for the fungicide sensitivity assay, growth was highest in Sabouraud medium, and so it was selected for growing liquid cultures for nucleic acid extraction. Similarly, a temperature of 18°C was selected based on previously discussed data.

A lag phase of growth was observed up to day 6 for both isolates. Therefore for gene expression analyses, any treatments should be applied and samples taken from day 7 onwards, once the culture has entered linear growth. This is consistent with the observations of Caldwell (1937) that *R. secalis* growing in culture takes 4-5 days "to become macroscopically evident". An upper time limit of 10-12 days ensures that cultures are in linear growth, and not entering a stationary or declining phase. Lebedeva (2005) also observed a lower growth rate in colony diameter at 14-21 days compared to 0-14 days. Furthermore, an upper time limit of ten days for culture growth for nucleic acid extractions avoided the accumulation of putative secondary metabolites, which results in a melanised appearance, correlated with poor PCR performance of extracted DNA, for older cultures. The melanisation of older cultures was also described by Schein and Kerelo (1956) and Owen (1958), with the age at which cultures become melanised depending on the growth media used.

Chapter 3

QoI fungicide sensitivity in *R. secalis*

3.1 Introduction

The Quinone Outside Inhibitors (QoIs), including the strobilurins, inhibit cytochrome *b* in the electron transfer chain of mitochondrial respiration. Target-site resistance has been found in many species (FRAC 2010b), often due to the G143A substitution. Isolates of *B. graminis* f.sp. *tritici* with a G143A-encoding mutation and a 200-fold reduction in QoI sensitivity were found in 1998 (Sierotzki *et al.* 2000b), but no target-site resistance was reported in *R. secalis* for the following ten years (Torriani *et al.* 2009a). In 2008, target-site resistance to QoIs in *R. secalis* was reported in field isolates from one site in France, and this was found to be due to the G143A substitution (FRAC QoI Working Group 2008). So far this has not been found in the UK, but alternative oxidase (AOX) activity has been implicated in the reduced *in vitro* QoI sensitivity of some isolates (McCartney 2006).

AOX can act as an alternative electron acceptor from ubiquinone, circumventing cytochrome *b* (Joseph-Horne and Hollomon 2000). However, its role *in planta* is not clear, as it may be inhibited by plant secondary metabolites, or the reduced ATP generation resulting from the alternative respiratory pathway may be insufficient for some stages of fungal growth (Wood and Hollomon 2003). McCartney (2006) reported a ten-fold reduction in QoI sensitivity of some *R. secalis* isolates *in vitro* when AOX inhibitors were added, but this was not investigated at the molecular level. *AOX* expression in *M. oryzae* was induced following the addition of a QoI fungicide (Yukioka *et al.* 1998), and inter-specific differences in QoI sensitivity between *F. graminearum* and *M. nivale* correlated with induced expression of *AOX* (Kaneko and Ishii 2009), but so far there are no published reports of intraspecific differences in *AOX* expression in relation to QoI fungicide sensitivity.

Huh and Kang (2001) reported the presence of two *AOX* paralogues in *C. albicans*. One paralogue was expressed constitutively. Expression of the other was induced by complex III inhibitors, and varied with growth stage. Tanton *et al.* (2003) reported the presence of two *AOX* paralogues in *N. crassa*. One paralogue was constitutively expressed at a low level, and increased expression was induced by addition of a complex III inhibitor; the other was not expressed under the conditions used. McDonald and Vanlerberghe (2006) list multiple *AOX*

genes in several other fungal species, including the leotiomycete *B. cinerea*. It is not clear whether these represent independent duplications within a few lineages, or a basal duplication into two fungal *AOX* paralogues.

This chapter describes QoI sensitivity testing in *R. secalis*, and the effect of adding the AOX inhibitor SHAM. The *cytochrome b* QoI target site encoding gene was sequenced for a selection of isolates with a range of QoI sensitivities. The *R. secalis AOX* gene was identified, and phylogenetic analyses were carried out to investigate the occurrence of multiple fungal *AOX* paralogues. The *AOX* gene was sequenced for a selection of *R. secalis* isolates, and expression levels analysed.

3.2 Materials and Methods

3.2.1 Sensitivity testing

Isolates used for initial tests comprised reference isolates from a range of locations, and field isolates collected by J. Fountaine from field trials in 2001-2002, stored in silica gel at -80°C. Additional isolates were obtained from Syngenta Crop Protection, the Scottish Agricultural College and BASF. Isolates used are shown in Table 2.1.

Sensitivity testing was carried out using the method developed in Chapter 2. In brief, isolates were grown on Czapek Dox agar with mycological peptone (Czapek Dox broth mix 33.25 g l^{-1}, mycological peptone 5 g l^{-1}, agar 20 g l^{-1}), with an inoculum density of 1.25 x 10^5 spores per 90mm Petri dish, at 18°C for 10 days. Spores were harvested and suspended in sterile distilled water. Fungicide sensitivity assays were carried out in 96-well microtitre plates (TPP, Switzerland). Fungicide-amended 2x Sabouraud medium (100µl) and concentration-adjusted spore suspension (100µl at 2.5 x 10^5 spores ml^{-1}) was added to each well. Columns 1-12 contained increasing concentrations of technical-grade azoxystrobin, as shown in Table 3.1. Rows A-H comprised two pseudo-replicate rows for each of four isolates. Each assay was repeated with 10µl ml^{-1} of 8mg ml^{-1} SHAM (salicylhydroxamic acid; Sigma-Aldrich, Germany) solution in DMSO added to the medium, to give a final concentration of 40 µg ml^{-1} SHAM, to inhibit AOX activity. Plates were sealed with parafilm and incubated in darkness at 18°C for seven days.

Table 3.1. Final fungicide concentrations used in strobilurin sensitivity bioassays, carried out in 96-well microtitre plates.

Microtitre plate column	1	2	3	4	5	6	7	8	9	10	11	12
Final azoxystrobin or pyraclostrobin concentration ($\mu g\ ml^{-1}$) (3 s.f.)	0	0.00169	0.00508	0.0152	0.0457	0.137	0.412	1.235	3.70	11.1	33.3	100

After 7 days, fungal growth was measured by optical absorbance at 620nm using an Optima Fluostar plate reader (BMG Labtech, Germany), in well-scanning mode with a 4x4 matrix of scanning points within a 3mm diameter. The accompanying software was used to fit a dose-response curve (4-parameter fit) and calculate EC_{50} values. Correlation between EC_{50} values with and without SHAM, and between QoI and triazole sensitivity (chapter 4), was tested by least squares linear regression analysis in GenStat 13th Edition (VSN International, Hertfordshire, UK).

3.2.2 SHAM matrix assays

The assay procedure was carried out as described above, but with a single isolate per plate. Spore suspensions were diluted to 5×10^5 spores ml^{-1}, and 50μl was added to each well. SHAM was dissolved in DMSO at 432 mg ml^{-1}, then 10 μl ml^{-1} was added to sterile distilled water. Six serial dilutions were carried out at a dilution factor of 1/3, and 50μl was added to each well to give the following final concentrations: 0μg ml^{-1} (50μl distilled water only), then 1.48 μg ml^{-1}, 4.44 μg ml^{-1}, 13.3μg ml^{-1}, 40 μg ml^{-1}, 120 μg ml^{-1}, 360 μg ml^{-1} and 1080 μg ml^{-1}. Isolates SAC 09/943/186, SAC 09/943/73, SAC 09/943/115 and RS 219 were tested. Plates were incubated and read as described in section 3.2.1.

The assay was then carried out for isolates R.s. 2310 4.2, R.s. 2313 4.2 and R.s. 2314 4.2. SHAM was dissolved in DMSO at 16 mg ml^{-1}, then 10 μl ml^{-1} was added to sterile distilled water, and six serial dilutions carried out at a dilution factor of 1/3, to give the following final concentrations: two rows at 0μg ml^{-1} (50μl distilled water only), then one row each at 0.123 μg ml^{-1}, 0.494 μg ml^{-1}, 1.48 μg ml^{-1}, 4.44 μg ml^{-1}, 13.3 μg ml^{-1} and 40μg ml^{-1}. A subsequent experiment tested pyraclostrobin sensitivity of isolates R.s. 2310 4.2, R.s. 2313 4.2, R.s. 2314 4.2 and R.s. 2318 4.2 with the same concentrations of SHAM and fungicide, in order to

obtain EC_{50} values for the azoxystrobin-resistant isolates, since pyraclostrobin has greater intrinsic activity than azoxystrobin.

3.2.3 DNA extraction

Isolates QUB 30-10, 788, K1124, Rs 9528.4, GKII 18-2-3, RS 219, RS 783, R.s. 2310 4.2, R.s. 2318 4.2, R.s. 2313 4.2 and R.s. 2314 4.2 were selected for DNA extraction and sequencing as they had a range of QoI sensitivities.

Isolates were grown in Sabouraud liquid medium for ten days. Fungal material was harvested by filtration and freeze-dried. DNA was extracted by grinding for 45 seconds at 5 m/s in a FastPrep-24 homogeniser (MP Biomedicals, Ohio) with 700µl DNA extraction buffer (95ml 2x TEN (400mM Trizma hydrochloride, 50mM EDTA, 500mM sodium chloride), 95ml 2% SDS, 0.39g phenanthroline monohydrate $C_{18}H_8N \cdot H_2O$ (Sigma), 4.0g polyvinylpyrrolidone (Sigma), 9.5ml 1% β-mercaptoethanol). Samples were incubated at 65°C for 20 minutes, 350µl ice-cold ammonium acetate was added, samples incubated on ice for 20 minutes then centrifuged at 13200 rpm for 15 minutes. The supernatant was added to 900µl ice-cold isopropanol, incubated at room temperature for 15 minutes and centrifuged for 15 minutes. The supernatant was discarded, the pellet washed twice with 400µl 70% ethanol, dried on a heat block at 65°C and re-suspended in 200µl sterile distilled water. DNA was quantified with a NanoDrop spectrophotometer (NanoDrop products, Detroit), then diluted to 20ng µl^{-1} in sterile distilled water.

3.2.4 Target site sequence analysis

A PCR reaction was carried out using Red Hot Taq (ABgene, Epsom, UK) according to manufacturer's instructions, with 1.5mM $MgCl_2$, 0.2µM dNTPs, 0.2µM primers and 2ng µl^{-1} template, with *cytochrome b* primers Forwards 1 and Reverse 1 (Table 3.2). Primers were designed in Vector NTI (Invitrogen Corporation), based on the *cytochrome b* sequence of *R. secalis* isolate Sy1-14. The PCR programme comprised 2 minutes at 94°C; followed by 40 cycles of 30 seconds at 94°C, 1 minute at 58°C and 90 seconds at 72°C; followed by 5 minutes at 72°C.

Table 3.2. Primers used for amplification and expression analysis of *cytochrome b* and *AOX* genes of *R. secalis*

Gene	Primer name	Primer sequence
cytochrome b	Forward 1	5'-CGATTCACCACAACCATCTAATCTAAG-3'
	Reverse 1	5'-AAGGCGAAGAATAGTCTATTGACGTGTT-3'
AOX	Forward 1	5'-GAACACCAGGTTTGACTCCCAATCAT-3'
	Reverse 1	5'-CATCTTAGTCTTTCCGTCGTGTTCAT-3'
	Expression F	5'-TCTCCACAACACCTCGAACACAACTA-3'
	Expression R	5'-AAGGCTTGGTTTCCGTAACAGCATT-3'
β-tubulin (RT-qPCR endogenous control)	Expression F	5'-GTGCAGTCACTGTTCCAGAGTTGACC -3'
	Expression R	5'-GCGGTTTGGACATTGGTGGG -3'

Products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Wisconsin, USA), eluting into 50µl sterile distilled water. Purified PCR products were ligated into the pGEM-t Easy plasmid vector (Promega) using T4 DNA ligase (Promega) according to manufacturer's instructions. JM109 competent cells (Promega) were transformed according to manufacturer's instructions, using blue-white screening for plasmids with inserts. White transformant colonies were suspended in 50µl sterile distilled water. PCR reactions to screen colonies were set up as described above, with 10% volume transformant suspension as template. The PCR programme started with 2.5 minutes at 94°C to lyse the bacterial cells, followed by 40 cycles of 30 seconds at 94°C, 60 seconds at 58°C and 60 seconds at 72°C, followed by 5.5 minutes at 72°C. One transformant for each PCR product was selected for sequencing. The bacterial suspension was added to 5ml LB broth and incubated overnight at 37°C with shaking. Plasmid DNA was then extracted and purified using the GeneElute (Sigma) or QIASpin (Qiagen) mini-prep kits, according to pack instructions. DNA samples were sequenced by Eurofins MWG (Germany). Sequences were assembled in the ContigExpress module of Vector NTI (Invitrogen Corporation), vector sequence was removed and amino acid sequences predicted in Vector NTI, and sequences aligned using the ClustalW algorithm implemented in the AlignX module of Vector NTI.

3.2.5 *AOX* sequence analysis

A text search was carried out for *AOX* and "Alternative Oxidase" on GenBank (http://www.ncbi.nlm.nih.gov/genbank/), within the Leotiomycetes (Goodwin 2002), followed by BLAST cross-searching. A tBLASTn search of the translated *S. sclerotiorum*, *B. cinerea* and *B. graminis* f.sp. *tritici* sequences was carried out against the *R. secalis* genome currently in preparation at the Scottish Crop Research Institute (Anna Avrova, personal communication).

The *R. secalis AOX* gene was amplified from genomic DNA, extracted as described in section 3.2.3, for isolates K1124, RS 783 and SCRI 13-13. Additionally, for isolates K1124 and RS 783, *AOX* was amplified from cDNA, synthesised as described in section 3.2.6. PCR reactions were carried out using Easy-A High-Fidelity PCR Cloning Enzyme (Stratagene, California, USA) according to manufacturer's instructions, with 50 ng genomic or 1% cDNA template. The PCR programme comprised 2 minutes at 95°C; followed by 30 cycles of 40 seconds at 95°C, 30 seconds at 65°C and 2 minutes at 72°C; followed by 7 minutes at 72°C. PCR was carried out with primers *AOX* Forward 1 and Reverse 1 (Table 3.2). Primers were designed based on the *AOX* BLAST match from the *R. secalis* genome (Section 3.3.4) in Vector NTI (Invitrogen Corporation). PCR products were purified, cloned and sequenced as described in section 3.2.4.

Sequences were assembled in the ContigExpress module of Vector NTI (Invitrogen Corporation), vector sequence was removed and amino acid sequences predicted in Vector NTI, and sequences aligned using the ClustalW and MUSCLE algorithms implemented in BioEdit (Hall 1999). The *R. secalis* AOX predicted amino acid sequence was analysed for possible mitochondrial import using MitoProt II (Claros and Vincens 1996).

Phylogenetic analyses were carried out with *R. secalis AOX* sequences and the *AOX* sequences from GenBank, along with *AOX* sequences from selected fungal genomes, as shown in Figure 3.8 - Figure 3.10. tBLASTn searches were carried out on fungal genomes using the predicted protein sequences of both *C. albicans* AOX paralogues as query sequences. Amino acid sequences were aligned using the ClustalW algorithm implemented in BioEdit (Hall 1999). Corresponding alignments of coding nucleotide sequences were created in PAL2NAL (Suyama *et al.* 2006). Neighbour-joining and maximum parsimony trees were calculated from the PAL2NAL alignment of coding DNA sequences in PAUP* 4.0beta

(Swofford 1991), with 100 bootstrap replicates. A maximum likelihood tree was reconstructed from the coding DNA sequence alignment using the CodeML programme implemented in TOPALi, with the GTR+I+G substitution model selected according to the Akaike Information Criterion, and 1000 bootstrap runs. Bayesian analysis was carried out in MrBayes, with the GTR+I+G model, and other parameters on default settings including a random starting tree. A two-chain MCMC analysis was carried out for 500,000 generations, sampling every 100 generations, with a burn-in of 1250 samples, and a 50% strict consensus tree was constructed.

3.2.6 *AOX* expression analysis

To investigate constitutive and induced expression, isolates 788, K1124, RS 783, R.s. 2310 4.2, R.s. 2313 4.2, R.s. 2314 4.2 and R.s. 2318 4.2 were selected to cover a range of QoI sensitivities. Isolates were grown in 100ml Sabouraud liquid medium at 2.5×10^4 spores ml^{-1} in an orbital shaker at 150 rpm at 20°C, with two flasks per isolate. Azoxystrobin sensitivity assays with 0 and 40 μg ml^{-1} SHAM were set up as described above using the same inoculum. After seven days, 10μl of 10mg ml^{-1} azoxystrobin in acetone, giving a final azoxystrobin concentration of 1μg ml^{-1}, was added to one flask of each isolate, and 10μl of acetone only was added to the other flask. The cultures were harvested three days later.

Induced expression was then measured over a shorter time after fungicide addition. To investigate induced expression, it was not possible to add a concentration of azoxystrobin that would inhibit cytochrome *b* in isolates carrying the G143A substitution without killing all other isolates, so four pre-2008 isolates with a range of QoI sensitivities were used. Isolates 788, K1124, R 9528.4 and RS 783 were grown as described. After seven days, one flask per isolate was harvested before adding azoxystrobin. The remaining flasks were harvested 30 minutes, 1 hour, 2 hours and 4 hours after adding the fungicide.

Fungal material was harvested by vacuum filtration, immediately placed into liquid nitrogen then stored at -80°C before freeze-drying. RNA extraction was carried out with TRIzol Reagent (Invitrogen, California, USA), according to manufacturer's instructions, using a tissue homogeniser for 2 minutes, with two phase separation steps, and resuspending the pellet in 50μl TE. An equal volume of 8M lithium chloride was added, the mixture was incubated overnight at 4°C, then centrifuged at 4°C for 30 minutes and the supernatant discarded. The RNA pellet was washed with 70% ethanol, dried at room temperature,

resuspended in RNase-free water and stored at -80°C. The RNA was treated with the TURBO DNA-free Kit (Applied Biosystems, California, USA). cDNA was synthesised with the Superscript III first-strand synthesis system (Invitrogen, California, USA). End-point PCRs were carried out with Red Hot Taq as described in section 3.2.4 with *AOX* primers Expression F and Expression R (Table 3.2) to check for genomic DNA contamination.

Quantitative PCR was carried out using SYBR Green JumpStart Taq ReadyMix (Sigma), in 25µl reactions with 0.25µM primers and 0.2% cDNA template, with the 7500 Real Time PCR System (Applied Biosystems). *AOX* and *β-tubulin* primers Expression F and Expression R were used (Table 3.2), with *β-tubulin* used as the endogenous control. Primers were designed in Vector NTI (Invitrogen Corporation), based on the *AOX* BLAST match from the *R. secalis* genome (Section 3.3.4) and an *R. secalis β-tubulin* sequence provided by Bart Fraaije (unpublished data). The PCR cycle comprised 2 minutes at 95°C; followed by 40 cycles of 15 seconds at 95°C, 30 seconds at 58°C and 36 seconds at 72°C at which point the reading was taken; followed by a dissociation cycle of 15 seconds at 95°C, 1 minute at 60°C and 15 seconds at 95°C.

Results were analysed using the 7500 System software (Applied Biosystems). Relative gene expression was calculated as $2^{-\Delta\Delta C_T}$ (Livak and Schmittgen 2001), where C_T is the threshold cycle number and $\Delta\Delta C_T$ is the target C_T relative to the endogenous control gene and a calibrator sample. In this case *β-tubulin* was used as the endogenous control gene, and isolate 788 without fungicide was the calibrator sample for each experiment. Three technical replicates were carried out for each reaction.

SHAM potentiation was calculated as

$$P_{SHAM} = \frac{EC_{50}(Azoxystrobin)_{alone}}{EC_{50}(Azoxystrobin)_{+40\ \mu g\ ml^{-1}SHAM}}$$ (Wood and Hollomon 2003).

Correlation of SHAM potentiation with *AOX* expression was tested by least squares linear regression analysis in GenStat 13th Edition (VSN International, Hertfordshire, UK).

3.3 Results

3.3.1 Sensitivity testing

A full list of azoxystrobin EC_{50} values is given in Appendix 1. For pre-2008 isolates, EC_{50} values obtained without SHAM show considerable variability, ranging from 0.0135 to 2.58 $\mu g\ ml^{-1}$. The EC_{50} values obtained with 40 $\mu g\ ml^{-1}$ SHAM were generally lower than those without AOX inhibition, and 90% are within the range 0.015 to 0.15 $\mu g\ ml^{-1}$ (Figure 3.1). There was a significant ($P<0.01$) positive correlation between azoxystrobin EC_{50} values with and without 40 $\mu g\ ml^{-1}$ SHAM. However, on closer examination of the data, the correlation was significant ($P<0.01$) among those isolates with azoxystrobin EC_{50} values less than 0.06 $\mu g\ ml^{-1}$ without SHAM, but not significant ($P>0.1$) among those isolates with higher EC_{50} values (Figure 3.1). Of the 2008 isolates, R.s. 2310 4.2 and R.s. 2318 4.2 had azoxystrobin EC_{50} values within the ranges previously seen with and without 40 $\mu g\ ml^{-1}$ SHAM (Figure 3.1), whereas isolates R.s. 2313 4.2 and R.s. 2314 4.2 did not show a 50% reduction in growth even at the maximum tested concentration of 100$\mu g\ ml^{-1}$ azoxystrobin, with 0 or 40$\mu g\ ml^{-1}$ SHAM, and therefore could not be included in Figure 3.1.
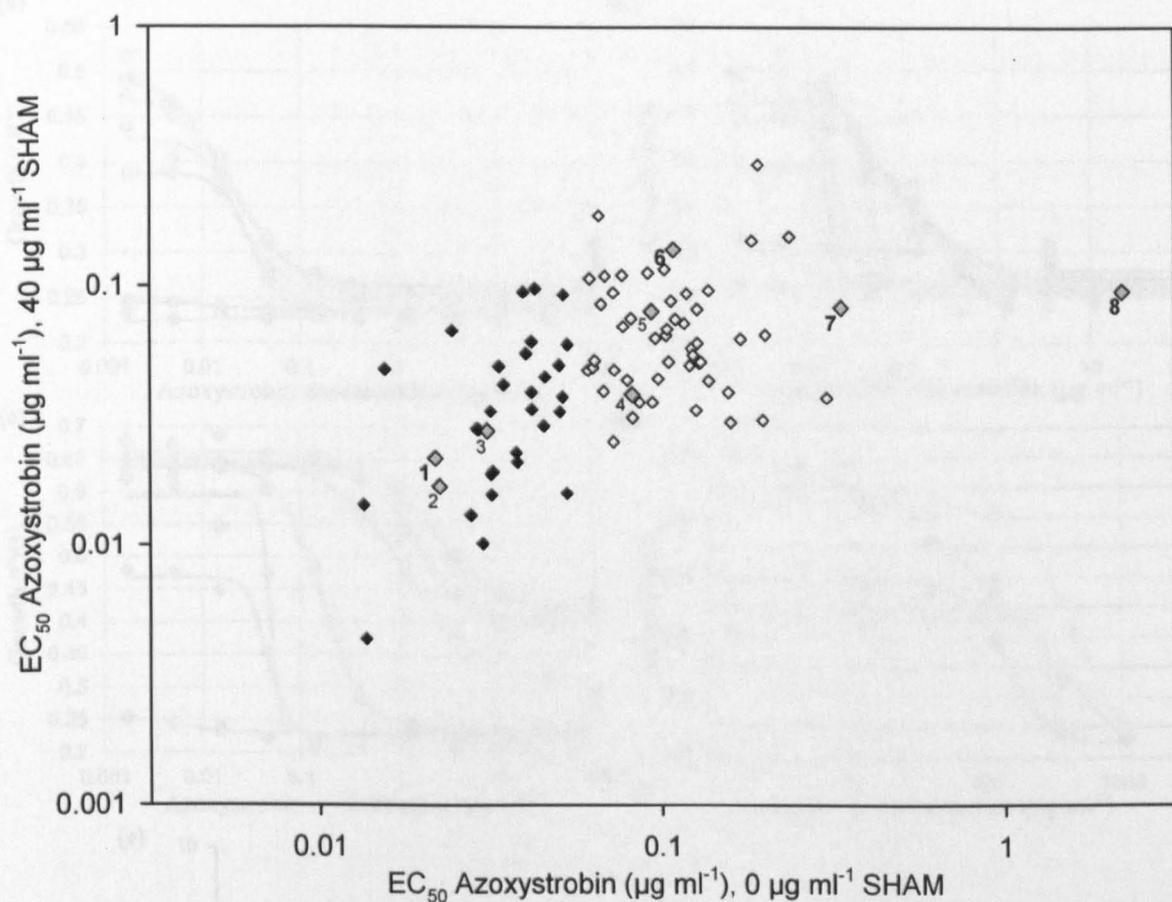
Figure 3.1. Scatter plots showing EC50 for azoxystrobin with 40 µg ml^{-1} SHAM against EC50 values for azoxystrobin without SHAM for *R. secalis* isolates. Black: isolates with azoxystrobin EC_{50} without SHAM less than 0.06 µg ml^{-1}; white: isolates with azoxystrobin EC_{50} without SHAM greater than 0.06 µg ml^{-1}; grey, isolates selected for sequence analysis. 1: 788; 2: R.s. 2310 4.2; 3: RS 219; 4: R.s. 2318 4.2; 5: GKII 18-2-3; 6: R9528.4; 7: K1124; 8: RS 783. Bold numbers indicate isolates used in the 4-hour *AOX* expression analysis experiment.

3.3.2 SHAM matrix assays

As SHAM concentration increased from 0 to 120 µg ml^{-1}, the azoxystrobin EC_{50} of isolate 788 remained in the range 0.014 to 0.030 µg ml^{-1} (Figure 3.2 a and e), whereas the azoxystrobin EC_{50} of isolates K1124 and RS 783 decreased from 0.094 to 0.017 µg ml^{-1} (Figure 3.2 b and e), and from 2.54 to 0.049 µg ml^{-1} (Figure 3.2 c and e), respectively. Isolate R9528.4 was intermediate between isolates 788 and K1124 (Figure 3.2 e). Above 120 µg ml^{-1}, SHAM itself was toxic, with EC_{50}s for SHAM alone ranging from 140-470 µg ml^{-1} (Figure 3.2 d).
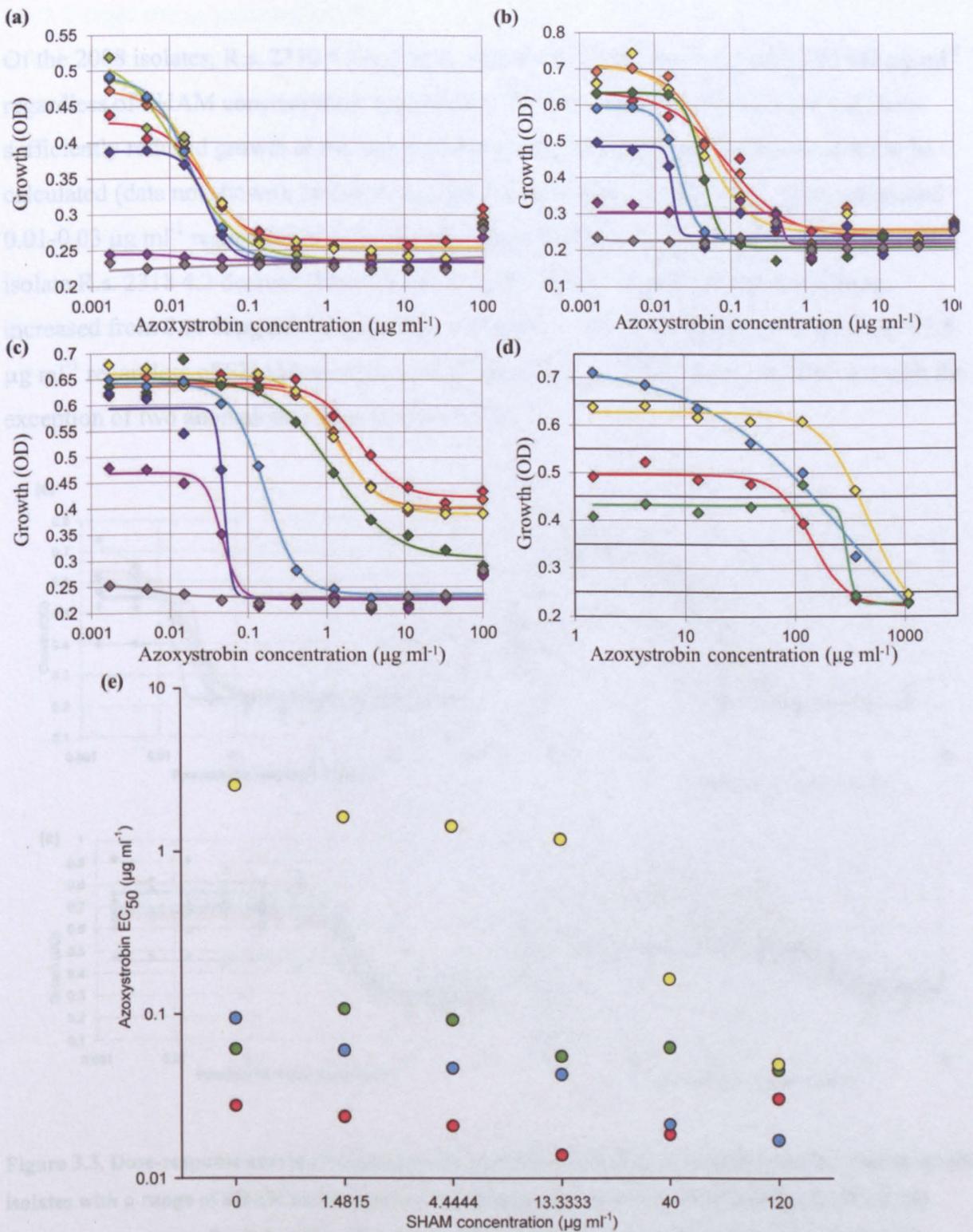
Figure 3.2. (a-c) Dose-response curves of fungal growth against azoxystrobin concentration, and azoxystrobin EC$_{50}$ values, for three *R. secalis* isolates, with a range of SHAM concentrations: (a) Isolate 788; (b) K1124; (c) RS 783. Red: 0 g ml^{-1} SHAM, orange: 1.48 μg ml^{-1}; yellow, 4.44 μg ml^{-1}; green, 13.3 μg ml^{-1}; blue, 40 μg ml^{-1}; dark blue, 120 μg ml^{-1}; purple, 360 μg ml^{-1}; grey, 1080 μg ml^{-1}. (d) Dose-response curves of fungal growth against SHAM without QoI fungicide. Red: isolate 788; blue: K1124; green: 9528.4; yellow: RS 783. (e) Comparison of azoxystrobin EC$_{50}$ values for different SHAM concentrations; isolates as in (d).

Of the 2008 isolates, R.s. 2310 4.2 had azoxystrobin EC_{50} values of around 0.02-0.04 μg ml^{-1} regardless of SHAM concentration. Isolates R.s. 2313 4.2 and R.s. 2314 4.2 did not show sufficiently reduced growth at the azoxystrobin concentrations tested for EC_{50} values to be calculated (data not shown). Isolate R.s. 2310 4.2 had pyraclostrobin EC_{50} values of around 0.01-0.03 μg ml^{-1} regardless of SHAM concentration (Figure 3.3a). Pyraclostrobin EC_{50} of isolate R.s. 2318 4.2 decreased from 0.145 to 0.026 μg ml^{-1} as SHAM concentrations increased from 0 to 40μg ml^{-1} (Figure 3.3b). Isolate R.s. 2314 4.2 had EC_{50} values of 1.5-2.5 μg ml^{-1} regardless of SHAM concentration (Figure 3.3c), as did isolate R.s. 2313 4.2 with the exception of two anomalous values (Figure 3.3d).
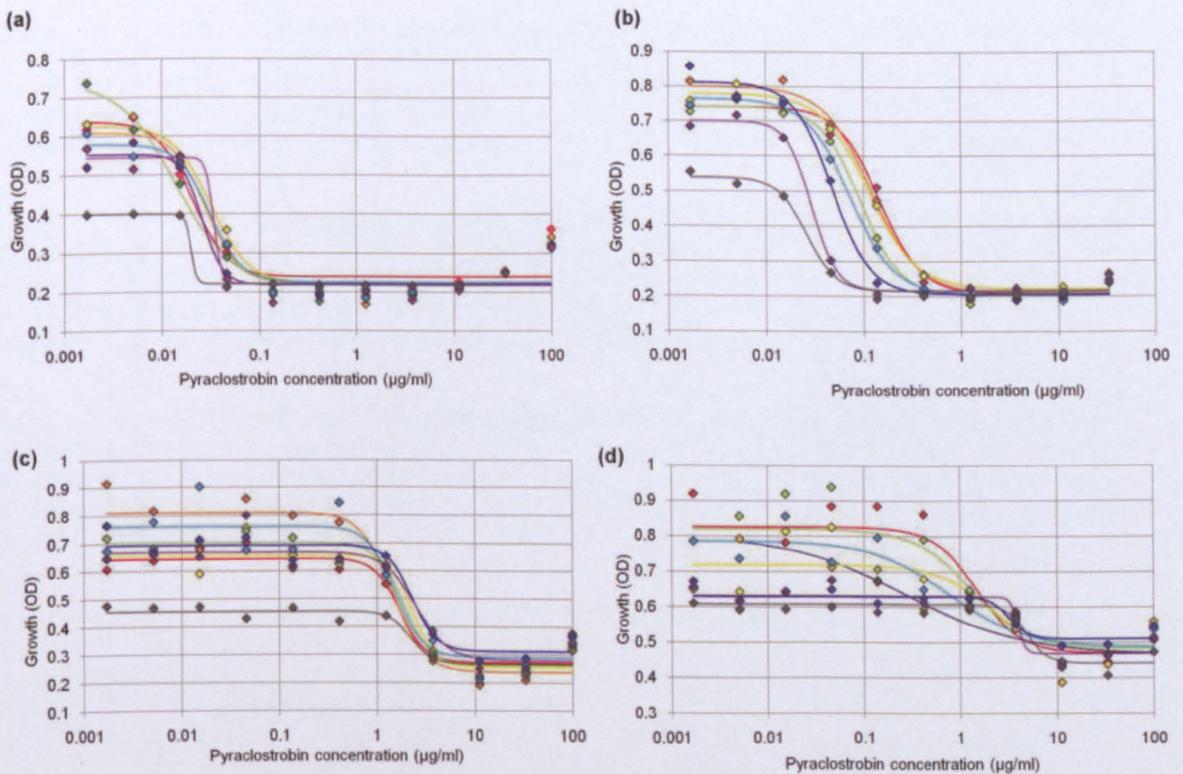
Figure 3.3. Dose-response curves of fungal growth against pyraclostrobin concentration for four *R. secalis* isolates with a range of SHAM concentrations: (a) Dose-response curves for isolate R.s. 2310 4.2; (b) Dose-response curves for isolate R.s. 2318 4.2; (c) Dose-response curves for isolate R.s. 2314 4.2; (d) Dose-response curves for isolate R.s. 2313 4.2; with a range of SHAM concentrations: Red: 0 μg ml^{-1}; orange: 0.0549 μg ml^{-1}; yellow, 0.165 μg ml^{-1}; green, 0.494 μg ml^{-1}; blue, 1.48 μg ml^{-1}; dark blue, 4.44 μg ml^{-1}; purple, 13.3 μg ml^{-1}; black, 40 μg ml^{-1}.

3.3.3 Target site sequence analysis

A 1118-base pair fragment of *cytochrome b*, encompassing base pairs 54-1171 of the genomic sequence, was amplified from the seven pre-2008 isolates. There were no differences in predicted amino acid sequence (Appendix 2). Of the 2008 French isolates, R.s. 2310 4.2 and R.s. 2318 4.2 had identical predicted amino acid sequences to the pre-2008 isolates, whereas R.s. 2313 4.2 and R.s. 2314 4.2 carried the G143A amino acid substitution (Figure 3.4), due to the substitution of a cytosine in place of a guanine at nucleotide position 428 (Figure 3.5).

```
                      1                                                           60
Sy1-14        (1)   MRIFKSHPLLKLVNSYIIDSPQPSNLSYLWNFGSLLAVCLAIQIVTGVTLAMHYNPSILE
R.s.2310 4.2  (1)   ------------------------------------------------------HYNPSILE
R.s.2313 4.2  (1)   ------------------------------------------------------HYNPSILE
R.s.2314 4.2  (1)   ------------------------------------------------------HYNPSILE
R.s.2318 4.2  (1)   ------------------------------------------------------HYNPSILE

                      61                                                          120
Sy1-14       (61)   AFNSIEHIMRDVNNGWLIRYLHSNTASFFFFLVYLHMGRGLYYGSYRAPRTLVWTIGTFI
R.s.2310 4.2  (9)   AFNSIEHIMRDVNNGWLIRYLHSNTASFFFFLVYLHMGRGLYYGSYRAPRTLVWTIGTFI
R.s.2313 4.2  (9)   AFNSIEHIMRDVNNGWLIRYLHSNTASFFFFLVYLHMGRGLYYGSYRAPRTLVWTIGTFI
R.s.2314 4.2  (9)   AFNSIEHIMRDVNNGWLIRYLHSNTASFFFFLVYLHMGRGLYYGSYRAPRTLVWTIGTFI
R.s.2318 4.2  (9)   AFNSIEHIMRDVNNGWLIRYLHSNTASFFFFLVYLHMGRGLYYGSYRAPRTLVWTIGTFI

                      121                                                         180
Sy1-14      (121)   FILMIVTAFLGYVLPYGQMSLWGATVITNLMSAIPWIGQDIVEFIWGGFSVNNATLNRFF
R.s.2310 4.2 (69)   FILMIVTAFLGYVLPYGQMSLWGATVITNLMSAIPWIGQDIVEFIWGGFSVNNATLNRFF
R.s.2313 4.2 (69)   FILMIVTAFLGYVLPYGQMSLWAATVITNLMSAIPWIGQDIVEFIWGGFSVNNATLNRFF
R.s.2314 4.2 (69)   FILMIVTAFLGYVLPYGQMSLWAATVITNLMSAIPWIGQDIVEFIWGGFSVNNATLNRFF
R.s.2318 4.2 (69)   FILMIVTAFLGYVLPYGQMSLWGATVITNLMSAIPWIGQDIVEFIWGGFSVNNATLNRFF

                      181                                                         240
Sy1-14      (181)   ALHFVLPFILAALVLMHLIALHDSAGSGNPLGVSGNYDRLPFAPYFLFKDLITIFLFIFV
R.s.2310 4.2(129)   ALHFVLPFILAALVLMHLIALHDSAGSGNPLGVSGNYDRLPFAPYFLFKDLITIFLFIFV
R.s.2313 4.2(129)   ALHFVLPFILAALVLMHLIALHDSAGSGNPLGVSGNYDRLPFAPYFLFKDLITIFLFIFV
R.s.2314 4.2(129)   ALHFVLPFILAALVLMHLIALHDSAGSGNPLGVSGNYDRLPFAPYFLFKDLITIFLFIFV
R.s.2318 4.2(129)   ALHFVLPFILAALVLMHLIALHDSAGSGNPLGVSGNYDRLPFAPYFLFKDLITIFLFIFV

                      241                                                         300
Sy1-14      (241)   LSLFVFFMPNVLGDSENYVVANPMQTPPAIVPEWYLLPFYAILRSIPNKLLGVIAMLSAI
R.s.2310 4.2(189)   LSLFVFFMPNVLGDSENYVVANPMQTPP---------------------------------
R.s.2313 4.2(189)   LSLFVFFMPNVLGDSENYVVANPMQTPP---------------------------------
R.s.2314 4.2(189)   LSLFVFFMPNVLGDSENYVVANPMQTPP---------------------------------
R.s.2318 4.2(189)   LSLFVFFMPNVLGDSENYVVANPMQTPP---------------------------------

                      301                                                         360
Sy1-14      (301)   LVILAMPFTDLSRSRGIQFRPLSKIAFYIFVANFLILMVLGAKHVESPFIEFGQISTVIY
R.s.2310 4.2(217)   ------------------------------------------------------------
R.s.2313 4.2(217)   ------------------------------------------------------------
R.s.2314 4.2(217)   ------------------------------------------------------------
R.s.2318 4.2(217)   ------------------------------------------------------------

                      361                             397
Sy1-14      (361)   FSHFLIIVPLVSLIENSLIDLNTSIDYSSPSVLEKA-
R.s.2310 4.2(217)   -------------------------------------
R.s.2313 4.2(217)   -------------------------------------
R.s.2314 4.2(217)   -------------------------------------
R.s.2318 4.2(217)   -------------------------------------
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Figure 3.4. Translated aligned sequences of cytochrome *b* from *R. secalis* isolates Sy1-14 (Torriani, 2004), R.s. 2310 4.2, R.s. 2318 4.2, R.s. 2313 4.2 and R.s. 2314 4.2. Position 143 is highlighted.

```
Sy1-14        (1)    ATGAGAATATTTAAGAGTCATCCTTTATTAAAATTGGTTAATTCCTATATAATCGATTCACCACAACCATCTAATCTAAGCTAC
R.s.2310 4.2  (1)    ----------------------------------------------------------------------------------
R.s.2313 4.2  (1)    ----------------------------------------------------------------------------------
R.s.2314 4.2  (1)    ----------------------------------------------------------------------------------
R.s.2318 4.2  (1)    ----------------------------------------------------------------------------------

Sy1-14        (85)   TTATGAAATTTTGGTTCTTTATTAGCCGTTTGTTTAGCTATACAAATAGTTACAGGTGTAACATTGGCTATGCATTACAACCCT
R.s.2310 4.2  (1)    -----------------------------------------------------------------------TGCATTACAACCCT
R.s.2313 4.2  (1)    -----------------------------------------------------------------------TGCATTACAACCCT
R.s.2314 4.2  (1)    -----------------------------------------------------------------------TGCATTACAACCCT
R.s.2318 4.2  (1)    -----------------------------------------------------------------------TGCATTACAACCCT

Sy1-14        (169)  AGTATATTAGAAGCGTTTAATTCCATAGAACATATTATGCGTGATGTAAATAACGGATGATTAATACGTTACTTACATAGTAAC
R.s.2310 4.2  (15)   AGTATATTAGAAGCGTTTAATTCCATAGAACATATTATGCGTGATGTAAATAACGGATGATTAATACGTTACTTACATAGTAAC
R.s.2313 4.2  (15)   AGTATATTAGAAGCGTTTAATTCCATAGAACATATTATGCGTGATGTAAATAACGGATGATTAATACGTTACTTACATAGTAAC
R.s.2314 4.2  (15)   AGTATATTAGAAGCGTTTAATTCCATAGAACATATTATGCGTGATGTAAATAACGGATGATTAATACGTTACTTACATAGTAAC
R.s.2318 4.2  (15)   AGTATATTAGAAGCGTTTAATTCCATAGAACATATTATGCGTGATGTAAATAACGGATGATTAATACGTTACTTACATAGTAAC

Sy1-14        (253)  ACTGCATCTTTTTTCTTCTTCCTAGTGTATTTACACATGGGTAGAGGTTTATATTATGGGTCATACAGAGCACCTAGAACATTA
R.s.2310 4.2  (99)   ACTGCATCTTTTTTCTTCTTCCTAGTGTATTTACACATGGGTAGAGGTTTATATTATGGGTCATACAGAGCACCTAGAACATTA
R.s.2313 4.2  (99)   ACTGCATCTTTTTTCTTCTTCCTAGTGTATTTACACATGGGTAGAGGTTTATATTATGGGTCATACAGAGCACCTAGAACATTA
R.s.2314 4.2  (99)   ACTGCATCTTTTTTCTTCTTCCTAGTGTATTTACACATGGGTAGAGGTTTATATTATGGGTCATACAGAGCACCTAGAACATTA
R.s.2318 4.2  (99)   ACTGCATCTTTTTTCTTCTTCCTAGTGTATTTACACATGGGTAGAGGTTTATATTATGGGTCATACAGAGCACCTAGAACATTA

Sy1-14        (337)  GTATGAACAATAGGTACATTTATATTCATATTAATGATCGTTACAGCATTCTTGGGTTATGTGCTTCCTTATGGACAGATGTCT
R.s.2310 4.2  (183)  GTATGAACAATAGGTACATTTATATTCATATTAATGATCGTTACAGCATTCTTGGGTTATGTGCTTCCTTATGGACAGATGTCT
R.s.2313 4.2  (183)  GTATGAACAATAGGTACATTTATATTCATATTAATGATCGTTACAGCATTCTTGGGTTATGTGCTTCCTTATGGACAGATGTCT
R.s.2314 4.2  (183)  GTATGAACAATAGGTACATTTATATTCATATTAATGATCGTTACAGCATTCTTGGGTTATGTGCTTCCTTATGGACAGATGTCT
R.s.2318 4.2  (183)  GTATGAACAATAGGTACATTTATATTCATATTAATGATCGTTACAGCATTCTTGGGTTATGTGCTTCCTTATGGACAGATGTCT

                                         ▼▼▼
Sy1-14        (421)  TTATGAGGTGCCACAGTTATAACTAATCTTATGAGTGCTATACCTTGAATAGGTCAAGACATTGTTGAGTTTATCTGAGGGGGT
R.s.2310 4.2  (267)  TTATGAGGTGCCACAGTTATAACTAATCTTATGAGTGCTATACCTTGAATAGGTCAAGACATTGTTGAGTTTATCTGAGGGGGT
R.s.2313 4.2  (267)  TTATGAGCTGCCACAGTTATAACTAATCTTATGAGTGCTATACCTTGAATAGGTCAAGACATTGTTGAGTTTATCTGAGGGGGT
R.s.2314 4.2  (267)  TTATGAGCTGCCACAGTTATAACTAATCTTATGAGTGCTATACCTTGAATAGGTCAAGACATTGTTGAGTTTATCTGAGGGGGT
R.s.2318 4.2  (267)  TTATGAGGTGCCACAGTTATAACTAATCTTATGAGTGCTATACCTTGAATAGGTCAAGACATTGTTGAGTTTATCTGAGGGGGT

Sy1-14        (505)  TTTTCTGTTAATAATGCAACTTTAAATAGATTCTTTGCATTACATTTTGTTTTACCGTTTATATTAGCTGCATTAGTATTAATG
R.s.2310 4.2  (351)  TTTTCTGTTAATAATGCAACTTTAAATAGATTCTTTGCATTACATTTTGTTTTACCGTTTATATTAGCTGCATTAGTATTAATG
R.s.2313 4.2  (351)  TTTTCTGTTAATAATGCAACTTTAAATAGATTCTTTGCATTACATTTTGTTTTACCGTTTATATTAGCTGCATTAGTATTAATG
R.s.2314 4.2  (351)  TTTTCTGTTAATAATGCAACTTTAAATAGATTCTTTGCATTACATTTTGTTTTACCGTTTATATTAGCTGCATTAGTATTAATG
R.s.2318 4.2  (351)  TTTTCTGTTAATAATGCAACTTTAAATAGATTCTTTGCATTACATTTTGTTTTACCGTTTATATTAGCTGCATTAGTATTAATG

Sy1-14        (589)  CACTTAATAGCCTTACACGATAGTGCAGGGTCAGGTAATCCTTTAGGTGTATCAGGTAATTACGATAGATTACCTTTTGCTCCT
R.s.2310 4.2  (435)  CACTTAATAGCCTTACACGATAGTGCAGGGTCAGGTAATCCTTTAGGTGTATCAGGTAATTACGATAGATTACCTTTTGCTCCT
R.s.2313 4.2  (435)  CACTTAATAGCCTTACACGATAGTGCAGGGTCAGGTAATCCTTTAGGTGTATCAGGTAATTACGATAGATTACCTTTTGCTCCT
R.s.2314 4.2  (435)  CACTTAATAGCCTTACACGATAGTGCAGGGTCAGGTAATCCTTTAGGTGTATCAGGTAATTACGATAGATTACCTTTTGCTCCT
R.s.2318 4.2  (435)  CACTTAATAGCCTTACACGATAGTGCAGGGTCAGGTAATCCTTTAGGTGTATCAGGTAATTACGATAGATTACCTTTTGCTCCT

Sy1-14        (673)  TACTTCTTATTCAAAGATTTAATAACTATCTTTTTATTTATCTTTGTATTAAGTTTATTCGTATTCTTCATGCCTAACGTATTA
R.s.2310 4.2  (519)  TACTTCTTATTCAAAGATTTAATAACTATCTTTTTATTTATCTTTGTATTAAGTTTATTCGTATTCTTCATGCCTAACGTATTA
R.s.2313 4.2  (519)  TACTTCTTATTCAAAGATTTAATAACTATCTTTTTATTTATCTTTGTATTAAGTTTATTCGTATTCTTCATGCCTAACGTATTA
R.s.2314 4.2  (519)  TACTTCTTATTCAAAGATTTAATAACTATCTTTTTATTTATCTTTGTATTAAGTTTATTCGTATTCTTCATGCCTAACGTATTA
R.s.2318 4.2  (519)  TACTTCTTATTCAAAGATTTAATAACTATCTTTTTATTTATCTTTGTATTAAGTTTATTCGTATTCTTCATGCCTAACGTATTA

Sy1-14        (757)  GGTGATAGTGAAAATTACGTTGTAGCTAACCCTATGCAAACTCCACCTGCGATAGTTCCGGAGTGATATTTACTACCTTTCTAT
R.s.2310 4.2  (603)  GGTGATAGTGAAAATTACGTTGTAGCTAACCCTATGCAAACTCCACCT-----------------------------------
R.s.2313 4.2  (603)  GGTGATAGTGAAAATTACGTTGTAGCTAACCCTATGCAAACTCCACCT-----------------------------------
R.s.2314 4.2  (603)  GGTGATAGTGAAAATTACGTTGTAGCTAACCCTATGCAAACTCCACCT-----------------------------------
R.s.2318 4.2  (603)  GGTGATAGTGAAAATTACGTTGTAGCTAACCCTATGCAAACTCCACCT-----------------------------------

Sy1-14        (841)  GCTATATTAAGATCTATACCTAACAAATTATTAGGTGTTATAGCTATGCTTAGTGCTATATTAGTTATATTAGCTATGCCATTT

Sy1-14        (925)  ACAGATTTAAGTAGATCTAGAGGTATACAATTTAGACCTTTAAGTAAAATAGCTTTTTATATTTTTGTTGCTAATTTCTTAATA

Sy1-14        (1009) TTAATGGTGTTAGGTGCTAAACACGTTGAATCACCATTCATAGAATTTGGACAAATAAGTACCGTAATATATTTCTCACACTTT

Sy1-14        (1093) TTAATCATAGTGCCTTTGGTTTCTTTAATAGAAAACAGTTTAATAGATTTAAACACGTCAATAGACTATTCTTCGCCTTCCGTT

Sy1-14        (1177) TTAGAAAAAGCGTAA (1191)
```

Figure 3.5. Aligned DNA sequences of *cytochrome b* from *R. secalis* isolates Sy1-14(Torriani, 2004), R.s. 2310 4.2, R.s. 2318 4.2, R.s. 2313 4.2 and R.s. 2314 4.2. Arrows indicate codon 143.

3.3.4 *AOX* sequence analysis

AOX sequences for *S. sclerotiorum*, *B. cinerea* and *B. graminis* f.sp. *hordei* were found on GenBank. A tBLASTn search of the predicted protein sequences against the *R. secalis* initial genome contigs gave the same three significant matches for each query sequence. The three fragments were all on one contig and appeared to be from three exons, separated by two introns. Genomic and cDNA sequences from isolates K1124 and RS 783 confirmed the predicted introns, with no genomic sequence differences between the three isolates (Figure 3.6). The first intron, between base pairs 251-307, is present in *R. secalis* and predicted in *S. sclerotiorum*, *B. cinerea* and *B. graminis* f.sp. *hordei*. A second predicted intron in *S. sclerotiorum* and *B. cinerea* is present between base pairs corresponding to 650-651 of the *R. secalis* gene, whereas a second intron in *R. secalis* is situated between base pairs 874-928 (Figure 3.7).

In a HMMER3 search of the Pfam protein families databases with the *R. secalis* predicted amino acid sequence, the Pfam A family "Alternative oxidase" as the only significant match, with an E-value of $3.1e^{-87}$. All the highly conserved regions identified by Berthold *et al.* (2000) are present, except the "LET" region comprises the fungal variant "LES" (Figure 3.7). Analysis with MitoProt II gave 0.9903% probability of the *R. secalis* AOX predicted peptide being mitochondrially imported, with a predicted cleavage site at residue 72, 11 base pairs downstream of the predicted site in *S. sclerotiorum*, *B. cinerea* and *B. graminis* f.sp. *hordei* (Figure 3.7).

```
13-13 Genomic    (1)   ATGTATGTCGCAAGGGTATCAACAAAGCTTCAACTCTCTAAGCAATCTGCTGCACAGCTCTCGAGAGCAGTGACTACTTGCTCCCAA
K1124 Genomic    (1)   ATGTATGTCGCAAGGGTATCAACAAAGCTTCAACTCTCTAAGCAATCTGCTGCACAGCTCTCGAGAGCAGTGACTACTTGCTCCCAA
K1124 cDNA       (1)   ATGTATGTCGCAAGGGTATCAACAAAGCTTCAACTCTCTAAGCAATCTGCTGCACAGCTCTCGAGAGCAGTGACTACTTGCTCCCAA
RS 783 Genomic   (1)   ATGTATGTCGCAAGGGTATCAACAAAGCTTCAACTCTCTAAGCAATCTGCTGCACAGCTCTCGAGAGCAGTGACTACTTGCTCCCAA
RS 783 cDNA      (1)   ATGTATGTCGCAAGGGTATCAACAAAGCTTCAACTCTCTAAGCAATCTGCTGCACAGCTCTCGAGAGCAGTGACTACTTGCTCCCAA

13-13 Genomic    (88)  TGCCATGGAGGCTCAACGAACGCAGCAGGTTTTCGTTTGACAGCTGCACAATCTACAATCGCGCCGCCAGTTCTCCACAACACCTCGA
K1124 Genomic    (88)  TGCCATGGAGGCTCAACGAACGCAGCAGGTTTTCGTTTGACAGCTGCACAATCTACAATCGCGCCGCCAGTTCTCCACAACACCTCGA
K1124 cDNA       (88)  TGCCATGGAGGCTCAACGAACGCAGCAGGTTTTCGTTTGACAGCTGCACAATCTACAATCGCGCCGCCAGTTCTCCACAACACCTCGA
RS 783 Genomic   (88)  TGCCATGGAGGCTCAACGAACGCAGCAGGTTTTCGTTTGACAGCTGCACATCTACAATCGCGCCGCCAGTTCTCCACAACACCTCGA
RS 783 cDNA      (88)  TGCCATGGAGGCTCAACGAACGCAGCAGGTTTTCGTTTGACAGCTGCACCTCTACAATCGCGCCGCCAGTTCTCCACAACACCTCGA

13-13 Genomic    (175) ACACAACTACGAGATATATTCCCTTCTCCAGAACATGAGCATATCAAGAAGACCGAAGCCGCTTGGCCTCATCCACCGTAAGCCTAT
K1124 Genomic    (175) ACACAACTACGAGATATATTCCCTTCTCCAGAACATGAGCATATCAAGAAGACCGAAGCCGCTTGGCCTCATCCACCGTAAGCCTAT
K1124 cDNA       (175) ACACAACTACGAGATATATTCCCTTCTCCAGAACATGAGCATATCAAGAAGACCGAAGCCGCTTGGCCTCATCCACC----------
RS 783 Genomic   (175) ACACAACTACGAGATATATTCCCTTCTCCAGAACATGAGCATATCAAGAAGACCGAAGCCGCTTGGCCTCATCCACCGTAAGCCTAT
RS 783 cDNA      (175) ACACAACTACGAGATATATTCCCTTCTCCAGAACATGAGCATATCAAGAAGACCGAAGCCGCTTGGCCTCATCCACC----------

13-13 Genomic    (262) CTGCCAACACTTTAGCAACTGCCACATGCTGATTCTTACCTATAGATACGATGGCGAAAAAATAAAGAATGATATCTACTACGCCCA
K1124 Genomic    (262) CTGCCAACACTTTAGCAACTGCCACATGCTGATTCTTACCTATAGATACGATGGCGAAAAAATAAAGAATGATATCTACTACGCCCA
K1124 cDNA       (252) --------------------------------------------ATACGATGGCGAAAAAATAAAGAATGATATCTACTACGCCCA
RS 783 Genomic   (262) CTGCCAACACTTTAGCAACTGCCACATGCTGATTCTTACCTATAGATACGATGGCGAAAAAATAAAGAATGATATCTACTACGCCCA
RS 783 cDNA      (252) --------------------------------------------ATACGATGGCGAAAAAATAAAGAATGATATCTACTACGCCCA

13-13 Genomic    (349) TCGAGAGCCCCAAGACTTCAGTGATAGAATAGCTCTCTTCATGGTTCGCTTACTTCGATTCGGAATGGATACAGCTACACGGTATAA
K1124 Genomic    (349) TCGAGAGCCCCAAGACTTCAGTGATAGAATAGCTCTCTTCATGGTTCGCTTACTTCGATTCGGAATGGATACAGCTACACGGTATAA
K1124 cDNA       (294) TCGAGAGCCCCAAGACTTCAGTGATAGAATAGCTCTCTTCATGGTTCGCTTACTTCGATTCGGAATGGATACAGCTACACGGTATAA
RS 783 Genomic   (349) TCGAGAGCCCCAAGACTTCAGTGATAGAATAGCTCTCTTCATGGTTCGCTTACTTCGATTCGGAATGGATACAGCTACACGGTATAA
RS 783 cDNA      (294) TCGAGAGCCCCAAGACTTCAGTGATAGAATAGCTCTCTTCATGGTTCGCTTACTTCGATTCGGAATGGATACAGCTACACGGTATAA

13-13 Genomic    (436) GCACGACGTCGAAACGCCCAAGAAAATCGGTGACAGCAATGCTGTTACGGAAACCAAGCCTTACGCAATGTCCGAGAAGAAATGGCT
K1124 Genomic    (436) GCACGACGTCGAAACGCCCAAGAAAATCGGTGACAGCAATGCTGTTACGGAAACCAAGCCTTACGCAATGTCCGAGAAGAAATGGCT
K1124 cDNA       (381) GCACGACGTCGAAACGCCCAAGAAAATCGGTGACAGCAATGCTGTTACGGAAACCAAGCCTTACGCAATGTCCGAGAAGAAATGGCT
RS 783 Genomic   (436) GCACGACGTCGAAACGCCCAAGAAAATCGGTGACAGCAATGCTGTTACGGAAACCAAGCCTTACGCAATGTCCGAGAAGAAATGGCT
RS 783 cDNA      (381) GCACGACGTCGAAACGCCCAAGAAAATCGGTGACAGCAATGCTGTTACGGAAACCAAGCCTTACGCAATGTCCGAGAAGAAATGGCT

13-13 Genomic    (523) CATCCGAATGGTATTTCTGAATCTGTTGCAGGTGTACCAGGGATGGTCGCTGGGATGGTTCGCCACCTTCATAGTTTGAGACGACT
K1124 Genomic    (523) CATCCGAATGGTATTTCTGAATCTGTTGCAGGTGTACCAGGGATGGTCGCTGGGATGGTTCGCCACCTTCATAGTTTGAGACGACT
K1124 cDNA       (468) CATCCGAATGGTATTTCTGAATCTGTTGCAGGTGTACCAGGGATGGTCGCTGGGATGGTTCGCCACCTTCATAGTTTGAGACGACT
RS 783 Genomic   (523) CATCCGAATGGTATTTCTGAATCTGTTGCAGGTGTACCAGGGATGGTCGCTGGGATGGTTCGCCACCTTCATAGTTTGAGACGACT
RS 783 cDNA      (468) CATCCGAATGGTATTTCTGAATCTGTTGCAGGTGTACCAGGGATGGTCGCTGGGATGGTTCGCCACCTTCATAGTTTGAGACGACT

13-13 Genomic    (610) CAAGAGAGACAATGGATGGATCGAAACACTACTGGAAGAAGCCTATAATGAGCGGATGCATCTTCTTACATTTCTCAAGATGGCAGA
K1124 Genomic    (610) CAAGAGAGACAATGGATGGATCGAAACACTACTGGAAGAAGCCTATAATGAGCGGATGCATCTTCTTACATTTCTCAAGATGGCAGA
K1124 cDNA       (555) CAAGAGAGACAATGGATGGATCGAAACACTACTGGAAGAAGCCTATAATGAGCGGATGCATCTTCTTACATTTCTCAAGATGGCAGA
RS 783 Genomic   (610) CAAGAGAGACAATGGATGGATCGAAACACTACTGGAAGAAGCCTATAATGAGCGGATGCATCTTCTTACATTTCTCAAGATGGCAGA
RS 783 cDNA      (555) CAAGAGAGACAATGGATGGATCGAAACACTACTGGAAGAAGCCTATAATGAGCGGATGCATCTTCTTACATTTCTCAAGATGGCAGA

13-13 Genomic    (697) GCCTGGCAAATTCATGAAGTTCATGATATTAGGAGCTCAGGGCGTTTTCTTCAACTCGATGTTTCTCTCCTACCTCATTTCGCCAAA
K1124 Genomic    (697) GCCTGGCAAATTCATGAAGTTCATGATATTAGGAGCTCAGGGCGTTTTCTTCAACTCGATGTTTCTCTCCTACCTCATTTCGCCAAA
K1124 cDNA       (642) GCCTGGCAAATTCATGAAGTTCATGATATTAGGAGCTCAGGGCGTTTTCTTCAACTCGATGTTTCTCTCCTACCTCATTTCGCCAAA
RS 783 Genomic   (697) GCCTGGCAAATTCATGAAGTTCATGATATTAGGAGCTCAGGGCGTTTTCTTCAACTCGATGTTTCTCTCCTACCTCATTTCGCCAAA
RS 783 cDNA      (642) GCCTGGCAAATTCATGAAGTTCATGATATTAGGAGCTCAGGGCGTTTTCTTCAACTCGATGTTTCTCTCCTACCTCATTTCGCCAAA

13-13 Genomic    (784) GACCTGTCACCGCTTTGTTGGCTATCTTGAGGAGGAGGCCGTCTTGACCTACTCACTTGCCATTCAAGATATTGAGGCTGGCAAGCT
K1124 Genomic    (784) GACCTGTCACCGCTTTGTTGGCTATCTTGAGGAGGAGGCCGTCTTGACCTACTCACTTGCCATTCAAGATATTGAGGCTGGCAAGCT
K1124 cDNA       (729) GACCTGTCACCGCTTTGTTGGCTATCTTGAGGAGGAGGCCGTCTTGACCTACTCACTTGCCATTCAAGATATTGAGGCTGGCAAGCT
RS 783 Genomic   (784) GACCTGTCACCGCTTTGTTGGCTATCTTGAGGAGGAGGCCGTCTTGACCTACTCACTTGCCATTCAAGATATTGAGGCTGGCAAGCT
RS 783 cDNA      (729) GACCTGTCACCGCTTTGTTGGCTATCTTGAGGAGGAGGCCGTCTTGACCTACTCACTTGCCATTCAAGATATTGAGGCTGGCAAGCT

13-13 Genomic    (871) CCCAGTAAGTTCAAGAAACTTCCGTCACTATCTGAAATCAAGTACTAACTTGCGTAGAAATGGTCTGATCCTAAATTTCAAATCCCT
K1124 Genomic    (871) CCCAGTAAGTTCAAGAAACTTCCGTCACTATCTGAAATCAAGTACTAACTTGCGTAGAAATGGTCTGATCCTAAATTTCAAATCCCT
K1124 cDNA       (816) CCCA------------------------------------------------------AAATGGTCTGATCCTAAATTTCAAATCCCT
RS 783 Genomic   (871) CCCAGTAAGTTCAAGAAACTTCCGTCACTATCTGAAATCAAGTACTAACTTGCGTAGAAATGGCTGATCCTAAATTTCAAATCCCT
RS 783 cDNA      (816) CCCA------------------------------------------------------AAATGGCCTGATCCTAAATTTCAAATCCCT

13-13 Genomic    (958) GCGCTGGCAGTCAATTACTGGAAGATGCCTGAGGGGTCCCGAACTATGAAGGATCTCCTGCTGTACATCAGAGCAGATGAAGCCAAG
K1124 Genomic    (958) GCGCTGGCAGTCAATTACTGGAAGATGCCTGAGGGGTCCCGAACTATGAAGGATCTCCTGCTGTACATCAGAGCAGATGAAGCCAAG
K1124 cDNA       (850) GCGCTGGCAGTCAATTACTGGAAGATGCCTGAGGGGTCCCGAACTATGAAGGATCTCCTGCTGTACATCAGAGCAGATGAAGCCAAG
RS 783 Genomic   (958) GCGCTGGCAGTCAATTACTGGAAGATGCCTGAGGGGTCCCGAACTATGAAGGATCTCCTGCTGTACATCAGAGCAGATGAAGCCAAG
RS 783 cDNA      (850) GCGCTGGCAGTCAATTACTGGAAGATGCCTGAGGGGTCCCGAACTATGAAGGATCTCCTGCTGTACATCAGAGCAGATGAAGCCAAG

13-13 Genomic   (1045) CATCGCGAAGTCAACCATACACTTGGCAATCTTGACCAAAATGAAGATTCGAACCCATTCGTAAGCGAATACAAAGACACCGACCTC
K1124 Genomic   (1045) CATCGCGAAGTCAACCATACACTTGGCAATCTTGACCAAAATGAAGATTCGAACCCATTCGTAAGCGAATACAAAGACACCGACCTC
K1124 cDNA       (937) CATCGCGAAGTCAACCATACACTTGGCAATCTTGACCAAAATGAAGATTCGAACCCATTCGTAAGCGAATACAAAGACACCGACCTC
RS 783 Genomic  (1045) CATCGCGAAGTCAACCATACACTTGGCAATCTTGACCAAAATGAAGATTCGAACCCATTCGTAAGCGAATACAAAGACACCGACCTC
RS 783 cDNA      (937) CATCGCGAAGTCAACCATACACTTGGCAATCTTGACCAAAATGAAGATTCGAACCCATTCGTAAGCGAATACAAAGACACCGACCTC

13-13 Genomic   (1132) CCGCATCCAGGCAAGGGTATCGAGCATATTAAGCCGTTGGGATGGGAAAGAAAAGATGTCATTTGA
K1124 Genomic   (1132) CCGCATCCAGGCAAGGGTATCGAGCATATTAAGCCGTTGGGATGGGAAAGAAAAGATGTCATTTGA
K1124 cDNA      (1024) CCGCATCCAGGCAAGGGTATCGAGCATATTAAGCCGTTGGGATGGGAAAGAAAAGATGTCATTTGA
RS 783 Genomic  (1132) CCGCATCCAGGCAAGGGTATCGAGCATATTAAGCCGTTGGGATGGGAAAGAAAAGATGTCATTTGA
RS 783 cDNA     (1024) CCGCATCCAGGCAAGGGTATCGAGCATATTAAGCCGTTGGGATGGGAAAGAAAAGATGTCATTTGA
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Figure 3.6. Genomic sequence of *AOX* from *R. secalis* isolate 13-13, and genomic and cDNA sequences from isolates K1124 and RS 783.

```
                      1                                                        60
B. graminis      (1) ---MYLSRLPPRSIPCR-SINQISRVVARSSKSRLEFVGSYQNSLTTNQTRCLRPFSSTS
B. cinerea       (1) MTTMYVSRVSTRPLSAQ-SAAQLSKAVAFFAQSYGLSSN-----ACTAPTPSRRAFTSAS
S. sclerotiorum  (1) MTSMYVSRVSKRNLSNQ-STAQLSKAVAFFAQSYGLSS-------TCTAHVQSRRAFTSGS
R. secalis       (1) ---MYVARVSTKLQLSKQSAAQLSRAVTTCSQCHGGSTNAAGFRLTAAHLQSRRQFSTTP
Consensus        (1) MTSMYVSRVSTR LSAQ SAAQLSKAVAFFAQSYGLSSNA   LTTAHTQSRRAFSSTS
                      61                                /                      120
B. graminis     (57) RSQL--RDYFPEADHEHIKKTEAAWPHPN/YPKEQMLTNISYAHRTPKDFSDRIALYLVRF
B. cinerea      (55) KIQVKGRDLFPEPEHGQIKRTEPAWPHPP/YTAEQMRSKVYFAHRKPRDFSDRVALGMVRF
S. sclerotiorum (54) KIQVKGRDLFPEPEHGQIKKTEPAWPHPP/YTADQMRSKVYFAHRKPRDFSDRVALCMVRF
R. secalis      (58) RTQL--RDIFPSPEHEHIKKTEAAWPHPN/YDGEKIKNDIYYAHREFQDFSDREELFMVRL
Consensus       (61) KIRVQLRDLFPEPEHGQIKKTEPAWPHPP/YTAEQMRSKIYFAHRKERDFSDREELFMVRF
                      121                                                     180
B. graminis    (115) LRFSTDLATGYKHDPVTITENGEK---VLKKPYRMSERKWLIRMVELESVAGVPGMVAGM
B. cinerea     (115) LRWCTDFATGYKHNVEAPKKASDSNALTATKPYQMSERKWLIRYVELESVAGVPGMVAGM
S. sclerotiorum(114) LRWCTDFATGYKHNVEEPKKASDSNAVAATKPYQMSERKWLIRYVELESVAGVPGMVAGM
R. secalis     (116) LRFGMDTATRYKHDVETPKKIGDSNAVTETKPYAMSEKKWLIRMVELESVAGVPGMVAGM
Consensus      (121) LRFCTDFATGYKHNVETPKKASDSNAVTATKPYQMSERKWLIRYVELESVAGVPGMVAGM
                      181                          ▼    ▼                      240
B. graminis    (172) LRHLHSLRRLKRDNGWIETLLEEA-YNERMHLLTFLKMAKPGWELRSMLIGAQGVFFNSMF
B. cinerea     (175) LRHLRSLRGLKRDNGWIETLLEEA/YNERMHLLTFLKMYEPGIFMRTMILGAQGVFFNSFF
S. sclerotiorum(174) LRHLRSLRGLKRDNGWIETLLEEA/YNERMHLLTFLKMYEPGIFMRTMILGAQGVFFNSFF
R. secalis     (176) VRHLHSLRRLKRDNGWIETLLEEA-YNERMHLLTFLKMAEPGKFMKFMILGAQGVFFNSMF
Consensus      (181) LRHLRSLRRLKRDNGWIETLLEEA YNERMHLLTFLKMYEPGIFMKTMILGAQGVFFNSMF
                      241                    ▼                /                300
B. graminis    (232) LSYLISPRTCHRFVAYLEEEAVLTYSTAIQDIEAGLLP-KWTSPEFRIPDLAVQYWKIPEG
B. cinerea     (235) LCYLFSPRTCHRFVGYLEEEAVLTYTLSIQDLENGHLP-KWADPDFKAPDLAVEYWGMPEG
S. sclerotiorum(234) ICYLLSPRTCHRFVGYLEEEAVLTYTLSIQDLENGHLP-KWSDPNFKAPDLAIEYWGMPED
R. secalis     (236) LSYLISPRTCHRFVGYLEEEAVLTYSLAIQDIEAGKLP-KWSDPKFQIPALAVNYWKMPEG
Consensus      (241) LSYLISPRTCHRFVGYLEEEAVLTYSLAIQDIENGHLP KWSDPDFKIPDLAVEYWKMPEG
                      301                   ▼    ▼                             360
B. graminis    (292) NRTMRDLLLYIRADEAKHREVNHTLGNLDQNEDPNPFVSEYRDKAAPHPSKGIEHIRPTG
B. cinerea     (295) NRSMRDLLYYIRADEAKHREVNHTLGNLKQDEDPNPFVSEYGKERGEKPGKGIESLKPVG
S. sclerotiorum(294) QRSMRDLLYYIRADEAKHREVNHTLGNLRQDVDPNPFVSTYGKEVGEKPGKGIECLRPAG
R. secalis     (296) SRTMRDLLLYIRADEAKHREVNHTLGNLDQNEDSNPFVSEYKDTDLPHPGKGIEHIKPLG
Consensus      (301) NRSMRDLLYYIRADEAKHREVNHTLGNLDQNEDPNPFVSEYGKE GPKPGKGIEHIKPLG
                      361
B. graminis    (352) WERNEII*
B. cinerea     (355) WERDEVI*
S. sclerotiorum(354) WERDEVI*
R. secalis     (356) WERKDVI*
Consensus      (361) WERDEVI*
```

Figure 3.7. Predicted amino acid sequences for *AOX* genes from *Blumeria graminis* f.sp. *hordei* (GenBank AF327336), *Botrytis cinerea* (Broad Institute genome, gene BC1G_05703.1), *Sclerotinia sclerotiorum* (Broad Institute genome, SS1G_02882.1) and *R. secalis*. Shaded residues are conserved across the four sequences. Highly conserved regions identified by Berthold *et al.* (2000) are underlined; arrows indicate putative di-iron-binding sites. / indicates intron positions. Wavy underlining indicates predicted mitochondrial targeting regions.

Searches for *AOX* genes in a selection of fungal genomes found the sequences included in Figure 3.8. No *AOX* genes were found in the genomes of the other yeast species in the Génolevures database. Figure 3.8 shows the Neighbour-joining tree. The 50% majority-rule consensus of most parsimonious trees was mostly compatible with but less resolved than the trees generated by other methods, with a 9-way basal polytomy in the filamentous ascomycetes (data not shown). Figure 3.9 shows the Maximum Likelihood phylogenetic tree. Bayesian analysis had reached stationarity after 500,000 generations, with the average standard deviation of split frequencies <0.01, the plot of log probability against generation time showing no apparent trend, and PRSF values in the range 1.000-1.040. The 50% majority-rule consensus tree of the maximum posterior probability tree set is shown in Figure 3.10. Figure 3.11 shows the presence of *AOX* genes in sequenced yeast genomes, with gene loss and duplication events predicted based on the maximum likelihood *AOX* gene tree.
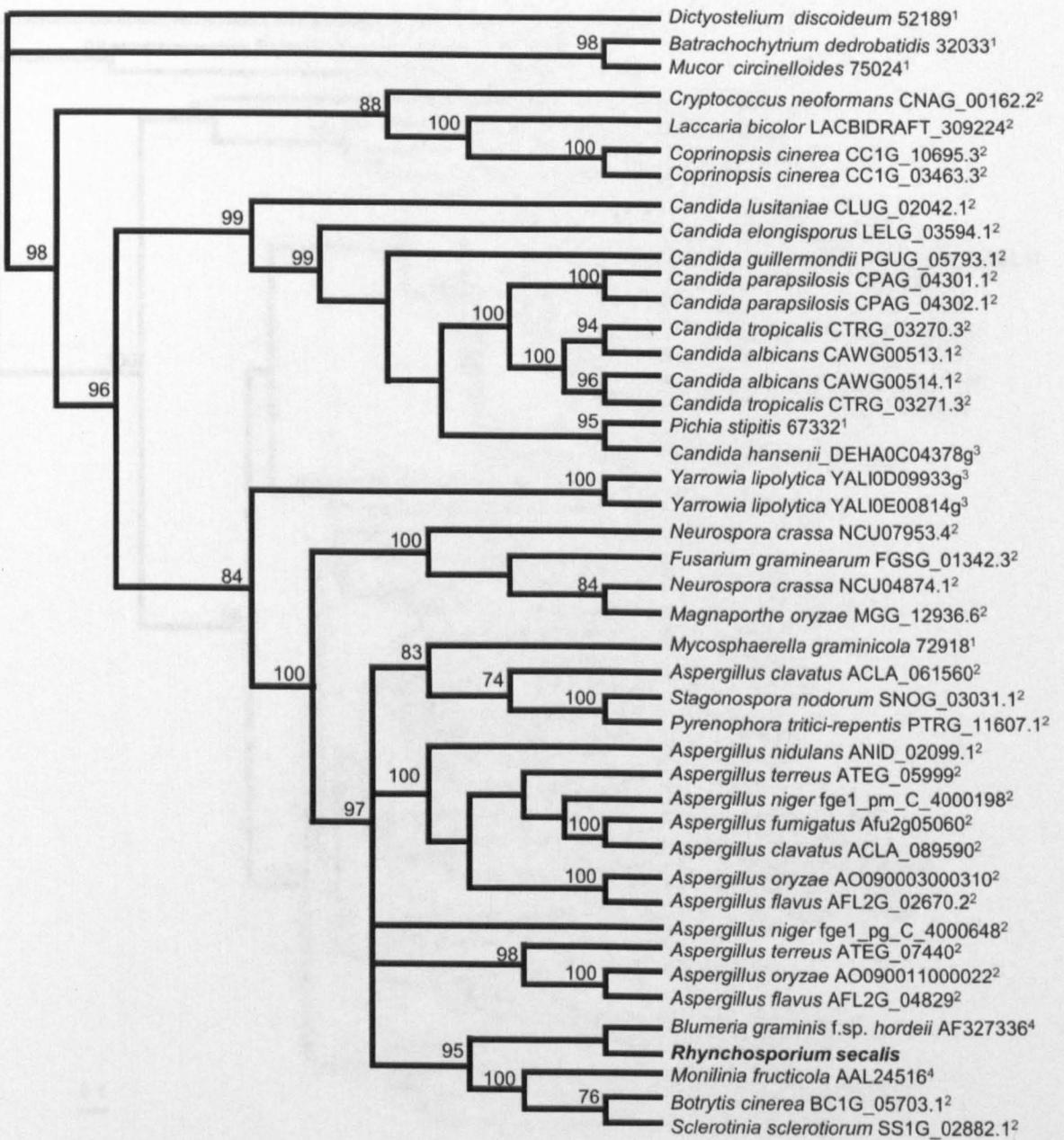
Figure 3.8. Neighbour-joining cladogram of coding DNA sequences of fungal *AOX* genes with *Dictyostelium discoideum* as an outgroup. 50% majority-rule consensus of 100 bootstrap replicates. Gene sources: 1. Joint Genome Initiative (http://www.jgi.doe.gov/), 2. Broad Institute (http://www.broadinstitute.org/scientific-community/data), 3. Génolevures (http://www.genolevures.org/), 4. GenBank (http://www.ncbi.nlm.nih.gov/genbank/). Numbers at nodes indicate bootstrap values over 70.
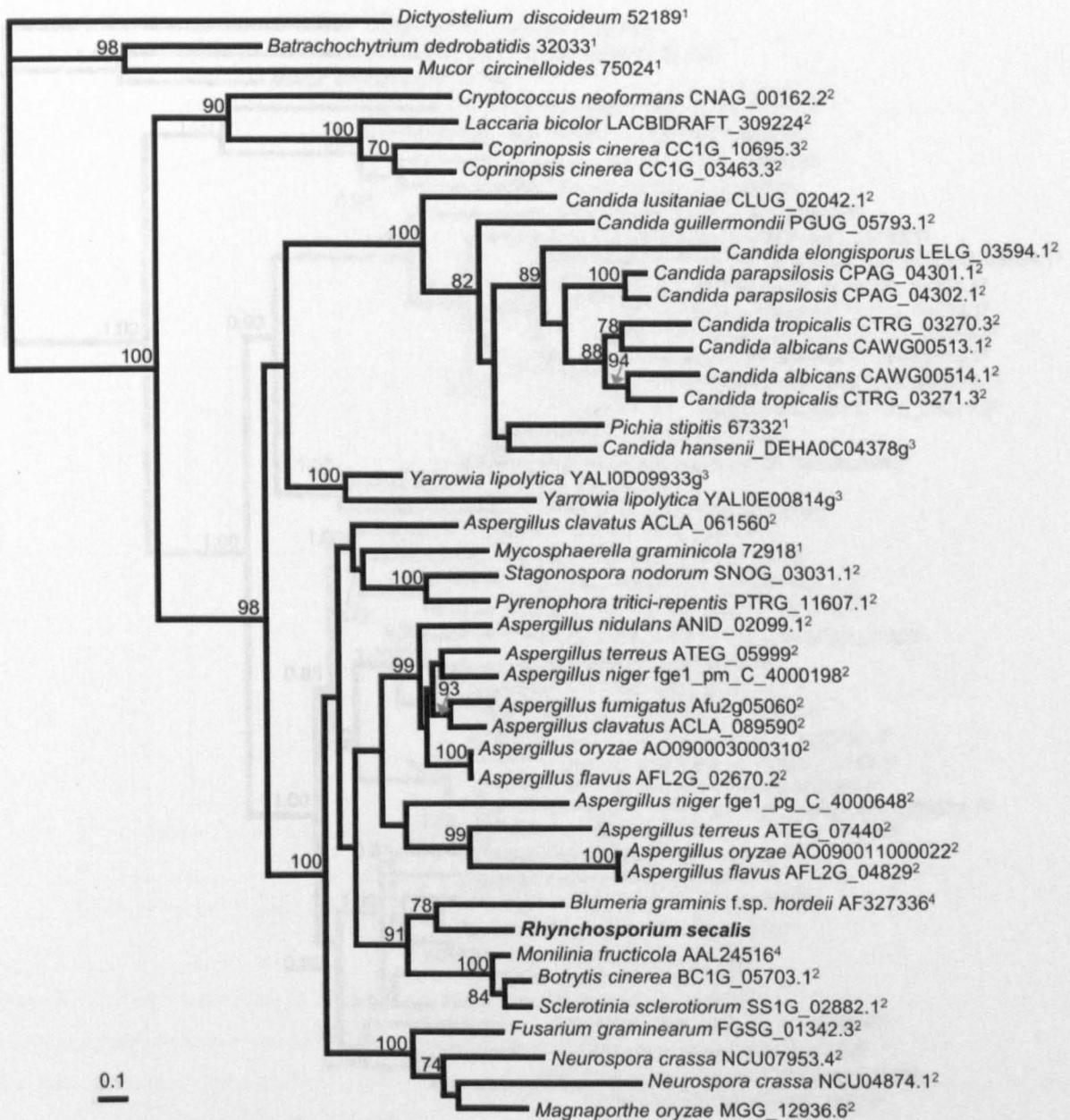
Figure 3.9. Maximum Likelihood phylogram of coding DNA sequences of fungal *AOX* genes with *Dictyostelium discoideum* as an outgroup. Gene sources: 1. Joint Genome Initiative (http://www.jgi.doe.gov/), 2. Broad Institute (http://www.broadinstitute.org/scientific-community/data), 3. Génolevures (http://www.genolevures.org/), 4. GenBank (http://www.ncbi.nlm.nih.gov/genbank/). Numbers at nodes indicate bootstrap values over 70.
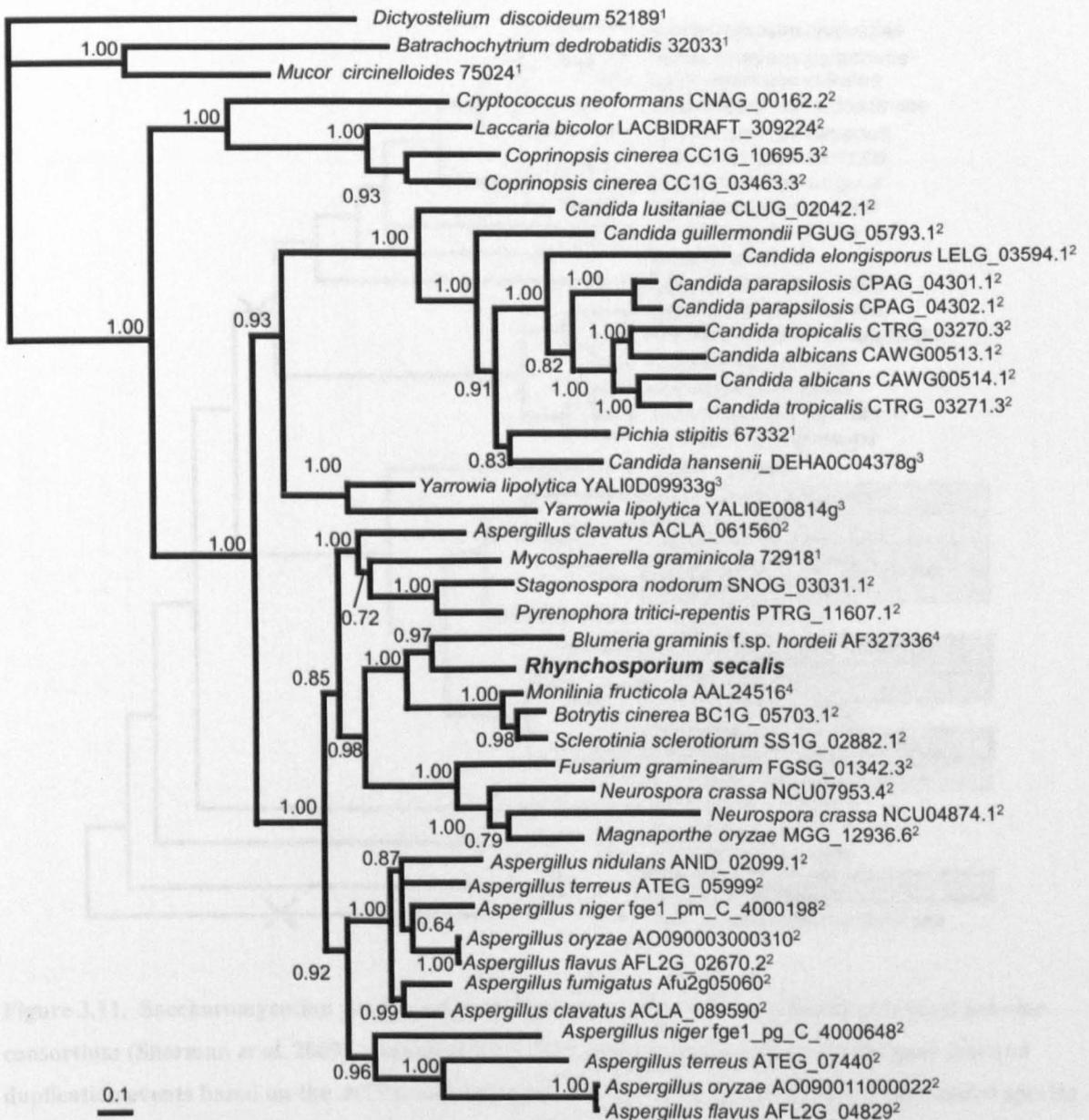
Figure 3.10. Bayesian phylogram (50% strict consensus) of coding DNA sequences of fungal *AOX* genes with *Dictyostelium discoideum* as an outgroup. Gene sources: 1. Joint Genome Initiative (http://www.jgi.doe.gov/), 2. Broad Institute (http://www.broadinstitute.org/scientific-community/data), 3. Génolevures (http://www.genolevures.org/), 4. GenBank (http://www.ncbi.nlm.nih.gov/genbank/). Numbers at nodes indicate posterior probabilities.

Figure 3.11. Saccharomycotina yeast species cladogram compiled by the Génolevures yeast genome consortium (Sherman *et al.* 2009), annotated with *AOX* gene presence, and predicted gene loss and duplication events based on the *AOX* gene phylogeny shown in Figure 3.8 - Figure 3.10. Shaded species have nuclear genomes (complete or draft) available for searching: Pale shading with strikethrough: no *AOX* genes; Mid-grey shading with single outline: one *AOX* gene, Dark shading with double outline: two *AOX* genes. Crosses indicate predicted gene loss events, arrows indicate predicted gene duplication events.

3.3.5 *AOX* expression analysis

AOX expression three days after addition of azoxystrobin is shown in Figure 3.12.
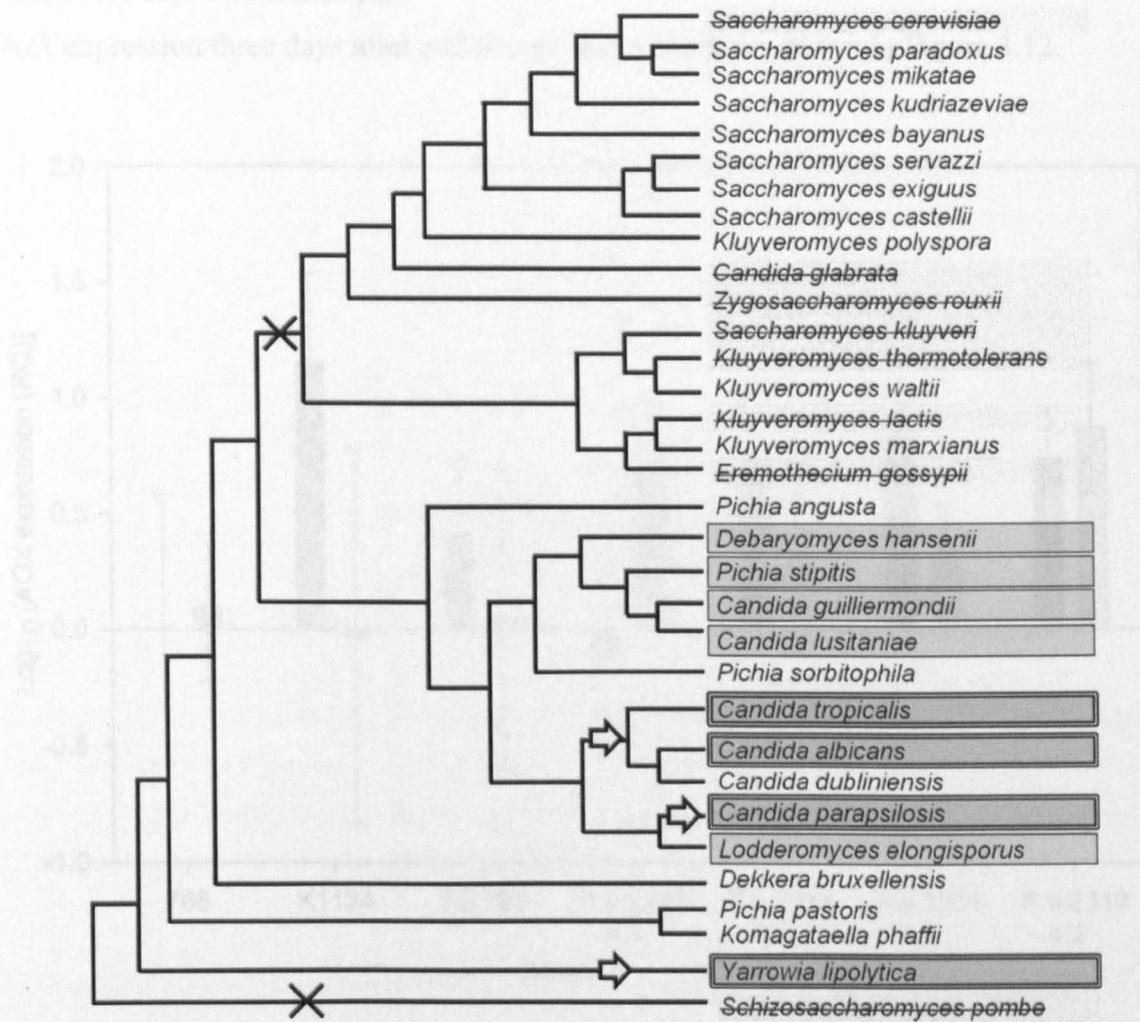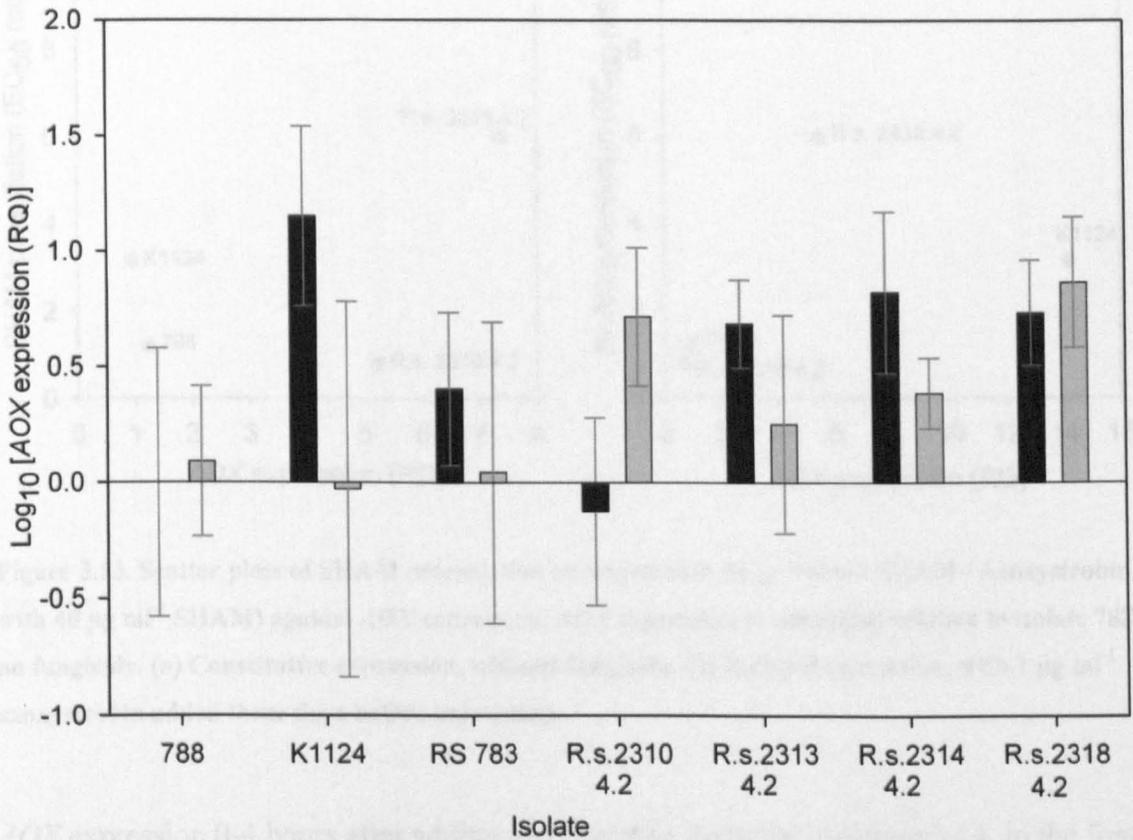
Figure 3.12. *AOX* **expression in** *R. secalis* **isolates three days after adding fungicide. Black: no fungicide; grey: azoxystrobin, final concentration 1 μg ml⁻¹.** *AOX* **expression is calculated relative to isolate 788 with no fungicide.**

There was no correlation between SHAM potentiation (section 3.2.6) and constitutive *AOX* expression, with only acetone added (P=0.995) (Figure 3.13a), nor between SHAM potentiation and induced *AOX* expression, three days after adding 1 μg ml⁻¹ azoxystrobin (P=0.845) (Figure 3.13b).

103

(a)

SHAM potentiation (EC$_{50}$ ratio)

12

10

8

6

4

2

0

● RS 783

R.s. 2318 4.2 ●

● K1124

● 788

● R.s. 2310 4.2

0 1 2 3 4 5 6 7 8

AOX expression (RQ)

(b)

SHAM potentiation (EC$_{50}$ ratio)

12

10

8

6

4

2

0

● RS 783

● R.s. 2318 4.2

K1124 ●

● 788

● R.s. 2310 4.2

0 2 4 6 8 10 12 14 16
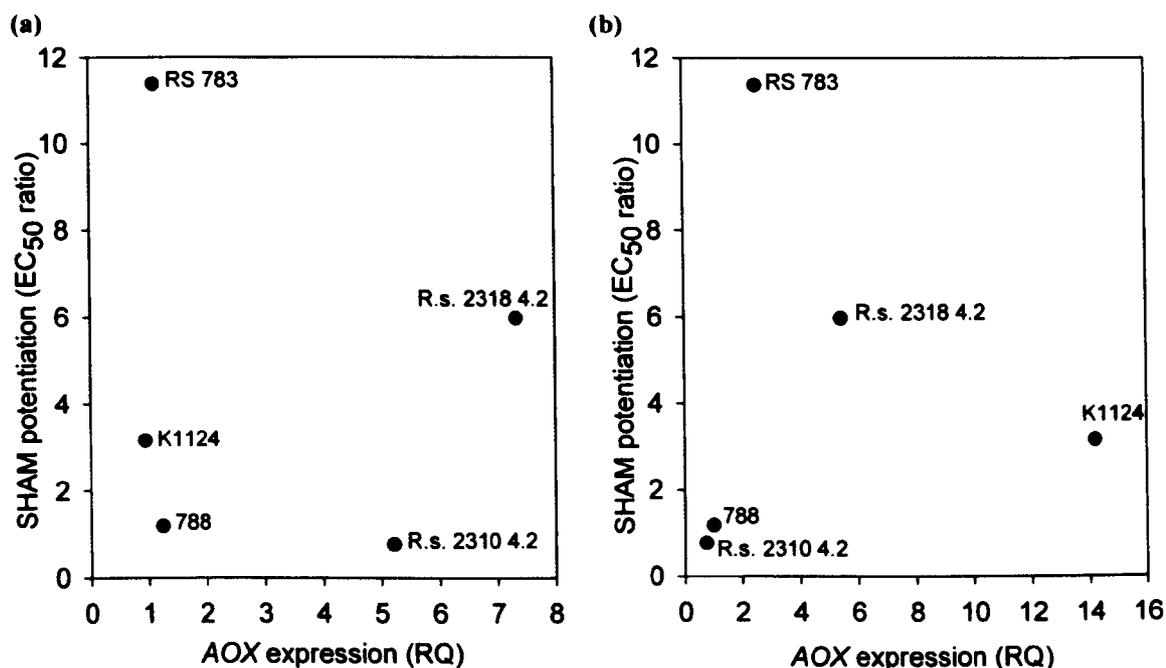
AOX expression (RQ)

Figure 3.13. Scatter plots of SHAM potentiation (Azoxystrobin EC$_{50}$ without SHAM / Azoxystrobin EC$_{50}$ with 40 µg ml^{-1} SHAM) against *AOX* expression. *AOX* expression is calculated relative to isolate 788 with no fungicide. (a) Constitutive expression, without fungicide. (b) Induced expression, with 1 µg ml^{-1} azoxystrobin added three days before harvesting.

AOX expression 0-4 hours after adding azoxystrobin is shown in Figure 3.14. In the first replicate, constitutive expression was similar for all isolates, and *AOX* expression increased in all isolates after the addition of azoxystrobin. Highest observed expression for isolates 788, R 9528.4 and RS 783 was at 120 minutes, and highest expression for isolate K1124 was at 60 minutes, after which transcript levels decreased again. The maximum expression level in isolates 788, K1124 and R 9528.4 was 5-8.5 times the reference (isolate 788 replicate 1 before adding fungicide), whereas the maximum expression for isolate RS 783 was 16.8 times the reference. In the second replicate, induced expression levels were lower in isolates 788, K1124 and R 9528.4, but higher in isolate RS 783. In isolate K1124, elevated constitutive expression was observed, after which expression decreased before a slight increase by 240 minutes. Expression in isolates 788 and R 9528.4 increased slowly after the addition of azoxystrobin throughout the time measured. Expression in isolate RS 783 increased greatly after the addition of azoxystrobin, reaching a peak of 512 times the reference at 60 minutes.
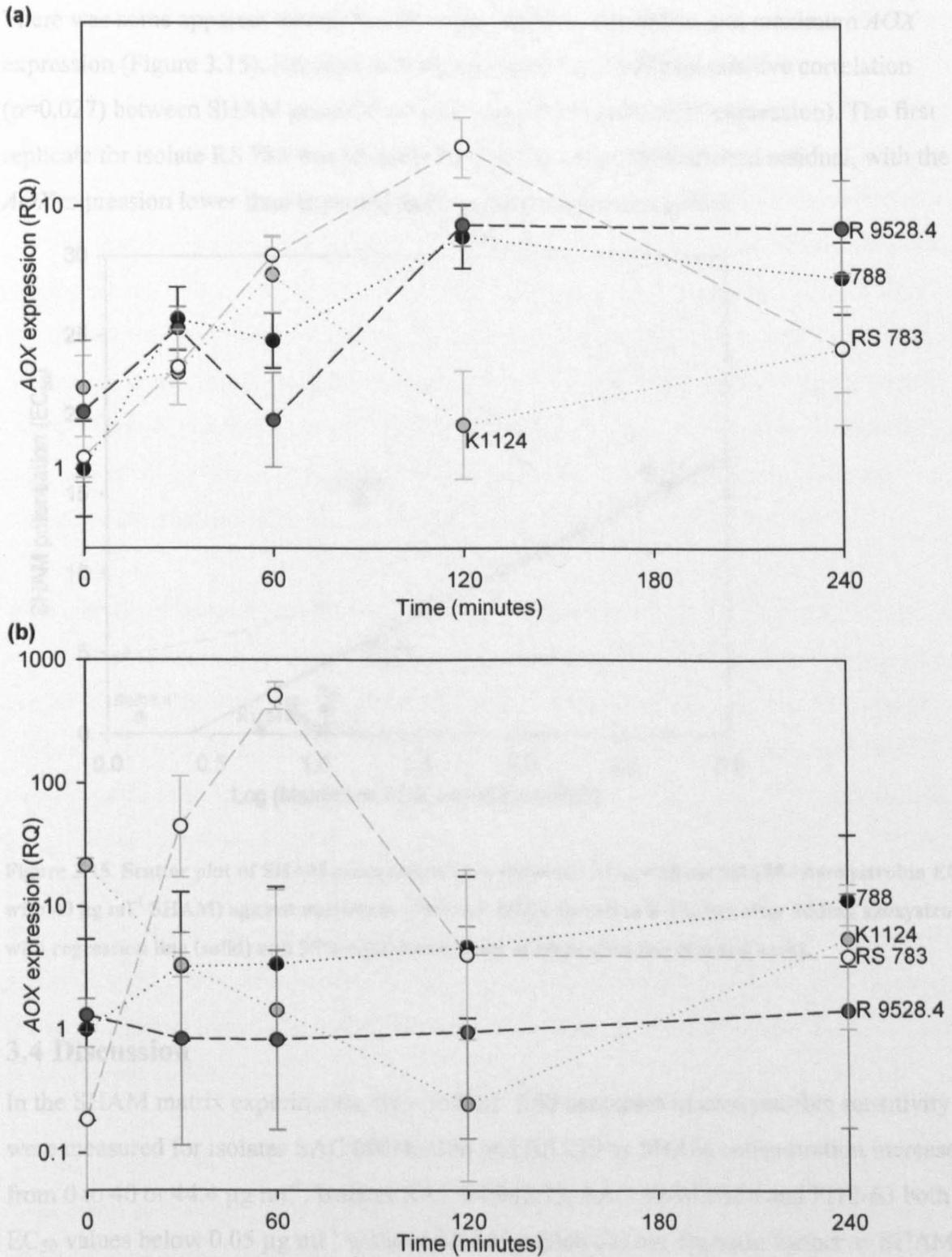
Figure 3.14. Induced *AOX* expression after addition of azoxystrobin to 7-day-old cultures of four *R. secalis* isolates. (a) Biological replicate 1, (b) Biological replicate 2. Black: isolate 788; light grey: isolate K1124; dark grey: isolate R 9528.4; white, isolate RS 783. *AOX* expression is calculated relative to isolate 788 with no fungicide for each set of biological replicates.

There was some apparent association between SHAM potentiation and maximum *AOX* expression (Figure 3.15). Regression analysis showed a significant positive correlation (p=0.027) between SHAM potentiation and Log_{10} (Maximum *AOX* expression). The first replicate for isolate RS 783 was identified as having a high standardized residual, with the *AOX* expression lower than expected under a linear regression model.
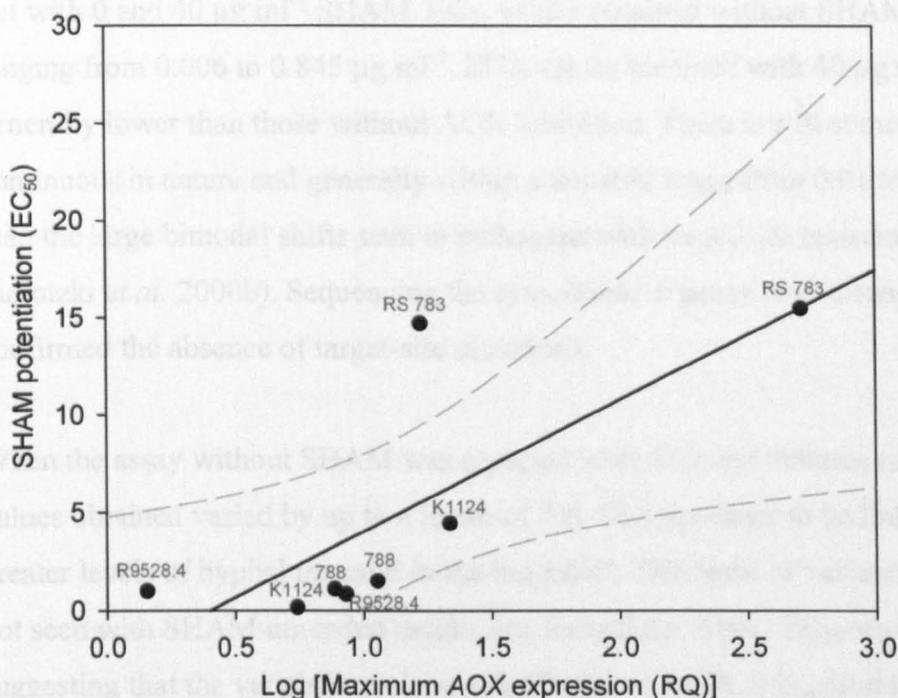
Figure 3.15. Scatter plot of SHAM potentiation (Azoxystrobin EC_{50} without SHAM / Azoxystrobin EC_{50} with 40 μg ml^{-1} SHAM) against maximum observed *AOX* expression 0-4 hours after adding azoxystrobin, with regression line (solid) and 95% confidence limits of regression line (dashed lines).

3.4 Discussion

In the SHAM matrix experiments, two- to four- fold decreases in azoxystrobin sensitivity were measured for isolates SAC 09/943/186 and RS 219 as SHAM concentration increased from 0 to 40 or 44.4 μg ml^{-1}. Isolates SAC 09/943/73, SAC 09/943/115 and FI12-63 both had EC_{50} values below 0.05 μg ml^{-1} without SHAM, which did not decrease further as SHAM concentration increased to 40 or 44 μg ml^{-1}. This is consistent with the results of McCartney (2006), who found that the EC_{50} of a less sensitive isolates decreased two- to three- fold as SHAM concentration increased from 0 to 55.55 μg ml^{-1}, whereas sensitive isolates had EC_{50} values below 0.1 ug ml^{-1} at all sub-lethal concentrations of SHAM. These results suggest that decreased *in vitro* strobilurin sensitivity in pre-2008 isolates is due to AOX activity.

For those isolates with azoxystrobin sensitivity affected by SHAM, EC_{50} values decreased as SHAM concentrations increased from 0 to 44.4 μg ml^{-1}, but at concentrations of 133 μg ml^{-1} and above, SHAM itself inhibited fungal growth. This confirms that 40 μg ml^{-1} is a suitable SHAM concentration to inhibit AOX activity in *R. secalis* azoxystrobin sensitivity assays in liquid medium. For screening pre-2008 isolates, azoxystrobin sensitivity assays were carried out with 0 and 40 μg ml^{-1} SHAM. EC_{50} values obtained without SHAM varied considerably, ranging from 0.006 to 0.845 μg ml^{-1}. EC_{50} values obtained with 40 μg ml^{-1} SHAM were generally lower than those without AOX inhibition. There is still some variability, but this is continuous in nature and generally within a ten-fold range from 0.01 to 0.1 μg ml^{-1}, rather than the large bimodal shifts seen in pathogens with target-site resistance to strobilurins (e.g. Sierotzki *et al.* 2000b). Sequencing the *cytochrome b* genes from a range of isolates confirmed the absence of target-site mutations.

When the assay without SHAM was repeated with different cultures of the same isolate, EC_{50} values obtained varied by up to a factor of 7.6. This appeared to be linked to the presence of greater levels of hyphal material in the inoculum. This level of variability of EC_{50} values was not seen with SHAM-amended media, nor for isolates where EC_{50} was unaffected by SHAM, suggesting that the variability relates specifically to AOX. It is possible that AOX facilitates the growth of established mycelium more than that of spores, due to the reduced efficiency of the alternative respiratory pathway and high energy demands of spore germination. This is also consistent with suggestions that AOX utilisation varies with developmental stage (Wood and Hollomon 2003). This variability means when comparing EC_{50} against *AOX* expression, the same batch of inoculum, and not just the same isolate, must be used for sensitivity assays and RNA extraction.

SHAM potentiation values, i.e. the EC_{50} without SHAM divided by the EC_{50} with SHAM (Wood and Hollomon 2003; section 3.2.6), for 40 μg ml^{-1} SHAM, varied from around 1 up to 15.1, but most were in the range of 1 to 5. This is consistent with the results of McCartney (2006), who observed a potentiation factor of 2.5 with 55.6 μg ml^{-1} SHAM in a less sensitive isolate. However, many isolates with AOX activity indicated by SHAM potentiation also showed trailing growth, a low level of residual growth above the EC_{50} value. This is also absent in the presence of SHAM, implying that this, too, is a result of AOX activity. This could have a role in the emergence of more resistant isolates, since it would allow a low level

of fungal growth to continue in the presence of strobilurin fungicides, but only if the effect is seen *in planta*.

No correlations between azoxystrobin $\log(EC_{50})$ values with or without 40 µg ml^{-1} SHAM and $\log(EC_{50})$ values for the four triazoles tested in Chapter 4 were significant at 1%. This is consistent with triazoles and strobilurins having different modes of action, and not showing cross-resistance. EC_{50} values for azoxystrobin with SHAM and tebuconazole were correlated at 5% significance, but this was a far weaker correlation than between the pairs of triazoles, and probably a result of separate mechanisms conferring multiple decreases in sensitivity between the least and most sensitive isolates.

There was a significant positive correlation between azoxystrobin EC_{50} values with and without 40 µg ml^{-1} SHAM, but the relationship was only present for those isolates with the lowest azoxystrobin EC_{50} values in the absence of SHAM. Therefore slight variation in azoxystrobin sensitivity amongst the most sensitive isolates is not affected by SHAM at the concentration used, but among less sensitive isolates, further increases in azoxystrobin EC_{50} are negated by the addition of SHAM and therefore likely to be due to AOX activity.

Of the isolates collected in France in 2008, R.s. 2310 4.2 had EC_{50} values within the sensitive range of pre-2008 isolates. Isolate R.s. 2318 4.2 had EC_{50} values consistent with AOX activity as seen in the pre-2008 isolates, with a SHAM potentiation factor of 5.58 for pyraclostrobin. Growth of isolates R.s. 2313 4.2 and R.s. 2314 4.2 was not sufficiently inhibited by azoxystrobin at the concentrations used to calculate EC_{50} values, so assays were repeated with pyraclostrobin, which has a higher intrinsic activity. Pyraclostrobin sensitivity of isolates R.s. 2313 4.2 and R.s. 2314 4.2 was reduced by a hundred-fold relative to sensitive isolates. This was far outside the range seen in the pre-2008 isolates, and closer to the 200-fold shift found in *B. graminis* f.sp. *tritici* isolates with the G143A substitution (Sierotski *et al.* 2000). Sequencing the *cytochrome b* gene from these *R. secalis* isolates confirmed that isolates R.s. 2313 4.2 and R.s. 2314 4.2 had a cytosine in place of a guanine at nucleotide position 428, resulting in the G143A amino acid substitution.

However, unlike in other pathogens including *B. graminis* f.sp. *tritici* and *M. graminicola*, in which the G143A mutation arose and spread, G143A has not been detected again in *R. secalis* in 2009 or 2010 (FRAC QoI Working Group 2009; FRAC QoI Working Group 2010).

Prior to 2008, when the G143A mutation had not been detected, it was suggested that the lack of G143A in *R. secalis* was due to low mutation rate (Gisi *et al.* 2002), or the structure of the *cytochrome b* gene making a G143A mutation lethal (Grasso *et al.* 2006). McCartney (2006) noted that *R. secalis cytochrome b* did not contain an intron after codon 143, so G143A-encoding mutations would not caused a lethal loss of splicing, but Fisher *et al.* (2004) demonstrated that G143A may be detrimental to protein function in some cytochrome *b* backgrounds. The comparatively long time for G143A to be detected in *R. secalis* may be due to lower mutation rates than some other pathogens. However, this does not explain why having arisen, the mutation has not yet spread. This may be partly due to absence of airborne ascospores in *R. secalis* (Fountaine *et al.* 2010). In 2008, two field isolates carrying the G143A substitution were found. These isolates appear to grow normally in culture, and are capable of SHAM-insensitive respiration. This demonstrates that *R. secalis* cytochrome *b* with G143A is a functional protein, although more subtle *in planta* fitness costs, such that the mutation is only favoured under strong selection by QoI fungicides, cannot be discounted. However, in the case of the MBC fungicides, slow emergence and erratic early spread of resistant mutants was followed by spread of resistance resulting in loss of disease control (Cooke and Locke 2002). Therefore, resistance management for the QoI fungicides remains important.

SHAM potentiation of azoxystrobin sensitivity in isolates without *cytochrome b* mutations suggested AOX activity. Searching the *R. secalis* genome with *AOX* genes of other leotiomycetes gave one significant match. The sequence contained two predicted introns, subsequently confirmed by cDNA sequences. Analysis of the predicted amino acid sequence with MitoProt found a potential mitochondrial targeting sequence. The predicted cleavage site was 11 base pairs downstream of that for other leotiomycete and most other filamentous ascomycete sequences. This is because MitoProt identifies the last possible cleavage site before other required properties of a mitochondrial targeting site are violated, and the *R. secalis* sequence contains an additional R-X-(F/I/L) motif (Gavel and von Heijne 1990) due to the isoleucine at residue 64. However, it also contains the predicted cleavage site present in the other leotiomycetes, with the R-X-(F/I/L) motif present at residues 51-53. Therefore the exact site at which the protein is cleaved in unclear, especially since the sites in other species are only predictions, but the protein is likely to be mitochondrially imported. Searching the predicted protein against the Pfam database confirmed that it is an Alternative Oxidase.

Maximum likelihood and Bayesian phylogenetic trees were largely congruent, apart from relationships between the Pezizomycotina classes. Neighbour-joining and maximum parsimony trees were similar but less resolved. The *R. secalis AOX* gene fell within the leotiomycete clade, as expected. Multiple *AOX* genes have been reported in some species. Two *AOX* genes were reported in *C. albicans*, one constitutively expressed and the other inducible (Huh and Kang 2001). Tanton *et al.* (2003) reported two *AOX* genes in *N. crassa*, one expressed at a low level constitutively and at a higher level when induced, and the other not expressed under studied conditions.

McDonald and Vanlerberghe (2006) list *B. cinerea* as containing two *AOX* genes, but the sequences listed were found to be two different fragments from a cDNA library, both of which fall within the same gene (BC1G_05703.1) in the *B. cinerea* genome. Similarly, the two listed sequences for *Coccidioides posadasii* are fragments of one gene (CPAG_01812.1 in isolate RMSCC 3488, genome at www. broadinstitute.org). The second sequence listed for *Podospora anserina* (AF252256) was incomplete, gave no significant matches from the *P. anserina* genome (http://podospora.igmors.u-psud.fr), and the only fungal *AOX* gene returned from a BLAST search of GenBank was from *Penicillium chrysogenum*. Therefore, it is not clear whether this represents a second *AOX* gene in some strains of *P. anserina* or a contaminant.

In addition to *C. albicans* and *N. crassa*, two *AOX* genes were found in *Candida tropicalis*, *C. parapsilosis*, *Yarrowia lipolytica*, *C. cinerea*, *Aspergillus clavatus*, *A. oryzae*, *A. flavus*, *A. terreus* and *A. niger*. Phylogenetic analysis shows that these represent several relatively recent duplications, rather than a basal duplication followed by widespread losses. The two *C. cinerea AOX* genes group together, indicating a recent duplication within the *C. cinerea* lineage. Within the Saccharomycotina yeasts, the *AOX* phylogeny supports separate duplications in the *C. tropicalis/C. albicans* lineage and in the *C. parapsilosis* lineage, rather than a single duplication followed by a loss in *Lodderomyces elongisporus*, and a separate duplication in *Y. lipolytica*, rather than a basal duplication followed by repeated losses. Furthermore, the Saccharomycotina *AOX* genes fall as expected within the fungi, supporting the presence of *AOX* as the ancestral state, with secondary losses in the *Schizosaccharomyces* and *Saccharomyces/Kluyveromyces* lineages. Within the Pezizomycotina, the duplication leading to the second *AOX* gene in *N. crassa* appears to have originated within the Sordariomycetidae, but within this subclass, resolution between the *M. oryzae* and *N. crassa*

genes is poorly supported. Therefore it is not possible to say whether the duplication took place within the *N. crassa* lineage, or in the common ancestor of *N. crassa* and *M. oryzae* followed by a loss from *M. oryzae*. Within *Aspergillus* sp., there appears to have been a duplication in the common ancestor followed by losses in *A. fumigatus* and *A. nidulans*, although the placement of the second *A. clavatus* gene with the Dothidiomycetes cannot be explained at present. There is no evidence of any *AOX* gene duplications ancestral to *R. secalis*, as the divergence of paralogues with different expression patterns is confined to within other lineages, and therefore the single *AOX* gene in *R. secalis* is descended from an undifferentiated *AOX* orthologue.

The *AOX* genomic DNA sequences of the three *R. secalis* isolates tested were identical. Therefore, apparent differences in AOX activity between isolates are not due to mutations in coding DNA, but may be due to differences in gene expression. Initially, *AOX* expression levels were measured three days after adding the QoI fungicide azoxystrobin. Only in isolate R.s. 2310 4.2 was *AOX* expression significantly higher with azoxystrobin than in the control, and this isolate had a low SHAM potentiation. SHAM potentiation was not correlated with constitutive (p=0.995) or induced *AOX* expression (p=0.845) among isolates without target-site resistance. Kaneko and Ishii (2009) reported *AOX* expression in *F. graminearum* reaching a peak 60 minutes after the addition of azoxystrobin, then falling back towards the constitutive expression level. Therefore, the experiment was repeated with cultures harvested immediately before, and 30, 60, 120 and 240 minutes after, addition of azoxystrobin. Induced expression was observed within 30-120 minutes, and in isolate RS 783, the isolate showing the greatest upregulation, expression was decreasing again by 240 minutes. Therefore, induced expression of *AOX* in *R. secalis* should be measured within 1-2 hours of fungicide addition, as was shown by Kaneko and Ishii (2009) for *F. graminearum*. Yukioka *et al.* (1998) reported that *AOX* expression in *M. oryzae* increased 20-60 minutes after addition of metominostribin, but only measured up to 60 minutes so it is not known how long the increased transcript levels lasted. Sierotzki *et al.* (2000a) report that in *M. fijiensis* isolates with a SHAM potentiation factor of 130 for azoxystrobin, *AOX* expression appears not to be inducible by trifloxystrobin. However, the trifloxystrobin was added 12 hours prior to harvesting the cultures, so given the current results and those of Kaneko and Ishii (2009), upregulation may have taken place more rapidly after QoI addition, with transcript levels returning to basal levels within 12 hours.

Constitutive *AOX* expression, before addition of azoxystrobin, was similar for all isolates, but there was variation in induced expression 30-240 minutes after addition of azoxystrobin. The greatest upregulation was observed in isolate RS 783, the isolate with the greatest SHAM potentiation in azoxystrobin sensitivity. There was a positive correlation (p=0.027) between Log_{10}(Maximum *AOX* expression) and SHAM potentiation. Kaneko and Ishii (2009) showed that greater induced *AOX* expression was associated with lower intrinsic azoxystrobin sensitivity in *F. graminearum* compared to *M. nivale*. Here, it is demonstrated that in *R. secalis*, differences in induced *AOX* expression may be associated with intraspecific differences in QoI sensitivity in the absence of SHAM.

The first biological replicate for isolate RS 783 appears anomalous, with the maximum *AOX* expression lower than expected given the SHAM potentiation value under the fitted linear regression model, and thirty times lower than the observed *AOX* expression for the second biological replicate. It is possible that the maximum *AOX* expression was between measured time points. The maximum observed *AOX* expression in the first biological replicate of isolate RS 783 was at 120 minutes, whereas in the second biological replicate the maximum observed expression was at 60 minutes. Due to the heterogeneous growth of *R. secalis* in liquid culture, it is not practicable to take representative aliquots. Consequently, a separate culture must be grown for each time point, limiting the number of time points for which measurements can be made. For future work, the use of promoter-reporter fluorescence gene constructs will allow repeated non-destructive measurements of expression over time. Furthermore, only *β-tubulin* was used as an endogenous control. The use of additional endogenous control genes would reduce the risk that apparent changes in relative expression of the target gene are actually the result of confounding changes in the expression of the control.

Expression analysis provides preliminary evidence for transcriptional upregulation of *AOX*, whether by increased transcription or reduced transcript degradation (Yukioka *et al.* 1998), in some *R. secalis* isolates. The *R. secalis* genome sequence upstream of the *AOX* coding sequence did not contain the induction motif identified in *N. crassa* (Chae *et al.* 2007), nor any of the putative transcriptional elements identified in *M. oryzae* (Yukioka *et al.* 1998). This is not surprising, since the induction motif in *N. crassa* was only found within the Sordariales and not in *M. oryzae*, a more distant Sordariomycete, and *R. secalis* as a Leotiomycete is more taxonomically distant from *N. crassa* and *M. oryzae* than they are from

each other. When the *R. secalis* genome is released and annotated, it may be possible to further investigate the regulatory pathways involved in *AOX* upregulation in this species.

It has been suggested that increased AOX activity may facilitate the evolution of target-site resistance, by enabling slow growth in the presence of QoI fungicides, under selective pressure for resistance and where mitochondrial mutation rates may be elevated due to high levels of reactive oxygen species (Avila-Adame and Köller 2003b). This could be further investigated by assessing *in planta* growth, including microscopic assessment of symptomless infection, of isolates with different levels of *AOX* upregulation or an *AOX* knockout strain, on QoI-treated plants. Alternatively, *in vitro* experimental evolution by serial transfer on QoI-amended media could be carried out, to investigate the effects of SHAM, flavones or *AOX* gene deletion on the arising of QoI-resistant strains. Since *AOX* over-expression was only observed when induced by QoIs, it was not possible to tell whether this upregulation was present in isolates with the G143A mutation, since it was not possible to achieve an inhibitory dose of fungicide for these isolates without killing all other isolates. If the molecular mechanisms for upregulation are identified in the future, it may be possible to test isolates for the corresponding genotype in order to establish whether the G143A mutation arose in a genetic background with *AOX* overexpression.

Chapter 4

Triazole sensitivity in *R. secalis*

4.1 Introduction

The triazoles are demethylation inhibitor (DMI) fungicides, inhibiting lanosterol 14α-demethylase in ergosterol synthesis (Buchenauer 1977), resulting in ergosterol depletion and build-up of toxic sterol intermediates such as 14-methyl-3,6-diol (Ragsdale and Sisler 1973). Lanosterol 14α-demethylase is a cytochrome P450, CYP51.

Reduced triazole sensitivity due to mutations in the target-site-encoding *CYP51* genes has been reported in several plant and clinical pathogens, including *M. graminicola* (Cools and Fraaije 2008), *B. graminis* f.sp. *hordei* (Wyand and Brown 2005) and *C. albicans* (Edlind 2008). These mutations have varying, interacting, quantitative effects on triazole sensitivity, in contrast to the qualitative QoI resistance conferred by the G143A substitution in cytochrome *b*. Furthermore, cross-resistance patterns between azoles (triazoles and imidazoles) vary for different mutations. For example, in clinical *C. albicans* isolates, substitutions S405F, G464S and R467K confer similar reductions in sensitivity to fluconazole and ketoconazole, whereas Y132H affects ketoconazole sensitivity fourfold more than fluconazole sensitivity (Sanglard *et al.* 1998). In *M. graminicola*, Y137F specifically affects triadimenol sensitivity, whereas I381V reduces sensitivity to tebuconazole (Cools and Fraaije 2008).

Some fungal species possess two paralogues of *CYP51*, designated *CYP51A* and *CYP51B* (Mellado *et al.* 2001). Mutations associated with reduced triazole sensitivity in clinical isolates of *A. fumigatus* are found in the *CYP51A* paralogue (Diaz-Guerra *et al.* 2003). The phylogeny of *CYP51* paralogues is investigated, and the nomenclature explained, in chapter 5.

Some fungal isolates with reduced triazole sensitivity do not possess any mutations in *CYP51*. Non-target-site mechanisms include increased efflux of the fungicide out of fungal cells by ABC transporter (de Waard *et al.* 2006) or major facilitator proteins (Tenreiro *et al.* 2000). Different transporters may confer reduced sensitivity to a single compound or multiple

triazoles (Sanglard and Odds 2002), or to triazoles and unrelated compounds such as QoIs (Reimann and Deising 2005).

In some cases, reduced azole sensitivity has been linked to specific transporter-encoding genes, such as *BcatrD* in *B. cinerea* (Hayashi *et al.* 2002), or reduced intracellular azole accumulation has been measured directly with radio-labelled fungicides (Hayashi *et al.* 2001; Roohparvar *et al.* 2008). In other cases, enhanced efflux has been inferred from the restoration of fungicide sensitivity by the addition of putative efflux inhibitors. A range of drugs known to affect mammalian efflux transporters and other transmembrane protein targets have been tested for efflux inhibition. These include the phenothiazines, calcium-calmodulin antagonists in clinical use as antipsychotics, which increased triazole sensitivity of less-sensitive isolates of both *C. albicans* (Marchetti *et al.* 2000) and *M. graminicola* (Roohparvar *et al.* 2008).

Reduced triazole sensitivity has been reported in field populations of *R. secalis* (Jones 1990; Kendall *et al.* 1993; Cooke *et al.* 2004). Sensitivity shifts against triadimenol were first reported in a survey of UK field isolates in 1987 (Jones 1990). By 1990, triadimenol sensitivity had declined further, resulting in loss of field control. A correlated but smaller sensitivity shift was seen for propiconazole and tebuconazole, but these compounds remained effective in the field (Kendall *et al.* 1993). In 2000-2002, sensitivity shifts were seen against the newly-introduced epoxiconazole (Oxley 2003). In 2005-2007, further shifts in epoxiconazole sensitivity were reported, with some evidence of cross-resistance with the newer prothioconazole (Oxley *et al.* 2008). Current monitoring of *R. secalis* in the UK suggests some reductions in azole sensitivity, but epoxiconazole and prothioconazole still provide field control, albeit at higher doses, or in more of a protectant capacity in the case of epoxiconazole (Oxley and Burnett 2010). The mechanisms causing triazole sensitivity shifts in *R. secalis* have not yet been identified.

This chapter describes investigations into triazole sensitivity in *R. secalis* and whether changes in sensitivity are due to mutations in *CYP51* genes, or associated with efflux pump activity. Sensitivity to propiconazole, tebuconazole, epoxiconazole and prothioconazole was tested for 80 *R. secalis* isolates, and cross-resistance patterns investigated. Sensitivity testing was also carried out for an additional set of 31 isolates collected in six European countries in 2009 by Syngenta Crop Protection, and cross-resistance patterns compared. Field isolates

were collected from experimental plots at Rothamsted Research in 2009 and their sensitivity compared to the reference and Syngenta isolate sets. The full sequence of *CYP51A* was found by genome-walking, and *CYP51A* and *CYP51B* sequences were analysed for 12 isolates with a range of triazole sensitivities. A *CYP51A* pseudogene, *CYP51A-P*, was also discovered and was sequenced for all isolates. Further sensitivity assays were carried out with the addition of putative efflux inhibitors to investigate the possible role of efflux pump activity in triazole sensitivity.

4.2 Materials and Methods

4.2.1 Triazole sensitivity testing

Sensitivity testing of the isolates listed in Table 2.1 was carried out as described in section 3.2.1, but with the fungicides and concentrations shown in Table 4.1. An additional set of 31 isolates, collected from several sites in the Czech Republic, Germany, France, Spain, the United Kingdom and Ireland in 2009, provided by Syngenta Crop Protection, were also tested for fungicide sensitivity.

Table 4.1. Final fungicide concentrations used in triazole sensitivity bioassays, carried out in 96-well microtitre plates.

Microtitre plate column	1	2	3	4	5	6	7	8	9	10	11	12
Fungicide	Final fungicide concentration ($\mu g\ ml^{-1}$) (3 s.f.)											
Epoxiconazole	0	0.00524	0.0131	0.0327	0.0819	0.205	0.512	1.28	3.2	8	20	50
Prothioconazole, Tebuconazole, Propiconazole	0	0.00169	0.00508	0.0152	0.0457	0.137	0.412	1.24	3.70	11.1	33.3	100
Tebuconazole, Propiconazole (less sensitive isolates)	0	0.00508	0.0152	0.0457	0.137	0.412	1.24	3.70	11.1	33.3	100	300

Cross-resistance was assessed by testing for correlation (least squares linear regression, with pairwise deletions of missing values) between $\log_{10}(EC_{50})$ values for each possible pairing of the fungicides tested, and by Principal Componenets Analysis (PCA) using the correlation

matrix of $\log_{10}$ transformed EC_{50}s for all four fungicides, in GenStat 13th Edition (VSN International, Hertfordshire, UK).

4.2.2 Field conditions and *R. secalis* isolation

Saffron winter barley was grown in field Great Harpenden II at Rothamsted Research, Hertfordshire, UK, according to standard farm operations but untreated with fungicides or treated with two sprays at 1/3 full rate of epoxiconazole (Opus, BASF; 125g l^{-1}, 0.33 l ha^{-1}) or propiconazole (Bumper 250EC, Makhteshim Agan; 250 g l^{-1}, 0.16 l ha^{-1}) at T0 and T1. Infected leaves were collected three weeks after the T1 fungicide application.

Lesions were cut from leaves, surface-sterilised for 2 minutes in 70% ethanol and 5 minutes in 1/10 10% sodium hypochlorite, rinsed in sterile water, dried on autoclaved tissue paper then placed on CDM agar containing 125 units ml^{-1} penicillin and 125 μg ml^{-1} streptomycin (Sigma), and incubated at 18°C for seven days. *Rhynchosporium secalis* spores were harvested into sterile distilled water and streaked out onto CDM plates to obtain single-spore isolates. Fungicide sensitivity testing was carried out as described in section 4.2.1. PCA was carried out, using the correlation matrix of $\log_{10}$ transformed EC_{50}s for the four fungicides, in GenStat 13th Edition (VSN International, Hertfordshire, UK).

4.2.3 Sequencing of *R. secalis* CYP51A by genome-walking

CYP51B had been sequenced from *R. secalis* (Hans Cools, personal communication). A second *CYP51*, *CYP51A*, had been found in *R. secalis* and a partial sequence had been obtained with degenerate primers (Helge Sierotzki, personal communication). The remainder was found by genome-walking. Genomic DNA libraries were prepared by Hans Cools, using the GenomeWaker universal kit (Clontech, California, USA), with restriction enzymes *DraI* (two libraries), *PvuII* (two libraries), *EcoRV* and *StuII*. Three reactions in the 5' direction and one in the 3' direction were needed to obtain the complete coding sequence of the gene (Figure 4.1). For each genome-walking step, two nested PCR reactions were carried out: the first with AP1 and GSP1, the second with AP2 and GSP2. Adaptor primers (AP1 and AP2) were specified by the GenomeWalker Universal Kit (Clontech, California, USA). Genome-specific primers (GSP1 and GSP2) used in successive reactions are listed in Table 4.2, and were designed in Vector NTI (Invitrogen Corporation).

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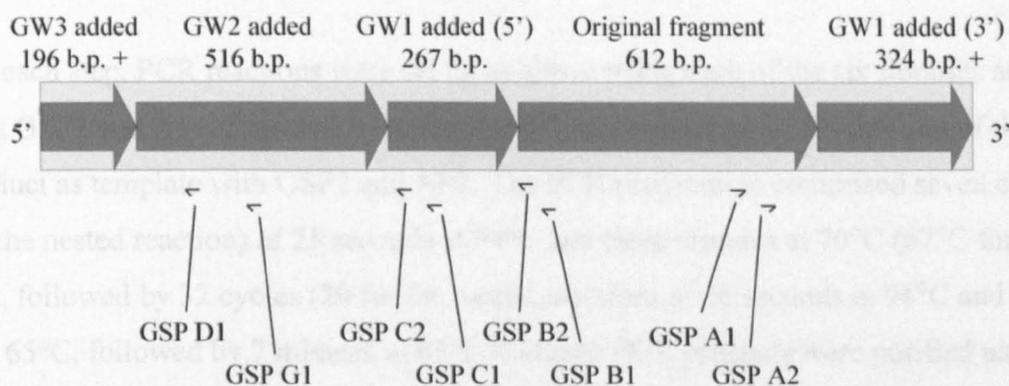
Figure 4.1. Primer-binding sites and sections added to the sequence of the second *CYP51* gene of *R. secalis* in successive genome walks. GSP = Gene-Specific Primer; primers listed in Table 4.2.

Table 4.2. List of gene-specific primers used in successive genome-walking reactions to obtain the full sequence of *R. secalis* *CYP51A*, and primers used for the amplification and sequencing of *CYP51* genes from *R. secalis* isolates

Gene	Primer name	Primer sequence
CYP51	A1: GW1 (3'), GSP1[a]	5'-TGGTGAAGGAGAC GCTCCGGCTCCATT-3'
Genome	A2: GW1 (3'), GSP2	5'-TGATGCGCGCAGT CAAGAACGACCTCC-3'
walking	B1: GW1 (5'), GSP1	5'- TTCTGAGGCAGTGG TAACCACGGAGCGAG -3'
	B2: GW1 (5'), GSP2	5'- ATTGGCCGGAACC CGTGGTCGAGATCA -3'
	C1: GW2 (5'), GSP1	5'- AGAGGGAGAGGTAG GCGATTGGGGAATGA -3'
	C2: GW2 (5'), GSP2	5'- TTCGAGTGCGCGTT GAGTGAGGCCGAA -3'
	G1: GW3 (5'), GSP1	5'- CGTTGACATCCTGCA ATCTTCCATTGAGGA -3'
	D2: GW3 (5'), GSP2	5'- TGTGCTGGAGGAAGA GGGAGGGTGAGAGA -3'
CYP51A	Forward 1	5'-ATGCTGGGTATCTTCTCGGTGCTAG -3'
	Reverse 1	5'- GACCCTCTTTTCCCATCTAACTCTCG-3'
	Internal	5'-TGCTAACTCGGCATATCTAGCTACACC -3'
	Nested F	5'-TCCGCTATGGATTCTACACCCTCA -3'
	Nested R	5'- CCGTCTTTGGGATCTGTCCTCC -3'
CYP51A-P	Forward 1	5'-AAAAGAAGAAGTCCGAACCGCCTC -3'
	Reverse 1	5'-CACCAAACGGTAAATAAGGAATCCTAATCT -3'
CYP51B	Forward 1	5'- GGAATTTTTGAGGCTGTTACAGTCCC-3'
	Reverse 1	5'- TTCTCTCTTCTCCCACTCAACCACC-3'
	Internal	5'- CCTTCAAGACTTACGTTCCAATCATTCAG-3'
	Nested F	5'- GCGGATTGGGCGTTGTGATC-3'
	Nested R	5'-CTCAACAACCTTCGAGCTTCCATC -3'

[a] GW: Genome Walk, GSP: Gene-Specific Primer

For each step, PCR reactions were set up as above using each of the six libraries as templates with GSP1 and AP1, followed by a nested PCR reaction using a 1/50 dilution of the first PCR product as template with GSP2 and AP2. The PCR programme comprised seven cycles (five for the nested reaction) of 25 seconds at 94°C and three minutes at 70°C (67°C for primer G1), followed by 32 cycles (20 for the nested reaction) of 25 seconds at 94°C and 3 minutes and 65°C, followed by 7 minutes at 65°C. Longest PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega, USA). Cleaned PCR products were ligated into the pGEM-t Easy plasmid vector (Promega, USA) using T4 DNA ligase (Promega, USA) according to manufacturer's instructions. JM109 competent cells were then transformed according to manufacturer's instructions, using blue-white screening for plasmids with inserts. PCR reactions were set up as described above, using 5μl of white transformant bacterial suspension as template, and primers AP2 and GSP2. The PCR programme started with 2 minutes 30 seconds at 94°C to lyse the bacterial cells; followed by 40 cycles of 30 seconds at 94°C, 60 seconds at 52°C and 60 seconds at 72°C; followed by 5 minutes 30 seconds at 72°C. One transformant for each PCR product was selected for sequencing. The bacterial suspension was added to 5ml LB broth and incubated overnight at 37°C with shaking. Bacterial pellets were collected from 3ml culture. Plasmid DNA was extracted and purified using the GeneElute (Sigma) or QIASpin (Qiagen) mini-prep kits and eluted into 50μl nuclease-free water, then sequenced by Eurofins MWG (Germany) and the sequences analysed in Vector NTI 10 (Invitrogen).

4.2.4 Sequencing of *CYP51* genes from *R. secalis* isolates

The *CYP51A* and *CYP51B* genes of isolates showing a range of triazole sensitivities were sequenced. These were fully-sensitive isolates K1124, FI12-63 and RS-219; intermediate isolates QUB 30-10, R 9528.4 and 9522.3; and less-sensitive isolates 788, SAC 1-4-8 (0003), GKII 18-2-3, GKII 18-3-2, SAC 09/943/14, QUB 12-3, OSA 28-2-2 and RS 783 (Figure 4.3).

PCR reactions were carried out using Phusion High-Fidelity DNA Polymerase (Finnzymes Oy, Espoo, Finland) according to manufacturer's instructions, in 30μl reactions with HF buffer, no DMSO, 0.5mM primers and 1.67 μg ml^{-1} template. The PCR programme comprised 2 minutes at 95°C; followed by 40 cycles of 10 seconds at 95°C, 20 seconds at 60°C (the annealing temperature) and 50 seconds at 72°C; followed by 4 minutes 10 seconds

at 72°C. PCR was carried out with primers Forwards 1 and Reverse 1 shown in Table 4.2 for *CYP51A* and *CYP51B* respectively. Primers were designed in Vector NTI (Invitrogen Corporation). PCR products were purified and sequenced by Eurofins MWG (Germany), with the Nested F, Nested R and Internal primers listed in Table 4.2 for *CYP51A* and *CYP51B* respectively.

CYP51A-P (see discussion, section 4.4.2) was sequenced for isolates QUB 30-10, 788, QUB 12-3, R 9528.4, GKII 18-2-3, RS 783, RS04CH6B NB32 and RS99CH1 H10B, in addition to isolates K1124, FI12-63 and RS 219 for which *CYP51A-P* sequences had already been obtained during *CYP51A* sequencing. cDNA sequences of *CYP51B* and *CYP51A* of isolate 788, and *CYP51A-P* of isolate FI12-63, were amplified from cDNA prepared as described in section 5.2.5. PCR reactions were carried out using Easy-A High-Fidelity PCR Cloning Enzyme (Stratagene, California, USA) according to manufacturer's instructions, with 2 ng μl^{-1} genomic or 1% cDNA template. The PCR programme comprised 2 minutes at 95°C; followed by 30 cycles of 40 seconds at 95°C, 30 seconds at 58°C and 2 minutes at 72°C; followed by 7 minutes at 72°C. PCR was carried out with primers Forward 1 and Reverse 1 shown in Table 4.2 for *CYP51B*, *CYP51A* and *CYP51A-P*, designed in Vector NTI (Invitrogen Corporation). PCR products were purified, cloned and sequenced as described in section 4.2.3.

4.2.5 Efflux inhibitor assay

The assay procedure was carried out as described in section 4.2.1 for tebuconazole and prothioconazole and section 3.2.1 for azoxystrobin, but with a single isolate per plate. Spore suspensions were diluted to 5×10^5 spores ml^{-1}, and 50μl was added to each well. The phenothiazine compounds, trifluoperazine dihidrochloride 98% (Sigma) and fluphenazine dihydrochloride (Sigma), were dissolved in sterile distilled water at 2.5 mg ml^{-1}. Six serial dilutions were carried out at a dilution factor of 1/5, and 50μl was added to each well to give the following final concentrations: 0μg ml^{-1} (50μl distilled water only), 0.04 μg ml^{-1}, 0.2 μg ml^{-1}, 1 μg ml^{-1}, 5 μg ml^{-1}, 25 μg ml^{-1} 125 μg ml^{-1} and 625 μg ml^{-1}. Isolates K1124, R 9528.4, 788 and RS 783 were tested (Table 2.1). Plates were incubated and read as described in section 3.2.1.

Correlation between reduction in EC_{50} with 5μg ml^{-1} putative inhibitor, and EC_{50} value without the putative inhibitor, for each fungicide-phenothiazine combination, was tested by

least squares linear regression analysis in GenStat 13th Edition (VSN International, Hertfordshire, UK).

4.3 Results

4.3.1 Triazole sensitivity testing

The full data set of triazole EC_{50} values is given in Appendix 3.

Significant positive correlations ($r>1$, $P<0.01$) were found between EC_{50} values for all pairs of triazoles. Scatter plots for all pairwise comparisons are shown in Figure 4.2.
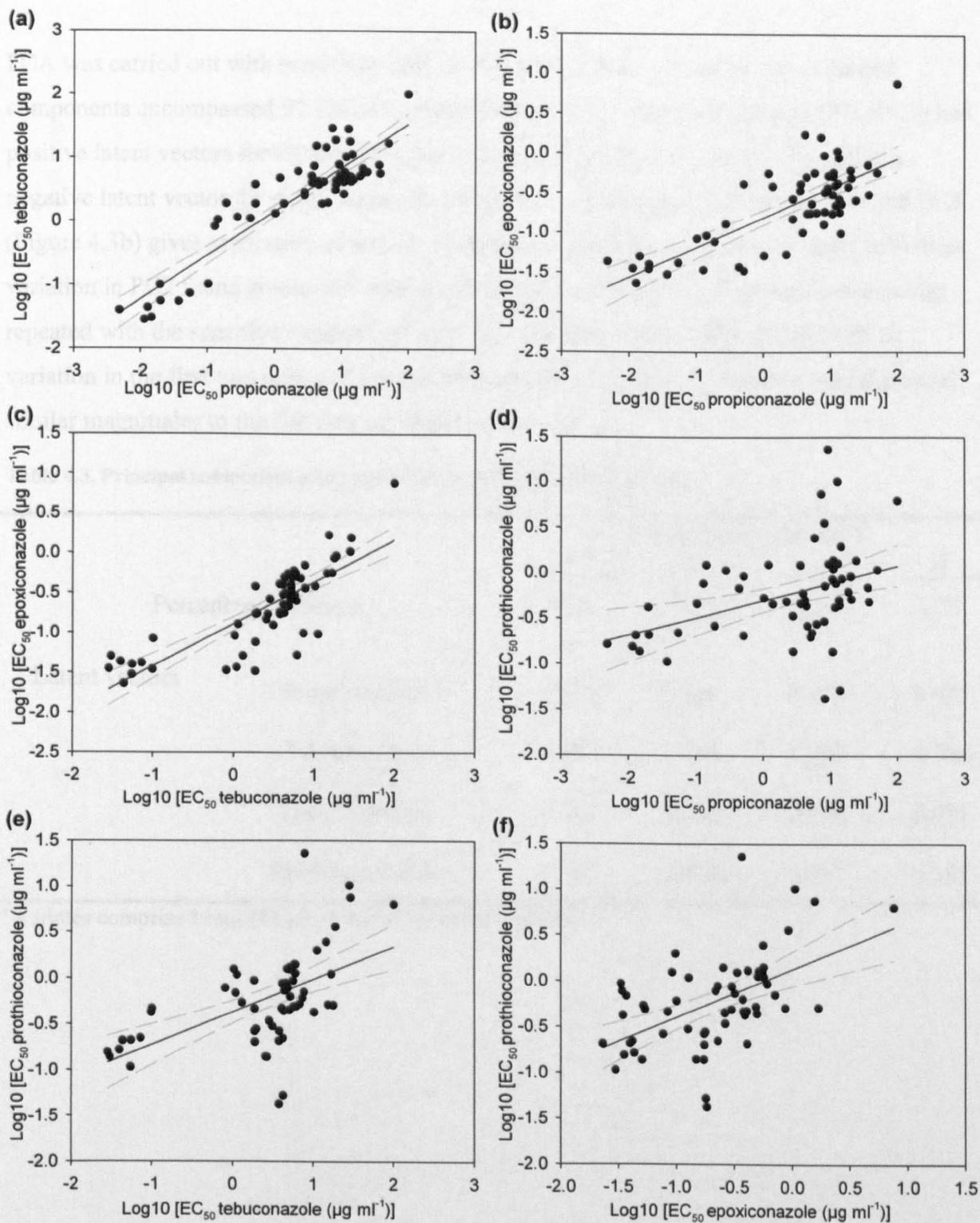
Figure 4.2. Scatter plots showing log(EC50) values of *R. secalis* isolates for each pairwise comparison of the four triazoles tested: (a) tebuconazole against propiconazole; (b) epoxiconazole against propiconazole; (c) prothioconazole against propiconazole; (d) epoxiconazole against tebuconazole; (e) prothioconazole against tebuconazole; (f) prothioconazole against epoxiconazole. Solid lines indicate linear regression; dashed lines indicate 95% confidence limits of regression line (calculated in SigmaPlot, Systat Software Inc.)

PCA was carried out with sensitivity data for the four triazoles. The first two principal components encompassed 92.3% of variation (Figure 4.3a). Principal component 1 (PC1) had positive latent vectors for all four variates, whereas principal component 2 (PC2) had a negative latent vector for prothioconazole (Table 4.3). Plotting isolates against PC1 and PC2 (Figure 4.3b) gives separation of sensitive, intermediate and less sensitive isolates, with most variation in PC2 found among the less-sensitive isolates. Principal component analysis was repeated with the sensitive isolates excluded. Results were similar, with almost 90% of variation in the first two principal components, and latent vectors having the same signs and similar magnitudes to the full data set (data not shown).

Table 4.3. Principal component analysis results for triazole Log(EC$_{50}$) data.

| | | Principal component | | | |
		1	2	3	4
Percentage variation		75.8	16.5	5.94	1.77
Latent vectors	Propiconazole [a]	0.521	0.400	0.428	0.621
	Tebuconazole	0.551	0.165	0.286	-0.766
	Epoxiconazole	0.521	0.098	-0.844	0.081
	Prothioconazole	0.391	-0.896	0.152	0.145

[a] Variates comprise Log$_{10}$ [EC$_{50}$ (μg ml^{-1})] for each fungicide
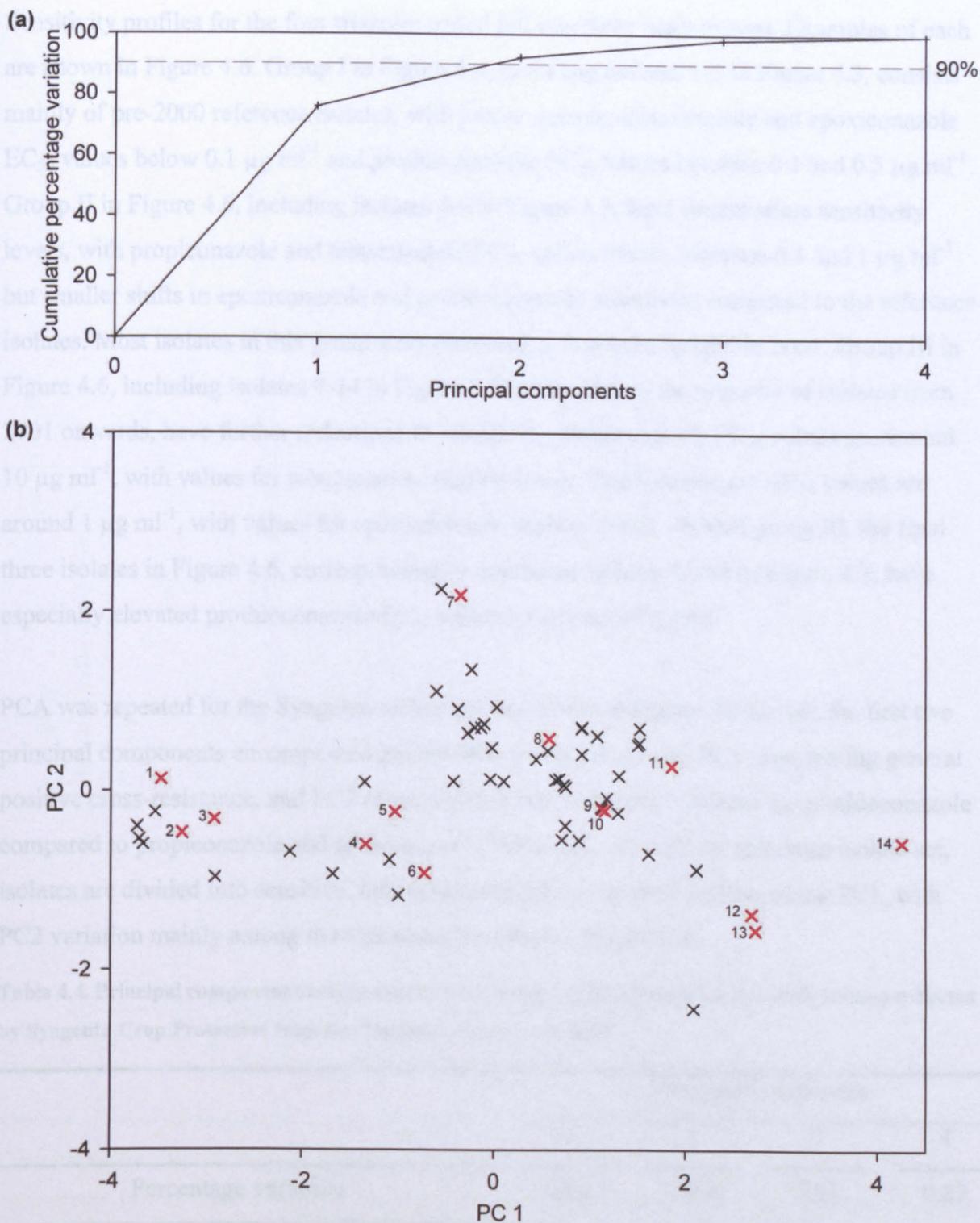
Figure 4.3. Principal component analysis of triazole EC$_{50}$ data for *R. secalis* isolates. (a) Cumulative percentage variation of principal components. (b) Principal component 2 against principal component 1. Red crosses indicate isolates selected for sequence analysis. 1: RS 219; 2: K1124; 3: FI12-63; 4: R 9528.4; 5: R 9522.3; 6: QUB 30-10; 7: GKII 18-3-2; 8: GKII 18-2-3; 9: 788; 10: SAC 0003 1.4.8; 11: SAC 09/943/14; 12: QUB 12-3; 13: OSA 28-2-2; 14: RS 783.

Sensitivity profiles for the four triazoles tested fall into three main groups. Examples of each are shown in Figure 4.6. Group I in Figure 4.6, including isolates 1-3 in Figure 4.3, consists mainly of pre-2000 reference isolates, with propiconazole, tebuconazole and epoxiconazole EC_{50} values below 0.1 µg ml^{-1} and prothioconazole EC_{50} values between 0.1 and 0.5 µg ml^{-1}. Group II in Figure 4.6, including isolates 4-6 in Figure 4.3, have intermediate sensitivity levels, with propiconazole and tebuconazole EC_{50} values mostly between 0.1 and 1 µg ml^{-1} but smaller shifts in epoxiconazole and prothioconazole sensitivity compared to the reference isolates. Most isolates in this group were collected in Northern Ireland in 2001. Group III in Figure 4.6, including isolates 7-14 in Figure 4.3, encompasses the majority of isolates from 2001 onwards, have further reductions in sensitivity. Propiconazole EC_{50} values are around 10 µg ml^{-1}, with values for tebuconazole slightly lower. Prothioconazole EC_{50} values are around 1 µg ml^{-1}, with values for epoxiconazole slightly lower. Within group III, the final three isolates in Figure 4.6, corresponding to numbered isolates 12-14 in Figure 4.3, have especially elevated prothioconazole EC_{50} values of around 10 µg ml^{-1}.

PCA was repeated for the Syngenta isolate set. As for the reference isolate set, the first two principal components encompassed around 90% of variation, with PC1 representing general positive cross-resistance, and PC2 revealing different sensitivity patterns for prothioconazole compared to propiconazole and tebuconazole (Table 4.4). As with the reference isolate set, isolates are divided into sensitive, intermediate and less-sensitive isolates along PC1, with PC2 variation mainly among the less-sensitive isolates (Figure 4.4).

Table 4.4. Principal component analysis results for triazole Log(EC_{50}) data for *R. secalis* isolates collected by Syngenta Crop Protection from six European countries in 2009.

		Principal component			
		1	2	3	4
Percentage variation		68.7	20.5	9.95	0.87
Latent vectors	Propiconazole [a]	0.557	-0.371	0.200	-0.715
	Tebuconazole	0.562	-0.267	0.382	0.683
	Epoxiconazole	0.506	0.105	-0.850	0.101
	Prothioconazole	0.343	0.883	0.301	-0.106

[a] Variates comprise Log_{10} [EC_{50} (µg ml^{-1})] for each fungicide
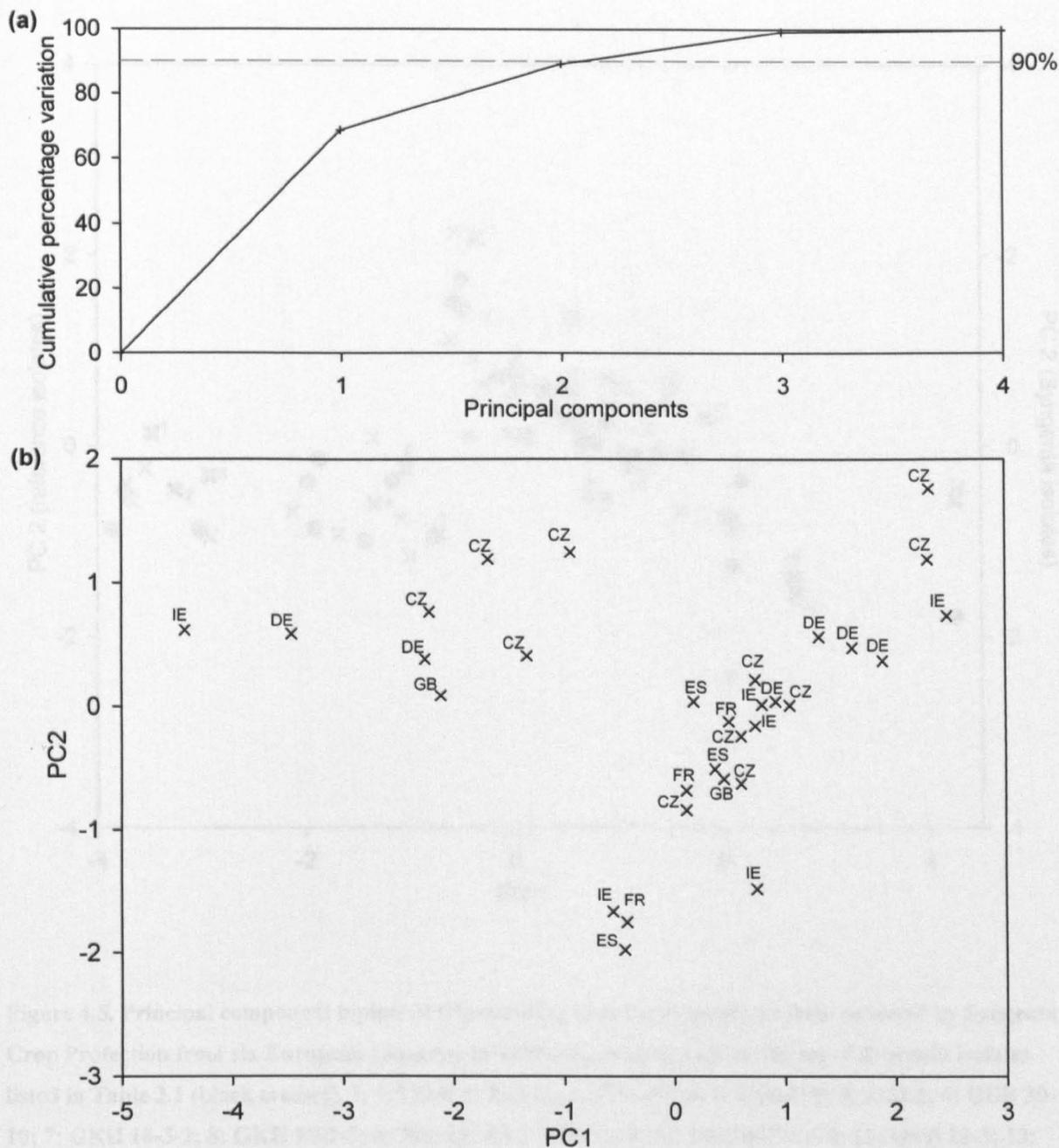
Figure 4.4. Principal component analysis of triazole EC$_{50}$ data for *R. secalis* isolates collected by Syngenta Crop Protection from six European countries in 2009. (a) Cumulative percentage variation of principal components. (b) Principal component 2 against principal component 1. Initials indicate country of origin: CZ: Czech Republic; DE: Germany; FR: France; ES: Spain; GB: United Kingdom; IE: Ireland.

Superimposing the biplots for the reference isolate set and the Syngenta isolate set shows that the sensitivity groupings match closely between the two isolate sets (Figure 4.5). The PC2 axis is reversed for the Syngenta isolate set, as the signs of the latent vectors are arbitrary.

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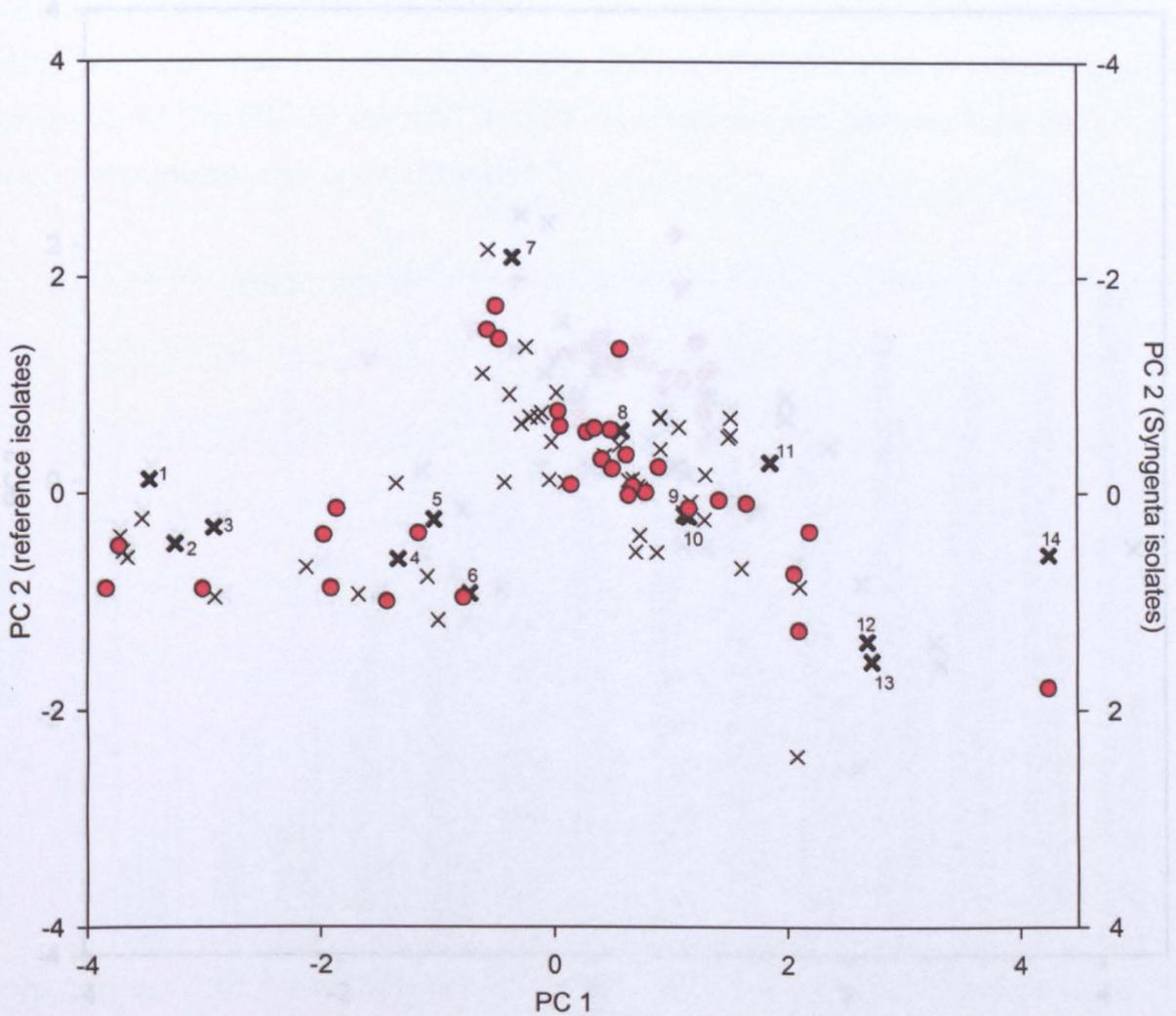
Figure 4.5. Principal component biplots of triazole EC$_{50}$ data for *R. secalis* isolates collected by Syngenta Crop Protection from six European countries in 2009 (red circles), and for the set of *R. secalis* isolates listed in Table 2.1 (black crosses). 1: RS 219; 2: K1124; 3: FI12-63; 4: R 9528.4; 5: R 9522.3; 6: QUB 30-10; 7: GKII 18-3-2; 8: GKII 18-2-3; 9: 788; 10: SAC 0003 1.4.8; 11: SAC 09/943/14; 12: QUB 12-3; 13: OSA 28-2-2; 14: RS 783.

4.3.2 Rothamsted field isolates

PC1 and PC2 were calculated from Rothamsted 2009 field isolate log$_{10}$[EC$_{50}$s] based on the coefficients obtained for the first isolate set. The 2009 isolates all fell within the lower end of the less-sensitive group, except one isolate which may have intermediate sensitivity (Figure 4.5). There was no difference between isolates from plots receiving different fungicide treatments.
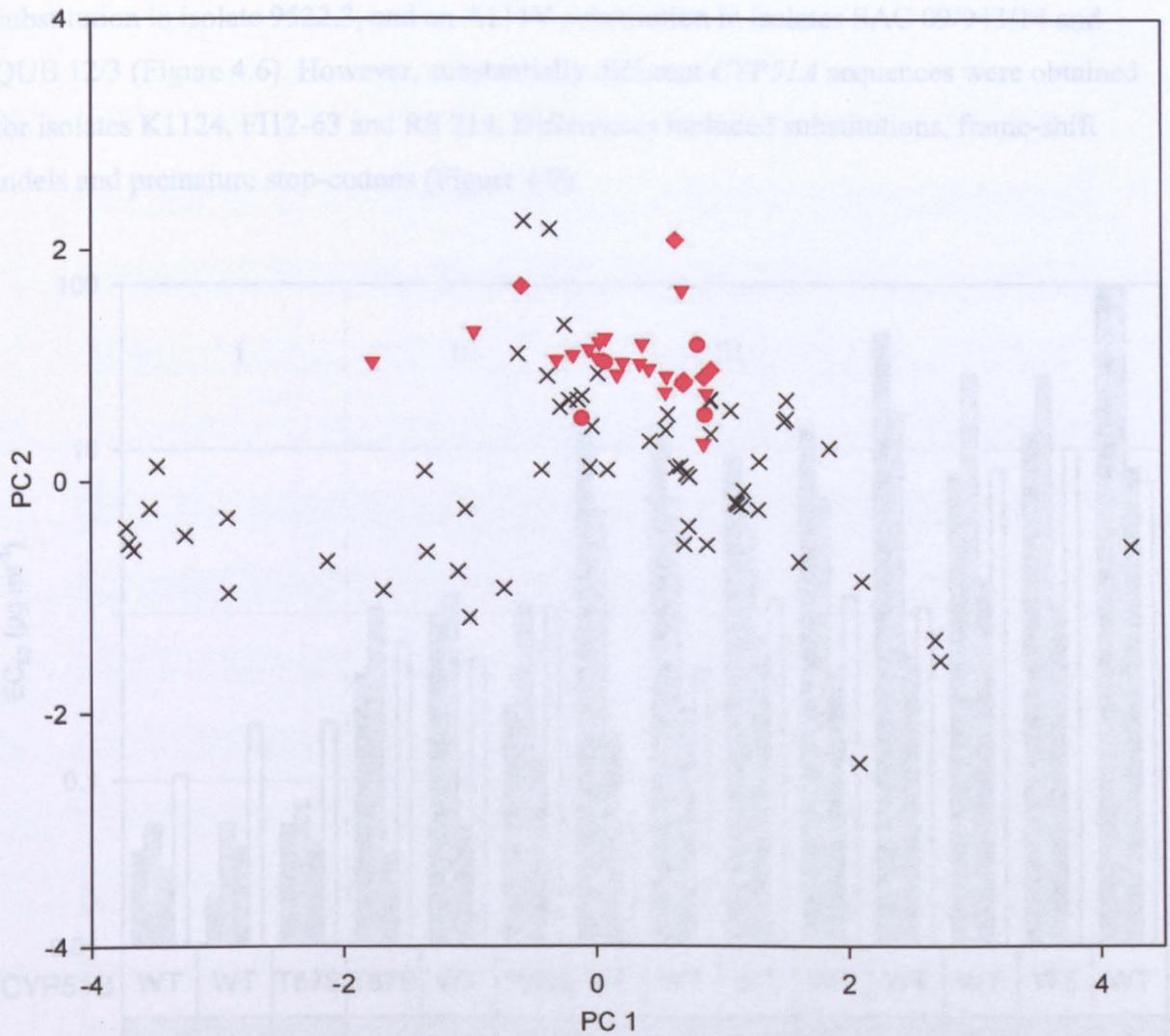
Figure 4.5. Principal component 2 plotted against principal component 1 for PCA of *R. secalis* log$_{10}$[EC$_{50}$] for triazole fungicides. Crosses represent the reference set of isolates. Red symbols represent isolates collected from Rothamsted Research in 2009: Diamonds: Untreated; Circles: Propiconazole treated; Triangles: Epoxiconazole treated.

4.3.3 *CYP51* sequence analysis

The complete *CYP51A* sequence was obtained. Intron positions at base pairs 173-309 and 511-569, confirmed by cDNA sequences, were removed and the sequence translated. The translated sequence of isolate 788 is shown in Figure 4.7. For *CYP51B*, cDNA sequences confirmed the presence of two introns, at base pairs 244-312 and 484-638.

CYP51B and *CYP51A* sequences for the selected *R. secalis* isolates are shown in Appendix 4(a) and (b). For *CYP51B*, the only non-synonymous mutation resulted in a T67S substitution in sensitive isolate FI12-63 and intermediate isolates QUB 30-10 and R9528.4 (Figure 4.6). For *CYP51A*, the only non-synonymous single nucleotide polymorphisms resulted in a P170S

substitution in isolate 9522.3, and an A111V substitution in isolates SAC 09/943/14 and QUB 12/3 (Figure 4.6). However, substantially different *CYP51A* sequences were obtained for isolates K1124, FI12-63 and RS 219. Differences included substitutions, frame-shift indels and premature stop-codons (Figure 4.7).

CYP51B	WT	WT	T67S	T67S	WT	T67S	WT	WT	WT	WT	WT	WT	WT	WT
CYP51A	*	*	*	WT	P170S	WT	WT	WT	WT	WT	A111V	A111V	WT	WT

Isolates: RS 219, K1124, FI12-63, R 9528.4, R 9522.3, QUB 30-10, GKII 18-3-2, GKII 18-2-3, 788, SAC 1-4-8 (0003), SAC 09/943/14, QUB 12-3, OSA 28-2-2, RS 783

Isolate

Figure 4.6. Propiconazole, tebuconazole, epoxiconazole and prothioconazole EC50 values of *R. secalis* isolates selected for target site sequence analysis. Black: propiconazole, Light grey: tebuconazole, Dark grey: epoxiconazole, White: prothioconazole. I: sensitive isolates; II: intermediate sensitivity; III: less sensitive. WT = Wild Type.

```
          1                                                                                                 100
788     ATGCTGGGTATCTTCTCGGTGCTAGTTTCTAGCATCCGCTATGGATTCTACACCCTCACTCTCCTTATTGTCACAGTCCTGCTCAACGCTCTACACCAAC
FI12-63 --------------------------------------------------------------------TCAATCCTCCTCAACGCTCTACACCAAA

          101                                                                                               200
788     TCCTCCCAAAGAAGAAGTCCGAACCGCCTTTGGTATGGCATTGGATCCCATTTATCGGGAATGCCATCGAATGTGAATCTCCCGTTTTCCTCCTTCCTAAC
FI12-63 TCCTCCAAAAGAAGAAGTCCGAACCGCCTCTGGTATGGCATTGGATCCCCTTTATTGGGAATGCCATCGAATGTGAATCTCCCGTTTTAGTCCTTCCGTAAC

          201                                                                                               300
788     CCTCTTGATCTTTTCTTTCTCTCACCCTTCGTCTTCCTGCAGCACACCTCCC-ATTATCAACCTCACATTCAGTAGAGCAAAAGATACTGACATGCAACC
FI12-63 CCTCTTGATCTTTCCTTTCTCTCACCCTTGCTCTTCCTCCACCACACCCCCCCATTATCAACGATCACATTCAGTAGAGCAAAAGTTACTGACATGCAACC

          301                                                                                               400
788     ATTTTTCTAGATGGCACCGACCCTCTAGCTTTCTACACAAAATGTCAACGGCAACATGGCCCGATCTTCACTTATATTCTTTTCGGCAAAAAAATGACTG
FI12-63 GTTTCTCTAGATGGCACCGATCCTCTAGCTTTCTACACAAAATGTCAACGGCACCATGGCCCGATCTTCACTCATATTCTTTTCGGCAGAAAAATGACTG

          401      ▼▼▼▼                                                                    ***               500
788     TTTATCTTGGGCTGGAAGGAAATGAGTTTATCCTCAATGGAAGATTGCAGGATGTCAACGCGGAAGATATTTATGGTCCA|CTGAGTGTGTAGTTCTCCAT
FI12-63 TTTATCTTGGGCTGGAAG----TGAATTTATCCTCAATGGTAGACTGCAGGATGTCAATGCAGAAGATATTTATGGTCCAC|TGAGTGTGTAATTCCAT

          501                                                                                               600
788     CAGCCTTCGTATCTCCCCTGACATGGACTCTCTCGAAATCGCTAAGCTTTCTATGTTCTCTATCCTGCTTGATAGGGAGTTCCTCACCAAAGTTATCCCAT
FI12-63 CAGCCTTCCTTCTCCCCTTCACATCCAGTCTCTTGAGATGGCTAAGCTCTCCATGTTCGCTATAAATGTTTGAATGGGGGACCCTCATCAAAGTTATCCCAT

          601                                                                                               700
788     GATAATTCGGGGAAAGCGATGCTAACTCGGCATATCTAGCTACACCAGTCTTCGGGCCCAACGTCATCTACGACTGCCCCAACTCCAAACTAATGGAACA
FI12-63 GACAAGTCAGGGAAAGCGATGCTAACTCGGCAGTTCTAGCTACACCAGTCTTCGGGCCCAACCTCATCTACGACTGCCCCAACTCCAAACTAATGGAACA

          701                                                                                               800
788     AAAGAAATTCGTCAAGTTCGGCCTCACTCAACGCGCACTCGAACAACACGTTCCCCTCATCGAAAACGAGTCCTCTCCTACATGGCTTCATCTCCGTCC
FI12-63 AAAGAAATTTGTCAAGTTCGGCCTCACCCAACGCGCACTCGAACAACACGTCGCTGTAATCGAGAACTAGGTCCTCTCCTACATGGCTTTATCTCCGTCC

          801                                                                                               900
788     TTCATTCCCCAATCGCCTACCTCTCCCCTCTTCCTCCGACCAAAGCACAACCAAAATGGAACAGAAACTTCAAACAGCCATAATAGACCTGCCTCACACCA
FI12-63 TTCATCTCCCAATCGCCTACCTCCCCCTCTTGTCTAACCAAGGCATAACCAAACGGAACAAAAACTTCAAACAGCCATAGTAGACCTTCCCCATACCA

          901                                                                                              1000
788     TGGCTCAGATCACAATCTTCACAGCCGGTCGCGCTCTTCAAGGCCCGGAGGTCCGCACTAAACTCACAGACGAATTCGCGGGTTTGTATGATGATCTCGA
FI12-63 TGTCTCAGATCACAATCTTCACAGCTGGTCGCGCTCTTCAAGGCCCGGAGGACTGCAGCAAACTCACAGACTAATTCGCGGATTTGTATGATGATCTCGA

          1001                                                                                             1100
788     CCACGGGTTCCGGCCAATCAACTTCCTCGCTCCGTGGTTACCACTGCCTCAGAACTGGAGAAGGGATAAAGCACATGCAAAGATGATGAGTGTGTATATG
FI12-63 CCACGGGTTTCGGCCAATCAACTTCCTCGCTCCGTGGCTACCGCTGCCGCAGAACTGGAGAAGAGATAAAGCACATGCAAAGATGATGAGTGTGTATATG

          1101                                                                                             1200
788     GACATCATATCACGACGACGTGAAAAGAGTGGTGGTGGAGTGGTGGGGGAGAGGAATGGAAAGGGAGGAGGAGATTGACATGATTGATAATCTCATGACTTGTG
FI12-63 GACATCATATCACGACGATGGGAAAAGAGTGGTGGTGGGGAGGTGGGGGAGAGGAATGGAGAGGGAGGAGGAGATTGACATGATTGACAATCTCATGACTTGTG

          1201                                                                                             1300
788     TCTATAAATCCGGAGAAACGATTCCAGACAGCGAGATTGCGTGTATGATGATCACGATCCTTATGGCGGGACA--ACATTCGTCGAGCAGTTCCTCGTGC
FI12-63 TCTATAAATCCGGAGAAACGATCCCAGATGGCGAAATTGCGTGTATGATGATCTCGATCCTTATGGCGGGAAAAAACACTCGTCCAGCAGTTCCTCGAGC

          1301                                                                                             1400
788     TGGATTATGCTCCATCTAGCCTCTCGCCCAGATCTTCAGGAGGAATTGTATCGCGAGCAACAGGATGCGAATCTCTACCTTGCCGGCAACAGAGGGCTAC
FI12-63 TGGATTATGCTGCATCTAGCCTCTCGCCCAGATCTTCAGGAGGAATTGTATCGCGAGCAACAGGATGCGAATCCTTATCTTGCCGGCAACAAAGGACTAT

          1401                                                                                             1500
788     AATACCAGGACTTAGAGAAATTGAAGTTGTTGAGTGACGTGGTGAAGGAGACGCTCCGGCTCCATTCTAGCATCCATTCTGTGATGCGCGCAGTCAAGAA
FI12-63 AATACCAGGACCTAGAGAAATTGACGTTGTTGGGTGACGTGGTGAAGGAGACGCTCCGGCTACATTCTAGCATCCATTCTATGATGCGCGCTATCAAGAA

          1501                                                                                             1600
788     CGACCTCCCGATTCCAGGGACACCATACGTCGTCACAACAGATAAAGTTCTGATTGCGAGTCCGCTTGTTACGCACCTCTCGCCCAGAACACTTTTCAGAC
FI12-63 CGACCTCCCGATTCCAGGGACACCATATGTTGTCACAACAGACAAAGTTCTGATCGCAGTCCGCTTGTTACGCACCTCTCGCCCAGAACACTTTTCAGAC

          1601                                                                                             1700
788     CCGGAGGAGTGGGATCCGTACCGCTGGGCTACACCGGGTGACATTGAGAAAGATGAAGAGGTGGATTATGGATATGGTGTGATTAGCAAGGGGACTAGGA
FI12-63 CCTGAGAAACGGGATCCGTACCGTTTGGGCTACCCTGGGTAACATTGAGAAAGATGAAGAGGCGGGTTATGGATATGGTGTGATTAGTAAGGAGATTAGGA

          1800
788     GTCCGTACTTACCGTTTGGTGCGGGCAGACATCGATGCATTGGGGAGAAGTTTGCATTTGTCAATTTATTGACGATTATAGCTACGCTGGTGAGGGGATA
FI12-63 TTCCTTATTTACCGTTTGGTGCGGGCAGACATCGGTGCATTGGGGAGAAGTTTGCATTCGTCAATTCATTGACGATTATAGCCACGCTTGTGAGGGGATA

          1801                                                                                             1900
788     TAAGTGGAGTCTTGTCCCTGGGGGAGAGGAAGGAGGACAGATCCCAAAGACGGATTATTCGAGTATGGTCTCTAGACCTATGCCGGGCTCGAGAGTTAGA
FI12-63 TAGGTGGAGTCT----------------------------------------------------------------------------------------

          1901         1918
788     TGGGAAAGAGGGTCTAG
FI12-63 -----------------
```

Figure 4.7. Alignment of *CYP51A* genomic DNA sequences from two *R. secalis* isolates. Introns are shown in grey. ▼indicates the four base pair frameshift indel, * indicates the stop codon in the FI12-63 sequence with | indicating the reading frame of each gene at that point.

Nucleotide sequences for *CYP51A-P* are given in Appendix 4(c). All sequences contained the 4 base pair deletion at base pairs 419-422, resulting in a premature stop codon at base pairs 482-484 (numbered according to functional *CYP51A* gene). cDNA sequences confirmed that the first intron is in the same position as in the functional *CYP51A* gene. The second intron starts two base pairs downstream but finishes in the same position as in the functional *CYP51A* gene. A third intron, with the splice site formed due to the presence of the sequence TAG instead of GAA at base pairs 764-766, was found at base pairs 712-766 (Figure 4.7).

4.3.4 Efflux inhibitor assays

Triazole EC_{50}s over the range of phenothiazine concentrations are shown in Figure 4.8. EC_{50}s for the phenothiazine compounds themselves in the absence of triazole fungicide were in the range 4.7-23.9 μg ml^{-1}, so triazole EC_{50} values at higher phenothiazine concentrations are not reliable. At 5 μg ml^{-1} phenothiazine, tebuconazole EC_{50}s were reduced by less than 2.05-fold (Table 4.5). Prothioconazole EC_{50}s were reduced by up to nine-fold, but this was not correlated with EC_{50} of the fungicide alone. Azoxystrobin EC_{50}s were reduced by up to 4.17, but this was not correlated with EC_{50} of the fungicide alone.
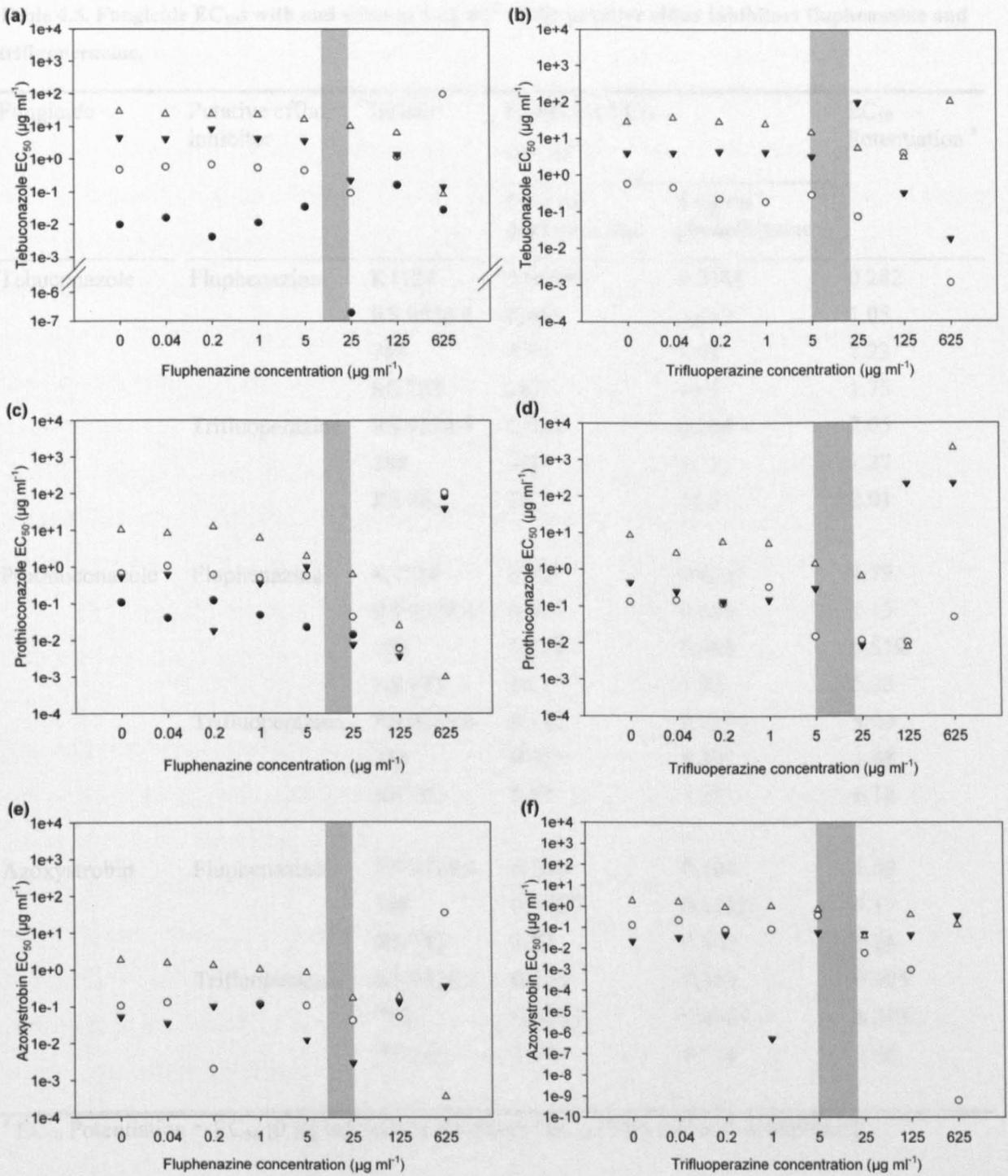
Figure 4.8. Tebuconazole and prothioconazole EC₅₀s of four *R. secalis* isolates over a range of concentrations of the putative efflux inhibitors fluphenazine and trifluoperazine. Black circles: isolate K1124; white circles: Rs 9528.4; black triangles: 788; white triangles, RS 783. Grey shading indicates the range of EC₅₀s of the putative efflux inhibitors alone for the four *R. secalis* isolates.

Table 4.5. Fungicide EC$_{50}$s with and without 5 µg ml^{-1} of the putative efflux inhibitors fluphenazine and trifluoperazine.

Fungicide	Putative efflux inhibitor	Isolate	Fungicide EC$_{50}$ (µg ml^{-1})		EC$_{50}$ Potentiation [a]
			0 µg ml^{-1} phenothiazine	5 µg ml^{-1} phenothiazine	
Tebuconazole	Fluphenazine	K1124	0.00969	0.0344	0.282
		RS 9528.4	0.465	0.442	1.05
		788	4.44	3.62	1.23
		RS 783	28.7	16.4	1.75
	Trifluoperazine	RS 9528.4	0.583	0.284	2.05
		788	4.01	3.15	1.27
		RS 783	28.7	14.3	2.01
Prothioconazole	Fluphenazine	K1124	0.107	0.0227	4.73
		RS 9528.4	0.664	0.586	1.13
		788	0.518	0.998	0.519
		RS 783	10.1	1.93	5.23
	Trifluoperazine	RS 9528.4	0.132	0.0147	9.00
		788	0.445	0.301	1.48
		RS 783	8.59	1.39	6.18
Azoxystrobin	Fluphenazine	RS 9528.4	0.104	0.104	1.00
		788	0.0507	0.0122	4.17
		RS 783	1.81	0.802	2.26
	Trifluoperazine	RS 9528.4	0.129	0.363	0.355
		788	0.0221	0.0564	0.393
		RS 783	1.91	0.716	2.66

[a] EC$_{50}$ Potentiation = EC$_{50}$ (0 µg ml^{-1} efflux inhibitor) / EC$_{50}$ (5 µg ml^{-1} efflux inhibitor)

4.4 Discussion

4.4.1 Triazole sensitivity testing

Sensitivity profiles against the four fungicides tested fall into three main groups. The first group, with the lowest EC$_{50}$ values for all four triazoles tested, consists mainly of pre-1997 isolates, which will be considered sensitive reference isolates for the triazoles being tested here, although isolates from the late 1980s onwards may come from populations that had already been subject to selection by triadimenol (Jones 1990).

The second group show some shifts in sensitivity levels, with propiconazole and tebuconazole EC_{50} values increased by around ten-fold, but smaller shifts in epoxiconazole and prothioconazole sensitivity relative to the reference isolates. Most isolates in this group were collected in Northern Ireland in 2001. Kendall *et al.* (1993) reported an eightfold shift in mean propiconazole sensitivity in field trials between 1988 and 1990, with some cross-resistance, but greater sensitivity, to tebuconazole. In this study, a similar shift in propiconazole sensitivity is apparent in this second group of isolates relative to the reference isolates, but with a greater shift in sensitivity to tebuconazole.

The third group of isolates, including the majority of isolates from 2001 onwards, are further reduced in triazole sensitivity. Propiconazole and tebuconazole EC_{50} values are around 100-fold higher than those of the sensitive reference isolates. Prothioconazole and epoxiconazole EC_{50} values are also increased relative to the sensitive reference isolates, but generally by less than ten-fold.

The decreased epoxiconazole sensitivity of isolates in group III, slightly less than ten-fold compared to group II, is consistent with shifts observed by Cooke *et al.* (2004), although actual sensitivity values are not comparable due to their use of MIC rather than EC_{50} values. Therefore, overall there is evidence of around a hundred-fold decrease in sensitivity to propiconazole and tebuconazole, around a ten-fold decrease in epoxiconazole sensitivity, and slightly less decline in prothioconazole sensitivity, for the majority of recent isolates compared to the reference isolates. Prothioconazole EC_{50} values appear somewhat elevated *in vitro*, compared to good control in the field (HGCA 2006), but the data still show that it is less affected by shifts in sensitivity than propiconazole and tebuconazole.

EC_{50} values for each pairing of the triazoles tested here showed a significant positive correlation, suggesting positive cross-resistance between all four compounds. This is consistent with the results of Girling *et al.* (1988) and Kendall *et al.* (1993), with resistance against newer triazoles found to be correlated with, but quantitatively lower than, resistance to older triazoles. Multivariate analysis largely supports this conclusion. The first principal component has positive latent vectors for all four variants, indicating that 75.7% of variance can be accounted for by general positive cross-resistance to all four triazoles tested. However, the second principal component, accounting for a further 16.6% of variance, has a negative

latent vector for prothioconazole but positive latent vectors for propiconazole and tebuconazole. This indicates a slightly different pattern of variation in prothioconazole compared to the other three fungicides. It has been shown that prothioconazole interacts with different regions of CYP51 from other triazoles (Parker *et al.* 2011). However, similar latent vectors were obtained when the most sensitive isolates were excluded from the analysis, and sensitivity differences in this subset of isolates are not due to target-site alterations.

The Syngenta isolate set had a similar pattern of triazole sensitivities, but with fewer sensitive isolates as they were all collected in 2009 whereas the first set included older isolates. As with the first isolate set, PC1, representing general positive cross-resistance and dividing isolates into sensitive, intermediate and less-sensitive isolates, and PC2, in which prothioconazole sensitivity varies differently from propiconazole and tebuconazole among the less-sensitive isolates, together encompass over 90% of variation.

Isolates collected from Rothamsted field plots in 2009 fell within the lower end of the less sensitive group. There was no separation of isolates from plots receiving different fungicide treatments, suggesting that the initial population at that site was already less-sensitive and no further selection took place due to fungicide use within the 2008-2009 growing season.

4.4.2 *CYP51* sequence analysis

No sequence differences detected in *CYP51B*, and no single-residue changes detected in *CYP51A*, correlated with differences in triazole sensitivity. Therefore, target-site resistance to triazoles, as found in *M. graminicola* (Cools and Fraaije 2008) and *B. graminis* (Wyand and Brown 2005), can be ruled out for current isolates of *R. secalis*.

However, *CYP51A* sequences obtained for isolates K1124, FI12-63 and RS 219 contained substitutions, frame-shift indels and premature stop-codons, suggesting that the gene is non-functional. Therefore this appears to be a pseudogene and is referred to as *CYP51A-P*. *CYP51A-P* gene sequences from the selected isolates contained some sequence differences, but all contained a 4 base pair deletion resulting in a stop codon at codon 114, and further premature stop codons downstream of codon 114. The presence of *CYP51* paralogues in sensitive and other isolates, and their possible role in azole sensitivity, is investigated in Chapter 5.

4.4.3 Efflux inhibitor assays

Since triazole sensitivity differences among intermediate and less-sensitive isolates are not explained by target-site sequence differences, a preliminary investigation into the possible role of enhanced efflux, using efflux inhibitors. However, the inhibitors used had very little effect on triazole sensitivity. At the highest concentration at which the efflux inhibitors themselves did not inhibit fungal growth, tebuconazole sensitivity was reduced around two-fold. This is less than the ten-fold variation seen between intermediate and less-sensitive isolates, and was similar for all isolates tested and not correlated with initial tebuconazole sensitivity. Greater reductions in prothioconazole sensitivity were seen in some isolates. The lowest potentiation of prothioconazole sensitivity by both phenothiazines was in isolate 788, which also had the highest score for Principal Component 2, indicating a lower prothioconazole $Log[EC_{50}]$ than expected from overall azole sensitivity, of the four isolates tested. However, these four isolates cover relatively little of the range of PC2 scores seen across all isolates. Therefore future tests on the effect of efflux inhibitors on prothioconazole sensitivity should include a selection of the isolates numbered 7-12 in Figure 4.3. However, potentiation in sensitivity to tebuconazole, prothioconazole or azoxystrobin was not correlated with initial fungicide EC_{50} for the isolate set tested here.

The inhibitors tested here were phenothiazines, which have previously been found to restore azole sensitivity in isolates of *C. albicans* (Marchetti *et al.* 2000) and *M. graminicola* (Roohparvar *et al.* 2008). Trifluoperazine was shown to be a non-competitive inhibitor of the yeast efflux pump Pdr5p (Kolaczkowski *et al.* 1996), although it may have different activity against different transporters (Wesołowska *et al.* 2009). McCartney (2006) reported that epoxiconazole and tebuconazole sensitivity in *R. secalis* were also unaffected by the efflux inhibitor ENT9811. However, efflux inhibition has been reported for a wide range of substances, including calcium channel blockers (Bulatova and Darwish 2008), tricyclic serotonin uptake inhibitors (Roohparvar *et al.* 2008), macrolide immunosuppressants (Hayashi *et al.* 2003), and milbemycin actinomycete metabolites (Lee *et al.* 2001). Therefore a role of efflux in reduced azole sensitivity in *R. secalis* cannot be categorically ruled out. Considering the wide range of putative efflux inhibitors, without clearly-defined structure-activity relationships except in some human and bacterial efflux inhibitors (Guz *et al.* 2000), it would not necessarily be possible to determine the role of efflux in sensitivity even if a wider range of substances were tested. Therefore, it would be preferable to measure fungicide accumulation directly, using [^{14}C] radiolabelled fungicides (Hayashi *et al.* 2003). Fungicide

accumulation would also be affected by different rates of azole uptake (Mansfield *et al.* 2010). Furthermore, when the *R. secalis* genome is released, it will be possible to search for predicted transporter genes, and in future microarrays or next generation sequence data may become available to identify genes that are upregulated in less-sensitive isolates or on addition of fungicides.

However, in the absence of evidence for increased efflux, other possible mechanisms should be investigated. Expression levels of the triazole target-site-encoding genes *CYP51A* and *CYP51B* are investigated in chapter 5.

Chapter 5

CYP51 paralogues and triazole sensitivity

5.1 Introduction

In the previous chapter, triazole sensitivity data were presented for *R. secalis*. Isolates fell into sensitive, intermediate and less sensitive groups, with further variation among the less-sensitive isolates. The target site encoding genes *CYP51A* and *CYP51B* and pseudogene *CYP51A-P* were sequenced for isolates from each sensitivity group. No point mutations correlated with triazole sensitivity, but in the case of the sensitive isolates, *CYP51A* could not be amplified and only *CYP51A-P* and *CYP51B* sequences were obtained. Further tests are needed to confirm whether *CYP51A* is present in these isolates.

Multiple *CYP51* genes have been identified from filamentous ascomycetes including *A. fumigatus* (Mellado *et al.* 2001), *Fusarium* spp. (Deng *et al.* 2007) and *Magnaporthe oryzae* (Yan *et al.* 2011). It is not known whether any functional divergence has taken place between *CYP51* paralogues. In the case of *A. fumigatus*, *CYP51* mutations and over-expression conferring reduced azole sensitivity have been reported in the *CYP51A* paralogue (Diaz-Guerra *et al.* 2003). However, knocking out *A. fumigatus CYP51A* increased the azole sensitivity of sensitive as well as less-sensitive isolates, suggesting that the presence of a wild-type *CYP51A* can confer reduced intrinsic sensitivity (Mellado *et al.* 2005).

While the presence of *CYP51A* may correlate with the initial shift from sensitive to intermediate sensitivity in *R. secalis*, no *CYP51* mutations correlate with further sensitivity shifts (Section 4.3.3). Furthermore, no evidence was found of a role of enhanced efflux in reduced triazole sensitivity. Another possible mechanism of reduced triazole sensitivity is target site over-expression (Hamamoto *et al.* 2000). This may be due to insertions (Schnabel and Jones 2001; Ma *et al.* 2006; Ghosoph *et al.* 2007) or tandem repeats (Hamamoto *et al.* 2000; Mellado *et al.* 2007) in the *CYP51* promoter, mutations in transcription factors (Dunkel *et al.* 2008; Heilmann *et al.* 2010) or chromosome duplication (Sionov *et al.* 2010). These mechanisms all result in constitutive over-expression of *CYP51*, but greater induced expression of an ABC transporter (Luo and Schnabel 2008) and AOX (Chapter 2) in response to fungicide exposure have been reported in fungal isolates with reduced sensitivity to

triazoles and QoIs, respectively. Furthermore, expression analysis of *CYP51A* and *CYP51B* may shed light on functional differences between the two paralogues.

This chapter investigates the evolution of fungal *CYP51* paralogues, and the role of the two *R. secalis CYP51* paralogues in triazole sensitivity. Sequenced fungal genomes were searched for *CYP51* genes, and phylogenetic analyses and selection tests were carried out. The designations *CYP51A* and *CYP51B* used for *R. secalis CYP51* paralogues in the previous chapter are explained with reference to the fungal *CYP51* gene tree obtained.

A PCR-RFLP assay was designed to detect *CYP51A* and *CYP51A-P* in *R. secalis* isolates, and a Southern blot was carried out to confirm the results. A yeast expression assay was carried out to test whether *R. secalis CYP51A* encodes a lanosterol demethylase capable of complementing *S. cerevisiae CYP51*. Expression analysis was carried out for *R. secalis CYP51A* and *CYP51B*. Constitutive expression and induced expression in the presence of tebuconazole were analysed for both *CYP51* paralogues. A pyrosequencing assay was designed to detect *CYP51A* in *R. secalis* populations, and samples from the Hoosfield spring barley experiment were analysed.

5.2 Materials and Methods

5.2.1 Analysis of *CYP51* paralogues in fungal genomes

Initially, a text search was carried out for annotated *CYP51* genes in fungal genomes on the Broad Institute server (http://www.broadinstitute.org/science/data). Subsequently, BLAST searches were carried out against each genome with *S. cerevisiae CYP51*, and with *CYP51A* and *CYP51C* from the nearest available relative for species where these paralogues were not found. A preliminary amino acid alignment was constructed using the ClustalW algorithm implemented in the AlignX module of Vector NTI (Invitrogen Corporation), and predicted introns and start and stop sites were checked manually.

Amino acid sequences obtained from fungal genomes, plus *CYP51* sequences from *Mycobacterium tuberculosis*, *Trypanosoma cruzi* and *Homo sapiens*, for which crystal structures have been solved, and additional outgroup sequences comprising *Arabidopsis thaliana CYP51G1* and *Mus musculus CYP51A1* and *CYP7A1*, were aligned using M-Coffee. The M-Coffee amino acid alignment was used to construct a corresponding coding nucleotide

alignment using PAL2NAL (Suyama *et al.* 2006). *Rhynchosporium secalis CYP51A-P* was then added manually according to its alignment with *R. secalis CYP51A* (Figure 4.7). Phylogenetic analyses were carried out with the full alignment, and with ambiguously-aligned regions excluded based on M-Coffee scores.

Model selection was carried out in jModelTest 0.1.1, selecting models by AICc. Neighbour-joining trees were reconstructed in MEGA5 (Tamura *et al.* 2011), using the TrN+G model for the full data set, with 100 bootstrap runs. Maximum likelihood phylogenetic reconstruction was carried out in PhyML (Guindon and Gascuel 2003) implemented through the TOPALi v2.5 platform (Milne *et al.* 2009), using the TVM+I+G model for the unambiguously aligned site set and the TrN+I+G model for the full data set, with 100 bootstrap runs. Nodes with under 50% bootstrap support were collapsed using the Phylo module of Biopython (Cock *et al.* 2009). Bayesian analysis was carried out in MrBayes (Ronquist and Huelsenbeck 2003), using the GTR+I+G model for the unambiguously aligned site set and the HKY+I+G model for the full data set. The mixed-chain Monte Carlo simulation was carried out for 1200000 generations until stationarity was reached (average standard deviation of split frequencies < 0.01), sampling every 100 generations, with a burn-in of 3000 samples. A 50% majority-rule consensus tree was calculated in PAUP* (Swofford 1991).

Intron sequences were too variable to align unambiguously so were not used for phylogenetic reconstruction, but intron presence/absence data were plotted onto the Maximum Likelihood tree generated from the full coding sequences for ascomycete *CYP51*s. Ancestral state reconstructions were carried out by Maximum Parsimony in Mesquite (Maddison and Maddison 2010).

Recombination detection was carried out using the DSS (Difference of Sums of Squares) method (McGuire and Wright 2000) in TOPALi v2.5. Selection testing was carried using the codon-based Z-test of selection implemented in MEGA 5 (Tamura *et al.* 2011). Further selection testing was carried out in the CodeML module of PAML 4.4 (Yang 2007), using branch-site new model A (model=2, NSsites=2; null model, $\omega_2=1$) with the branches leading to the *CYP51A*, *B* and *C* clades in turn set as the foreground branch, and in Diverge 2.0, using the Gu99 model, for each pairing of the yeast, A, B and C clades. Sequence logos for CYP51 substrate recognition sites (Podust *et al.* 2001) were made using WebLogo (Crooks *et al.*

2004). Selection testing was carried out for yeast and filamentous ascomycete *CYP51* clades, with *H. sapiens CYP51* as an outgroup. Since Diverge and WebLogo require a minimum of four sequences per group, *N. haematococca* (*Fusarium solani*) gene *e_gw1.5.672.1* was added to the alignment as a fourth *CYP51C*, and the tree reconstructed in PhyML from the full alignment using the TrN+I+G model.

Protein subcellular location prediction was carried out with TargetP 1.1 (Emanuelsson *et al.* 2000), SignalP 3.0 (Bendtsen *et al.* 2004), TMHHMM (Krogh *et al.* 2001) and Phobius (Kall *et al.* 2004) according to the protocol described by Emanuelsson *et al.* (2007), with additional analyses in WoLF PSORT (Horton *et al.* 2006), Protein Prowler (Hawkins and Boden 2006) and MultiLoc (Hoglund *et al.* 2006).

5.2.2 PCR-RFLP Assay

To test for the presence of *CYP51A* genes in *R. secalis* isolates, a PCR-RFLP (Polymerase Chain Reaction – Restriction Fragment Length Polymorphism) assay was developed. PCR reactions were carried out using Phusion High-Fidelity DNA Polymerase (Finnzymes Oy, Espoo, Finland) according to manufacturer's instructions, in 30µl reactions with HF buffer, no DMSO, 0.5mM primers and 1.67 µg ml^{-1} template. Two reactions were carried out for each template to detect *CYP51A* and *CYP51A-P*, with primers Frameshift F and Stop Codon R for *CYP51A* and *CYP51A-P*, respectively (Table 5.1). Primers were designed manually, with annealing temperatures and dimer formation calculated in Vector NTI (Invitrogen Corporation). The PCR programme comprised 2 minutes at 95°C; 40 cycles of 10 seconds at 95°C, 20 seconds at the annealing temperature and 50 seconds at 72°C; followed by 4 minutes 10 seconds at 72°C. Annealing temperature was 60°C for the first cycle, reduced in 1°C increments per cycle to 56°C, followed by 35 cycles with an annealing temperature of 55°C. Restriction digests were carried out using 5µl PCR product, in 10µl reactions with enzymes *PstI*, *HindIII* and *EcoRI* (Promega) according to manufacturer's instructions, incubating for 3 hours. Products were run on 1% agarose gel with 0.01% ethidium bromide.

Table 5.1. List of primers used to amplify *CYP51* genes from *R. secalis* for PCR-RFLP analysis, Southern blot probe synthesis, yeast construct synthesis, expression analysis and pyrosequencing.

Assay	Gene	Primer name	Primer sequence [a]
PCR-RFLP	*CYP51A*	Frameshift F	5'- AGGAAATGAGTTTATCCTCAATGGAAGAT -3'
		Stop codon R	5'- TGAAGCCATGTAGGAGAGGACTTCGTTT -3'
	CYP51A-P	Frameshift F	5'- CTGGAAGTGAATTTATCCTCAATGGTAGAC -3'
		Stop codon R	5'- TGGGTGAGGCCGAACTTGACA -3'
Southern	*CYP51A*	Southern F	5'- TGTATGATGATCTCGACCACGGGTT -3'
blot		Reverse 10D	5'- CACCAAACGGTAAGTACGGACTCCTAG -3'
	CYP51A-P	Southern F	as for functional *CYP51A*
		Reverse 31D	5'- CACCAAACGGTAAATAAGGAATCCTAATCT -3'
Yeast	*CYP51A*	pYES F2	5'- GA<u>GGTAC</u>|<u>C</u>ATGCTGGGTATCTTCTCGGTG -3'
construct		pYES R	5'- CT<u>GAGCT</u>|<u>C</u>GACCCTCTTTTCCCATCTAACTCT -3'
	CYP51B	pYES F	5'- GA<u>GGTAC</u>|<u>C</u>ATGGGAATTTTTGAGGCTGTTACAGT -3'
		pYES R	5'- CT<u>GAGCT</u>|<u>C</u>AACCTTAACTTTTTCTCTCTTCTCCCA -3'
		pYES mut	5'- [Phos]GAAGACGACTGTGTACCTTGG[C]ACCC ACGGAAACGAATTCA -3'
Expression	*CYP51A*	Expression F	5'- AGGAAATGAGTTTATCCTCAATGGAAGAT -3'
analysis		Expression R	5'- TGAAGCCATGTAGGAGAGGACTTCGTTT -3'
	CYP51B	Expression F	5'- ACCCCAGTCTTTGGAAAGGATGTGGT -3'
		Expression R	5'- GAGTGGTCGAATTTGTCTCGGCAA -3'
	B-tubulin	Expression F	5'-GTGCAGTCACTGTTCCAGAGTTGACC -3'
		Expression R	5'-GCGGTTTGGACATTGGTGGG -3'
Pyro-	*CYP51A*	Pyro F3	5'- CCTCTAGCTTTCTACACAAAATGTC -3'
sequencing	and	Pyro R2	5'- GTTGTTCGAGTGCGCGTTG -3'
	CYP51A-P	Del F1	5'- [Btn]TTTCGGCARAAAAATGACTGT -3'
		Del R1	5'- ACATCCTGCARTCTWCCATTGAG -3'
		Del S1	5'- TCTWCCATTGAGGATAAAYT -3'

[a] [Phos]: Phosphorylated nuceleotide; [Btn]: Biotinylated nucleoide; Underlining indicates restriction enzyme recognition sequences, | indicates cleavage site; [C]: mismatch

5.2.3 Southern Blot

10μg genomic DNA from isolates 788, K1124 and FI12-63 was digested with high concentration restriction enzymes *HindIII*, *EcoRI*, *PstI* and *EcoRV* (Promega) in 20μl reactions according to manufacturer's instructions. Products were run on a 1.2% agarose gel at 100V for 3 hours. The gel was washed in 0.25M hydrochloric acid for 15 minutes, rinsed

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in distilled water, and blotted onto Hybond N+ membrane (GE Healthcare) with 0.4M sodium hydroxide for 16 hours. The membrane was washed in 2x SSC buffer (Sigma), air dried and stored in dry paper at room temperature. The gel was soaked in ethidium bromide solution for 30 minutes to ensure all DNA had been transferred.

A PCR reaction was carried out using Phusion High-Fidelity DNA Polymerase (Finnzymes Oy, Espoo, Finland) according to manufacturer's instructions, in 30μl reactions with HF buffer, no DMSO and 0.5mM primers. Soithern Blot primers (Table 4.2) were designed manually, with annealing temperatures and dimer formation calculated in Vector NTI (Invitrogen Corporation). Template was 2.5 μl of 10^{-4} diluted plasmid containing cloned cDNA product of *CYP51* for isolate 788 and *CYP51-P* for isolate FI12-63, synthesised as described in section 4.2.4. The PCR programme comprised 2 minutes at 95°C; 40 cycles of 10 seconds at 95°C, 20 seconds at 55°C and 50 seconds at 72°C; followed by 4 minutes 10 seconds at 72°C. PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega, USA), and diluted to give 25ng DNA in 45μl TE buffer. The probe was radiolabelled with [^{32}P] dCTP (Perkin Elmer, Waltham, Massachusetts) using the Amersham Rediprime II Random Prime Labelling System (GE Healthcare, Buckinghamshire) according to manufacturer's instructions.

The membrane was incubated with 50ml hybridisation buffer (7% SDS, 500mM sodium phosphate buffer pH 7.0) at 65°C with rotation in a hybridisation oven (Techne, Bibby Scientific Limited, Staffordshire), then incubated with the *CYP51A* (isolate 788) probe in hybridisation buffer with 100 μg ml^{-1} boiled sheared DNA at 65°C with rotation in a hybridisation oven for 16 hours. The membrane was washed twice in 2 x SSC, 0.1% SDS solution preheated to 65°C, and twice in 0.1% SSC, 0.1% SDS solution preheated to 65°C, at 65°C with rotation in a hybridisation over for 20 minutes. The membrane was sealed in a transparent plastic bag and placed in an autoradiogram cassette for 3 days. The autoradiogram was viewed with a Typhoon 8600 phosphoimager (Amersham Pharmacia Biotech, Buckinghamshire). The membrane was stripped by adding boiling 0.1% SDS and leaving to cool to room temperature, three times, and the hybridisation repeated with *CYP51A-P* (isolate FI12-63) probe.

5.2.4 Yeast Complementation

The primer binding sites and restriction sites used to make *R. secalis CYP51* inserts for yeast constructs are shown in Figure 5.1.
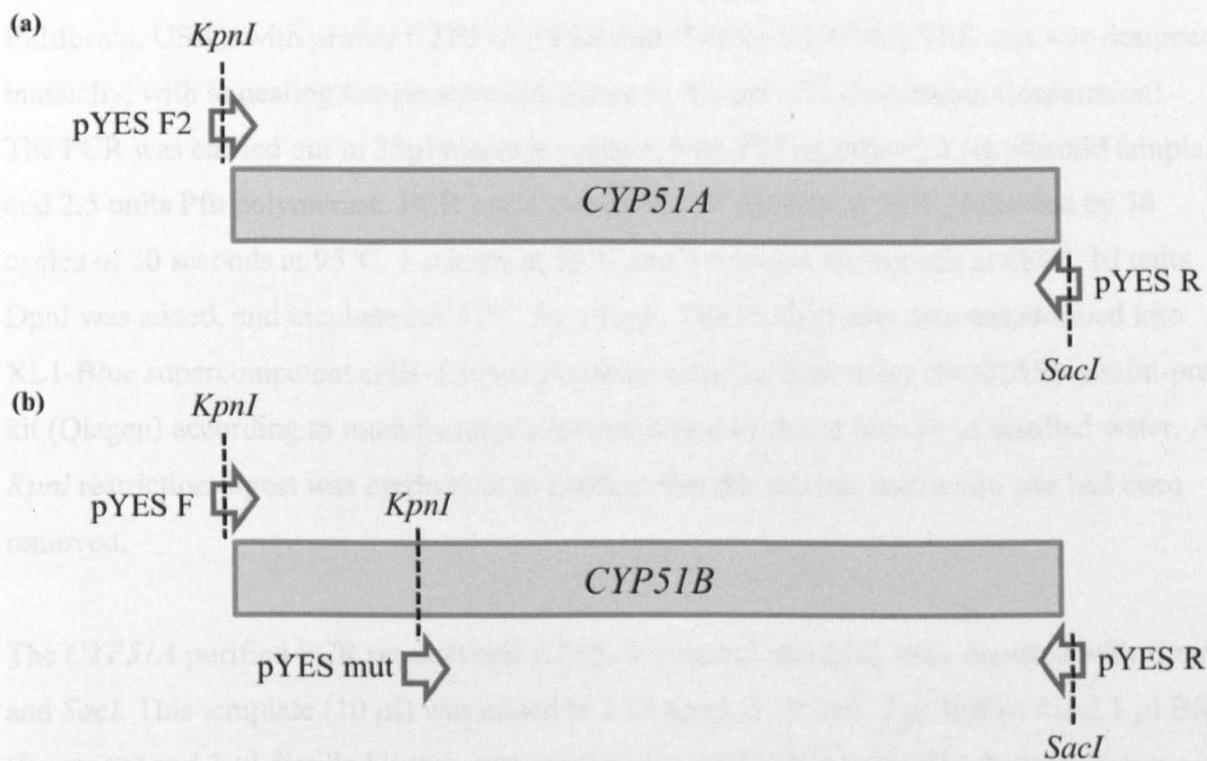
Figure 5.1. Primer binding sites and restriction sites for *R. secalis CYP51* inserts for yeast constructs: (a) *CYP51A*, (b) *CYP51B*.

A PCR reaction was carried out with Easy-A High-Fidelity PCR Cloning Enzyme (Stratagene, California, USA) according to manufacturer's instructions, with primers pYES F and pYES R for *CYP51B* and pYES F2 and pYES R for *CYP51A*. Primers were designed manually, with annealing temperatures and dimer formation calculated in Vector NTI (Invitrogen Corporation). PCR template was a 1/10 dilution of isolate 788 cDNA, synthesised as described in section 5.2.5. The PCR programme comprised 2 minutes at 95°C; followed by 30 cycles of 40 seconds at 95°C, 30 seconds at 62°C and 2 minutes at 72°C; followed by 7 minutes at 72°C. PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega, USA). For *CYP51A*, *PstI* and *HindIII* restriction digests were carried out as described in section 5.2.2 to confirm that the PCR product was *CYP51A* and not *CYP51A-P*.

The *CYP51B* PCR product was ligated into pGEM T-Easy plasmids, cloned in JM109 competent cells and plasmid DNA purified as described in section 4.2.3. Site-directed mutagenesis to remove the internal *KpnI* restriction site by synonymous substitution was carried out with the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies, California, USA), with primer *CYP51B* pYES mut. Primer *CYP51B* pYES mut was designed manually, with annealing temperature calculated in Vector NTI (Invitrogen Corporation). The PCR was carried out in 25 µl reaction volume, with 125 ng primer, 1 µg plasmid template and 2.5 units Pfu polymerase. PCR cycle comprised 30 seconds at 95°C, followed by 30 cycles of 30 seconds at 95°C, 1 minute at 55°C and 4 minutes 30 seconds at 68°C. 10 units DpnI was added, and incubated at 37°C for 1 hour. The product was then transformed into XL1-Blue supercompetent cells. Cloned plasmids were purified using the QIASpin mini-prep kit (Qiagen) according to manufacturer's instructions and eluted into 50 µl distilled water. A *KpnI* restriction digest was carried out to confirm that the internal restriction site had been removed.

The *CYP51A* purified PCR product and *CYP51B* mutated plasmids were digested with *KpnI* and *SacI*. This template (10 µl) was added to 2 µl *KpnI*, 2 µl *SacI*, 2 µl Buffer J and 1 µl BSA (Promega) and 3 µl distilled water, and incubated at 37°C for 3 hours. Products were run on a 1% agarose gel, insert bands were excised, and purified using the Wizard SV Gel and PCR Clean-Up System (Promega). Purified inserts were inserted into pYES2-CT vector. Ligations were carried out with T4 ligase (Promega) with 5:1 insert:vector, incubated at 4°C for 16 hours. Plasmids were then cloned into XL1-Blue supercompetent cells (Agilent) according to manufacturer's instructions but with the initial 1 hour incubation after heat-shocking carried out at 30°C rather than 37°C, and purified with the QiaSpin mini-prep kit (Qiagen).

Purified *CYP51A*, *CYP51B* and empty pYES2-CT plasmids were transformed into *S. cerevisiae* strain YUG37:*erg11*, in which the native *CYP51* is controlled by a doxycycline-repressible promoter. Transformation was carried out using the *S.c.* EasyComp Transformation Kit (Invitrogen) according to manufacturer's instructions. Selection plates comprised SD GAL+RAF agar medium, containing 2 g l^{-1} yeast nitrogen base without amino acids, 1.92 g l^{-1} yeast synthetic drop-out medium supplement without uracil, 20 g l^{-1} galactose and 20 g l^{-1} raffinose, with or without 3 µg ml^{-1} doxycycline.

In order to test for *CYP51* complementation, a growth assay was carried out with and without 3 μg ml^{-1} doxycycline. Yeast transformants were grown in SD GAL+RAF liquid medium at 30°C in an orbital shaker for 24 hours to induce *CYP51* expression. Yeast suspensions were diluted to 1.25 x 10^6 cells μl^{-1}, followed by 6 serial dilutions at 1/5 to give concentrations of 2.5 x 10^5, 5 x 10^4, 1 x 10^4, 2 x 10^3, 4 x 10^2 and 8 x 10^1 cells μl^{-1}. Five microlitre drops of spore suspension were placed on SD GAL+RAF plates with and without 3 μg ml^{-1} doxycycline, left to dry at room temperature, incubated at 30°C for 5 days then visually checked for growth.

5.2.5 *CYP51* Expression Analysis: Constitutive Expression

Isolates QUB 30-10, QUB 12-3, FI12-63, R 9528.4, GKII 18-2-3 and RS 783 were grown in 100ml Sabouraud liquid medium at 2.5 x 10^4 spores ml^{-1} in an orbital shaker at 150 rpm at 20°C for 10 days, at which point cultures were in the linear phase of growth as established in section 2.4.3. Fungal material was harvested by vacuum filtration, immediately placed into liquid nitrogen and stored at -80°C before freeze-drying.

RNA extraction was carried out with TRIzol Reagent (Invitrogen, California, USA), according to manufacturer's instructions, using a tissue homogeniser for 2 minutes, with two phase separation steps using BCP (Molecular Research Center Inc., USA), and resuspending the pellet in 50μl TE. An equal volume of 8M lithium chloride was added, the mixture was incubated overnight at 4°C, then centrifuged at 4°C for 30 minutes and the supernatant discarded. The RNA pellet was washed with 70% ethanol, dried at room temperature, resuspended in RNase-free water and stored at -80°C. The RNA was treated with the TURBO DNA-free Kit (Applied Biosystems, California, USA). cDNA was synthesised with the Superscript III first-strand synthesis system (Invitrogen, California, USA). End-point PCRs were carried out with Red Hot Taq as described in section 3.2.4 with *CYP51B* primers F1 and R1 to check for genomic contamination.

Quantitative PCR was carried out using SYBR Green JumpStart Taq ReadyMix (Sigma), in 25μl reactions with 0.25μM primers and 0.2% cDNA template, with the 7500 Real Time PCR System (Applied Biosystems). *CYP51B*, *CYP51A* and *β-tubulin* primers Expression F and Expression R (Table 5.1) were used, with *β-tubulin* used as the endogenous control. *CYP51B* and *β-tubulin* primers were designed in Vector NTI (Invitorgen Corporation); *CYP51A* primers were designed manually, with annealing temperature and dimer formation

calculated in Vector NTI. The PCR cycle comprised 2 minutes at 95°C; followed by 40 cycles of 15 seconds at 95°C, 30 seconds at 58°C and 36 seconds at 72°C at which point the reading was taken; followed by a dissociation cycle of 15 seconds at 95°C, 1 minute at 60°C and 15 seconds at 95°C. Data were analysed as described in Section 3.2.6, with *β-tubulin* as endogenous control and isolate QUB 30-10 as calibrator sample.

5.2.6 *CYP51* Expression Analysis: Induced Expression

Isolates QUB 30-10, 788, K1124, QUB 12-3, FI12-63, R 9528.4, GKII 18-2-3 and RS 783 were grown as described in section 5.2.5, with two cultures of each isolate. At day 7, tebuconazole solution in acetone was added to one culture of each isolate to give a final concentration of 1 μg ml^{-1}. An equal volume of acetone without tebuconazole was added to the other cultures. At day 10, cultures were harvested by vacuum filtration, immediately placed in liquid nitrogen and stored at -80°C before freeze-drying. RNA extraction and quantitative PCR was carried out as described in section 5.2.5. Two biological replicates were carried out. Data were analysed as described in Section 3.2.6, with *β-tubulin* as endogenous control and isolate R9528.4 without fungicide as calibrator sample. Correlation between EC$_{50}$ values, and gene upregulation following addition of tebuconazole, was tested by least squares linear regression analysis in GenStat 13th Edition (VSN International, Hertfordshire, UK).

5.2.7 Pyrosequencing Assay

Spring barley has been grown at Hoosfield at Rothamsted Research in Hertfordshire, UK, since 1852. DNA had been extracted from grain samples. *Rhynchosporium secalis* disease levels had been quantified by qPCR (Simon Atkins, unpublished data), and years with high levels were selected for pyrosequencing analysis.

A PCR reaction was carried out in 25μl reactions using Red Hot Taq (ABgene) according to manufacturer's instructions, with 1.5mM MgCl$_2$, 0.2μM dNTPs, 0.2μM primers and 1ng μl^{-1} template. Primers used were Pyro F3 and Pyro R3 (Table 5.1), designed using Pyrosequencing Assay Design Software (Biotage, Uppsala, Sweden). The PCR programme comprised 2 minutes at 94°C; followed by 40 cycles of 30 seconds at 94°C, 1 minute at 52°C and 90 seconds at 72°C; followed by 5 minutes at 72°C. The products were diluted 500-fold in nanopure water (Sigma), and a nested PCR carried out with the same conditions as the initial PCR but using primers Del F1 and Del R1 (Table 5.1), designed using Pyrosequencing Assay Design Software (Biotage, Uppsala, Sweden), and an annealing temperature of 58°C.

The Pyrosequencing assay was carried out using the PyroMark system (Qiagen), according to manufacturer's instructions. The bead mix was made with 3μl streptavidin-coated Sepharose beads, 37μl binding buffer, 25μl water and 15μl PCR product per well. Samples were processed with the Q96 vacuum workstation, and annealed to primer Del S1 (Table 5.1). Sequencing was carried out using the PSQ96 instrument (Biotage, Uppsala, Sweden), according to manufacturer's instructions.

5.3 Results

5.3.1 Analysis of *CYP51* paralogues in fungal genomes

CYP51 genes identified in genomes on the Broad Institute server are listed in Table 5.2. The full amino acid alignment is given in Appendix 5. For the alignment with ambiguously-aligned regions excluded, the TVM+I+G model was selected for phylogenetic reconstruction in PhyML, with GTR+I+G selected from the subset of jModeltest models available in MrBayes. For the full alignment of all sites, the TrN+I+G model was selected for phylogenetic reconstruction in PhyML, with HKY+I+G selected from the subset of jModeltest models available in MrBayes. All phylogenetic trees are shown in Figure 5.2.

Figure 5.2. Phylogenetic trees of *CYP51* sequences of *R. secalis* and from fungal genomes on the Broad Institute genome server, rooted with a *CYP7* outgroup. (a-d) Trees reconstructed from nucleotide alignments with ambiguously-aligned regions excluded based on M-Coffee alignment scores; (e-h) trees reconstructed from full nucleotide alignments. (a,e) Neighbour-joining phylograms: node labels indicate percentage bootstrap support, nodes collapsed if bootstrap support is under 50%. (b,f) 50% majority-rule consensus cladogram of most parsimonious trees: node labels indicate bootstrap support. (c,g) Maximum likelihood phylograms: node labels indicate percentage bootstrap support, nodes collapsed if bootstrap support is under 50%; numbers in brackets indicate omitted branch length. (d,h) 50% majority-rule consensus cladogram of Bayesian trees: node labels indicate percentage posterior probability.

(a)

(Figure 5.2)

(b)

(Figure 5.2)

(c)

(Figure 5.2)

(d)

(Figure 5.2)

(e)

(Figure 5.2)

(f)

CYP7 Homo sapiens
A1 Homo sapiens
G1 Arabidopsis thaliana
B1 Mycobacterium tuberculosis
E1 Trypanosoma cruzi
F5 Rhizopus oryzae
F1 Rhizopus oryzae
F1P Rhizopus oryzae
Puccinia triticina PTTG_05595.1
F1 Ustilago maydis
F1 Cryptococcus neoformans
Coprinopsis cinerea CC1G_07605
Coprinopsis cinerea CC1G_08364
F1 Schizosaccharomyces pombe
F1 Saccharomyces cerevisiae
F1 Candida guilliermondii
F1 Candida lusitaniae
F1 Debaryomyces hansenii
F1 Candida albicans
F1 Candida tropicalis
F1 Candida parapsilosis
F1 Lodderomyces elongisporus
F3 Fusarium graminearum
F3 Fusarium oxysporum
F3 Fusarium verticillioides
Mycosphaerella graminicola110231
Pyrenophora tritici-repentis PTRG_08691
Stagonospora nodorum SNOG_03702.1
Rhynchosporium secalis CYP51B
Botrytis cinerea BC1G_11853.1
Sclerotinia sclerotiorum SS1G_04805.1
F1 Magnaporthe oryzae
Chaetomium globosum CHGG_01652.1
F1 Neurospora crassa
F1 Fusarium graminearum
F1 Fusarium oxysporum
F1 Fusarium verticillioides
Colletotrichum graminicola GLRG_01612.1
Colletotrichum higginsianum CH063_04981.1
Verticillium albo-atrum VDBG_04839
Verticillium dahliae VDAG_04380
F1P Aspergillus terreus
F1 Aspergillus terreus
F1 Aspergillus nidulans
F1 Aspergillus clavatus
F1 Aspergillus fumigatus
F1 Neosartorya fischeri
F1 Aspergillus niger
F1 Aspergillus flavus
F1 Aspergillus oryzae
Paracoccidioides brasiliensis PAAG_00827
Blastomyces dermatitidis BDBG_01678.1
F1 Histoplasma capsulatum
F1 Coccidioides immitis
F1 Uncinocarpus reesii
Microsporum canis MCYG_07307.2
Microsporum gypseum MGYG_04803.2
Trichophyton rubrum TERG_01703.2
Trichophyton equinum TEQG_00435.2
Trichophyton tonsurans TESG_04364.2
Trichophyton verrucosum TRV_00734
Arthroderma benhamiae ARB_00950
Pyrenophora tritici-repentis PTRG_08691
Coccidioides immitis CIMG_07469
F7 Uncinocarpus reesii
Colletotrichum higginsianum CH063_09462.1
F2 Magnaporthe oryzae
Rhynchosporium secalis CYP51A
Rhynchosporium secalis CYP51A-p
F2 Fusarium graminearum
F2 Fusarium oxysporum
F2 Fusarium verticillioides
F2 Aspergillus nidulans
F2 Aspergillus terreus
F2 Aspergillus niger
F4 Aspergillus flavus
F4 Aspergillus oryzae
F2 Aspergillus clavatus
F2 Aspergillus fumigatus
F2 Neosartorya fischeri
F2 Aspergillus flavus
F2 Aspergillus oryzae
Microsporum canis MCYG_07010.2
F2 Histoplasma capsulatum
Blastomyces dermatitidis BDBG_04743.1
Microsporum gypseum MGYG_07916.2
Trichophyton equinum TEQG_02768.2
Trichophyton tonsurans TESG_02544.2
Trichophyton rubrum TERG_02984.2
Trichophyton verrucosum TRV_00734
Arthroderma benhamiae ARB_00950

Basidiomycete
Saccharomycotina
Pezizomycotina C
Pezizomycotina B
Pezizomycotina A

(Figure 5.2)

154

(g)

(Figure 5.2)

(h)

(Figure 5.2)

The presence of introns in ascomycete *CYP51* genes is shown in Figure 5.3. According to maximum parsimony ancestral state reconstructions, an intron after alignment position 357 (*C. albicans* base pair 235) was gained once with three subsequent losses. The intron after alignment position 566 (*C. albicans* position 432) was gained once with two subsequent losses. For the intron after position 1946 (*C. albicans* position 1518), two equally parsimonious reconstructions are possible, with one gain followed by three losses or three independent gains and one subsequent loss. Other introns are limited to single branches.

Filamentous ascomycete *CYP51* paralogues fall into three main clades, designated A, B and C (Figure 5.2). *CYP51* genes are listed, along with gene names according to P450 nomenclature (Nelson 2009), and the clade into which they fall in phylogenetic analyses, in Table 5.2.

The tree including *N. haematococca* gene e_gw1.5.672.1 was largely congruent with the maximum likelihood tree previously obtained from the full alignment, with the *N. haematococca* gene falling into the *CYP51C* clade as sister group to the other *Fusarium* spp. *CYP51F3* paralogues. Amino acid sequence variations between the three ascomycete CYP51 clades and yeast CYP51 in the predicted CYP51 substrate recognition sites (Podust *et al.* 2001) are shown in Figure 5.4.

Selection testing by the codon-based Z-test found no significant evidence of positive selection except in some comparisons with *Rhizopus oryzae CYP51F1-p*. The majority of pairwise comparisons showed evidence of purifying selection, even between paralogues.

Diverge results are listed in Table 5.3. All comparisons show significant evidence of Type I functional divergence. The greatest coefficient of functional divergence, θ_λ, is between yeast *CYP51* and *CYP51C*; the least functional divergence is between *CYP51A* and *CYP51B*. CodeML results are listed in Table 5.4. Likelihood is significantly greater (p<0.001) for new Branch-site model A with free ω_2 than with $\omega_2=1$ with the branch leading to the *CYP51A*, *CYP51B* or *CYP51C* clade as the foreground branch. $\omega_2 > 1$ was calculated as occurring at 6.6, 6.5 and 16.9% of sites on the branches leading to *CYP51A*, *CYP51B* and *CYP51C*, respectively.
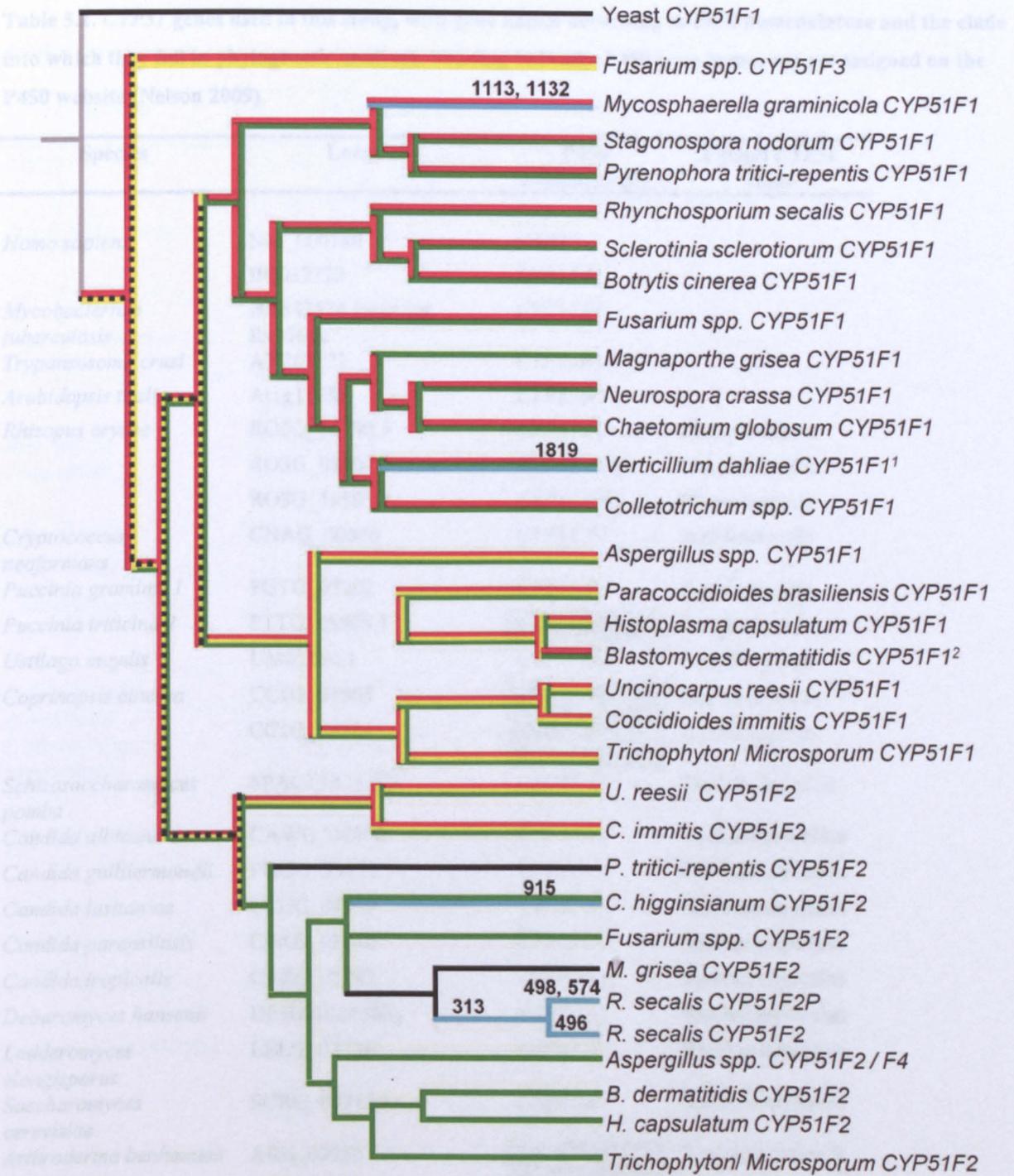
Figure 5.3. Introns present in ascomycete *CYP51* genes, shown on the maximum likelihood cladogram, with ancestral states reconstructed by maximum parsimony. Green: intron after alignment position 357; Red: intron after position 566; yellow: intron after position 1946; Blue: other introns, after the alignment position numbers indicated. Dashed lines: uncertainty due to two equally parsimonious solutions. [1]*V. albo-atrum* excluded due to poor sequence quality at the 3' end. [2] Intron after position 1946 was not predicted by GeneWise due to the absence of a guanine at the end of Exon 2, but this residue is not fully conserved in fungal introns, so presence or absence cannot be confirmed without transcript data.

Table 5.2. *CYP51* genes used in this study, with gene names according to P450 nomenclature and the clade into which they fall in phylogenetic analyses. Shading indicates P450 gene names not yet assigned on the P450 website (Nelson 2009).

Species	Locus ID	P450 nomenclature	Fungal *CYP51* clade
Homo sapiens	NM_000780	*CYP7*	
	BC032322	*CYP51A1*	
Mycobacterium tuberculosis	BX842574 locus tag Rv0764c	*CYP51B1*	
Trypansosoma cruzi	AY283023	*CYP51E1*	
Arabidopsis thaliana	At1g11680	*CYP51G1*	
Rhizopus oryzae	RO3G_11790.3	*CYP51 F1*	Mucoromycete
	RO3G_08504.3	*CYP51 F1-p*	Mucoromycete
	RO3G_16595.3	*CYP51 F5*	Mucoromycete
Cryptococcus neoformans	CNAG_00040	*CYP51 F1*	Basidiomycete
Puccinia graminis 1	PGTG_07202	*CYP51 F1*	Basidiomycete
Puccinia triticina 2	PTTG_05595.1	*CYP51 F1*	Basidiomycete
Ustilago maydis	UM03662.1	*CYP51 F1*	Basidiomycete
Coprinopsis cinerea	CC1G_07605	*CYP51 F1*	Basidiomycete
	CC1G_08364	Not yet designated	Basidiomycete
Schizosaccharomyces pombe	SPAC13A11.02c	*CYP51 F1*	Taphrinomcotina
Candida albicans	CAWG_04460	*CYP51 F1*	Saccharomycotina
Candida guilliermondii	PGUG_03415	*CYP51 F1*	Saccharomycotina
Candida lusitaniae	CLUG_04932	*CYP51 F1*	Saccharomycotina
Candida parapsilosis	CPAG_03310	*CYP51 F1*	Saccharomycotina
Candida tropicalis	CTRG_05283	*CYP51 F1*	Saccharomycotina
Debaromyces hansenii	DEHA0E20383g	*CYP51 F1*	Saccharomycotina
Lodderomyces elongisporus	LELG_03738	*CYP51 F1*	Saccharomycotina
Saccharomyces cerevisiae	SCRG_04712.1	*CYP51 F1*	Saccharomycotina
Arthroderma benhamiae	ARB_00950	*CYP51 F1*	Pezizomycotina B
	ARB_05314	*CYP51 F2*	Pezizomycotina A
Aspergillus clavatus	ACLA_005420	*CYP51 F1*	Pezizomycotina B
	ACLA_046180	*CYP51 F2*	Pezizomycotina A
Aspergillus flavus	AFL2G_06478	*CYP51 F1*	Pezizomycotina B
	AFL2G_02771	*CYP51 F2*	Pezizomycotina A
	AFL2G_10953	*CYP51 F4*	Pezizomycotina A
Aspergillus fumigatus	Afu7g03740	*CYP51 F1*	Pezizomycotina B
	Afu4g06890	*CYP51 F2*	Pezizomycotina A
Aspergillus nidulans	ANID_08283	*CYP51 F1*	Pezizomycotina B
	ANID_01901	*CYP51 F2*	Pezizomycotina A

Table 5.2 continued

Species	Locus ID	P450 nomenclature	Fungal *CYP51* clade
Aspergillus niger	est_fge1_pm_C_150008	*CYP51 F1*	Pezizomycotina B
	est_fge1_pm_C_40090	*CYP51 F2*	Pezizomycotina A
Aspergillus oryzae	AO090026000842	*CYP51 F1*	Pezizomycotina B
	AO090003000205	*CYP51 F2*	Pezizomycotina A
	AO090020000357	*CYP51 F4*	Pezizomycotina A
Aspergillus terreus	ATEG_02850	*CYP51 F1*	Pezizomycotina B
	ATEG_10302	*CYP51 F1-p*	Pezizomycotina B
	ATEG_05917	*CYP51 F2*	Pezizomycotina A
Blastomyces dermatitidis	BDBG_01678.1	*CYP51 F1*	Pezizomycotina B
	BDBG_04743.1	*CYP51 F2*	Pezizomycotina A
Botrytis cinerea	BC1G_11853.1	*CYP51 F1*	Pezizomycotina B
Chaetomium globosum	CHGG_01652.1	*CYP51 F1*	Pezizomycotina B
Coccidioides immitis	CIMG_07469	*CYP51 F1*	Pezizomycotina B
	CIMG_00573	*CYP51 F2/ CYP51 F7*	Pezizomycotina A
Colletotrichum graminicola	GLRG_01612.1	*CYP51 F1*	Pezizomycotina B
Colletotrichum higginsianum	CH063_04981.1	*CYP51 F1*	Pezizomycotina B
	CH063_09462.1	*CYP51 F2*	Pezizomycotina A
Fusarium graminearum	FGSG_01000	*CYP51 F1*	Pezizomycotina B
	FGSG_04092	*CYP51 F2*	Pezizomycotina A
	FGSG_11024	*CYP51 F3*	Pezizomycotina C
Fusarium oxysporum	FOXG_00394	*CYP51 F1*	Pezizomycotina B
	FOXG_11545	*CYP51 F2*	Pezizomycotina A
	FOXG_13138	*CYP51 F3*	Pezizomycotina C
Fusarium verticillioides	FVEG_01123	*CYP51 F1*	Pezizomycotina B
	FVEG_10277	*CYP51 F2*	Pezizomycotina A
	FVEG_12391	*CYP51 F3*	Pezizomycotina C
Histoplasma capsulatum (NAm1)	HCAG_04048.3	*CYP51 F1*	Pezizomycotina B
	HCAG_04481.3	*CYP51 F2*	Pezizomycotina A
Magnaporthe oryzae	MGG_04432.6	*CYP51 F1*	Pezizomycotina B
	MGG_04628.6	*CYP51 F2*	Pezizomycotina A
Microsporum canis	MCYG_07307.2	*CYP51 F1*	Pezizomycotina B
	MCYG_07010.2	*CYP51 F2*	Pezizomycotina A
Microsporum gypseum	MGYG_04803.2	*CYP51 F1*	Pezizomycotina B
	MGYG_07916.2	*CYP51 F2*	Pezizomycotina A
Mycosphaerella graminicola	110231	*CYP51 F1*	Pezizomycotina B
Neosartorya fischeri	NFIA_024690	*CYP51 F1*	Pezizomycotina B
	NFIA_109350	*CYP51 F2*	Pezizomycotina A

Table 5.2 continued

Species	Locus ID	P450 nomenclature	Fungal *CYP51* clade
Neurospora crassa	NCU02624	*CYP51 F1*	Pezizomycotina B
Paracoccidioides brasiliensis	PAAG_00827 (Pb01)	*CYP51 F1*	Pezizomycotina B
Pyrenophora tritici-repentis	PTRG_08691	*CYP51 F1*	Pezizomycotina B
	PTRG_09430	*CYP51 F2*	Pezizomycotina A
Rhynchosporium secalis	*CYP51B*	*CYP51 F1*	Pezizomycotina B
	CYP51A	*CYP51 F2*	Pezizomycotina A
	CYP51A-p	*CYP51 F2-P*	Pezizomycotina A
Sclerotinia sclerotiorum	SS1G_04805.1	*CYP51 F1*	Pezizomycotina B
Stagonospora nodorum	SNOG_03702.1	*CYP51 F1*	Pezizomycotina B
Trichophyton equinum	TEQG_00435.2	*CYP51 F1*	Pezizomycotina B
	TEQG_02768.2	*CYP51 F2*	Pezizomycotina A
Trichophyton rubrum	TERG_01703.2	*CYP51 F1*	Pezizomycotina B
	TERG_02984.2	*CYP51 F2*	Pezizomycotina A
Trichophyton tonsurans	TESG_04364.2	*CYP51 F1*	Pezizomycotina B
	TESG_02544.2	*CYP51 F2*	Pezizomycotina A
Trichophyton verrucosum	TRV_00734	*CYP51 F1*	Pezizomycotina B
	TRV_06889	*CYP51 F2*	Pezizomycotina A
Uncinocarpus reesii	UREG_07804.1	*CYP51 F1*	Pezizomycotina B
	UREG_00593.1	*CYP51F7*	Pezizomycotina A
Verticillium albo-atrum	VDBG_04839	*CYP51 F1*	Pezizomycotina B
Verticillium dahliae	VDAG_04380	*CYP51 F1*	Pezizomycotina B
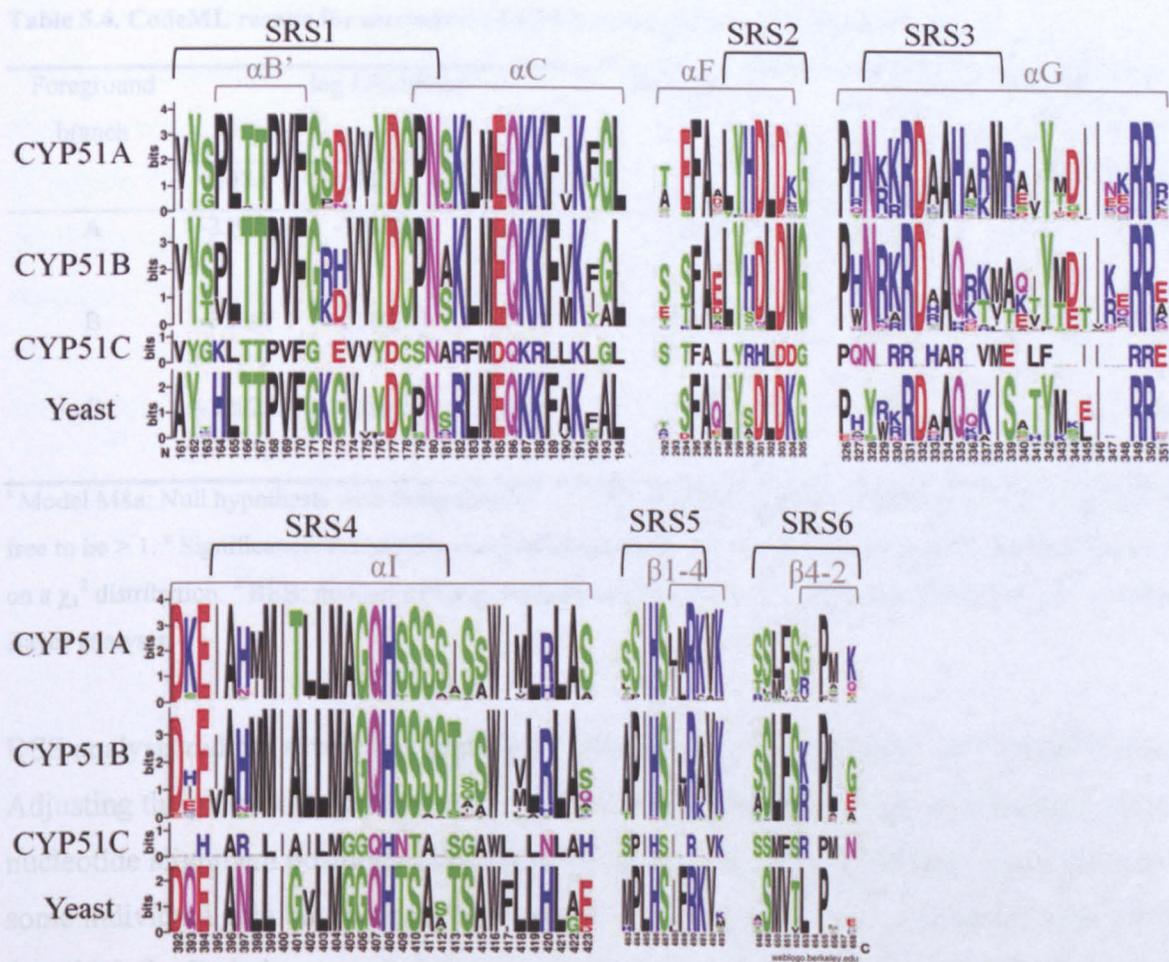
Figure 5.4. Amino acid sequence logos (Crooks *et al.* 2004) for filamentous ascomycete CYP51 groups A, B and C and yeast CYP51.

Table 5.3. Diverge results for ascomycete *CYP51* clades.

	Yeast/C	Yeast/A	Yeast/B	C/A	C/B	A/B
θ_λ (ML method) [a]	0.41 ±0.09	0.29 ±0.03	0.32 ±0.04	0.21 ±0.06	0.23 ±0.08	0.16 ±0.03
LRT θ_λ [b]	22.3 ***	72.1 ***	74.3 ***	13.6 ***	8.2 **	40.0 ***
Number of sites where P(F_1) > 50% [c]	78	46	58	11	9	21
Number of sites where P(F_1) > 67%	20	26	34	7	6	11

[a] Maximum likelihood estimate of $\theta \pm$ standard error. [b] Likelihood ratio test. ** significant at 1%, *** significant at 0.1%. [c] If P(F_1) > 50%, sites are more likely than not to have experienced functional divergence; if P(F_1) > 0.67, sites are twice as likely as not to have experienced functional divergence.

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Table 5.4. CodeML results for ascomycete *CYP51*s under the branch-site model.

Foreground branch	log Likelihood			Significance [c]	Foreground sites with ω > 1		
	M8a [a]	M8 [b]	Difference		Proportion	Number	BEB [d]
A	-32943.4	-32934.6	-8.8	>0.001	0.066	38	7
B	-32947.1	-32937.3	-9.8	>0.001	0.064	37	7
C	-32925.8	-32915	-10.8	>0.001	0.169	97	29

[a] Model M8a: Null hypothesis with foreground ω = 1. [b] Model M8: Alternative hypothesis with foreground ω free to be > 1. [c] Significance: Probability that likelihood under M8 is significantly greater than under M8a, based on a χ_1^2 distribution. [d] BEB: number of foreground sites with P (ω > 1) < 0.05 identified by Bayes Empirical Bayes analysis.

DSS analysis did not reveal any significant evidence of recombination at 95% significance. Adjusting the probability threshold to 50% resulted in three partitions, with breakpoints at nucleotide alignment positions 1150 and 1805, gave three trees differing in the placement of some individual taxa but retaining the main A, B, C and yeast clades except the final partition for which the B clade was split into two groups, as for trees generated from unambiguously aligned sites only, since the monophyly of the *CYP51B* clade is most strongly supported by a paralogue-specific region encoding the N-terminal region of the protein.

5.3.1.1 Protein localisation prediction

TargetP predicted most CYP51s as localising within the secretory pathway, although some CYP51Bs were predicted as 'other' (non-secretory, non-mitochondrial) (Table 5.5). Similar results were obtained by Protein Prowler. WolfPSORT also predicted locations within the secretory pathway (Plasma membrane, extracellular, endoplasmic reticulum or Golgi apparatus) for most CYP51s, and MultiLoc predicted all CYP51s to be located in the Plasma membrane, Golgi apparatus or endoplasmic reticulum (Table 5.5). The sequences were then analysed with SignalP. All CYP51B and CYP51C N-terminal peptides were predicted to form signal anchors, whereas most CYP51A N-terminal peptides were predicted to be cleaved signal peptides (Table 5.5).

However, TMHMM predicted transmembrane helices in the N-terminal regions of all fungal CYP51s. Since SingalP predicted a signal peptide and TMHHMM predicted a

transmembrane helix within the same region of most proteins, Phobius (Kall *et al.* 2004) was used to distinguish between the two (Emanuelsson *et al.* 2007). All CYP51 N-terminal regions were predicted as containing a transmembrane region and not a signal peptide, except *U. maydis* CYP51, *Schizosaccharomyces pombe* CYP51 and *C. cinerea* CC1G_08364, with predicted signal peptides upstream of the predicted transmembrane region, and *P. tritici-repentis* CYP51A and *Aspergillus niger* CYP51A, predicted as containing a signal peptide and not a transmembrane region (Table 5.5). The *R. secalis* CYP51A and CYP51B Phobius output plots are shown in Figure 5.5.

Table 5.5. Predicted subcellular localisation of fungal CYP51 peptides.

CYP51	TargetP [a]	Wolf PSORT [b]	Protein Prowler	MultiLoc [b]	SignalP	TMHM M Helix	Phobius
Cyp7 Human	Secretory	Golgi	Secretory	Golgi	Signal	5-24	TMH [c]
Mycobacterium tuberculosis B1	Other	Cyto	Other	Cyto	Non-secretory	None	None
Trypanosoma cruzi E1	Secretory	Plasma	Secretory	Golgi	Signal	None	Signal
Arabidopsis thaliana G1	Secretory	Cyto	Secretory	Golgi	Non-secretory	13-32	TMH
CYP51A1 Human	Secretory	Cyto	Other	Golgi	Signal	4-21, 28-50	Signal +TMH
Aspergillus clavatus B	Other	ER	Other	Golgi	Anchor	20-42	TMH
A.fumigatus B	Secretory	Plasma	Secretory	Golgi	Anchor	21-43	TMH
A. flavus B	Secretory	Plasma	Secretory	Plasma	Anchor	20-42	TMH
A. oryzae B	Secretory	Plasma	Secretory	Plasma	Anchor	20-42	TMH
A. niger B	Secretory	ER	Secretory	Plasma	Anchor	20-42	TMH
A. terreus B	Secretory	Plasma	Secretory	Plasma	Anchor	19-41	TMH
A. nidulans B	Secretory	Plasma	Secretory	Plasma	Anchor	20-42	TMH
A. terreus B	Other	ER	Other	Golgi	Anchor	20-42	TMH
Neosartorya fischeri	Secretory	Plasma	Secretory	Extra-cellular	Anchor	21-43	TMH
Blastomyces dermatitidis	Secretory	Plasma	Secretory	Extra-cellular	Anchor	20-42	TMH
Trichophyton rubrum	Other	Cyto	Secretory	Extra-cellular	Anchor	21-43	TMH
Microsporum canis	Other	Plasma	Other	Extra-cellular	Anchor	20-42	TMH
M. gypseum	Other	Plasma	Other	Extra-cellular	Anchor	21-43	TMH
Trichophyton equinum	Other	Cyto	Secretory	Extra-cellular	Anchor	21-43	TMH
T. tonsurans	Other	Cyto	Secretory	Extra-cellular	Anchor	21-43	TMH
T. verrucosum	Other	Cyto	Secretory	Extra-cellular	Anchor	21-43	TMH
Arthroderma benhamiae	Other	Cyto	Secretory	Extra-cellular	Anchor	21-43	TMH
Coccidioides immitis B	Secretory	Extra-cellular	Secretory	Golgi	Anchor	20-41	TMH
Uncinocarpus reesii B	Secretory	Extra-cellular	Secretory	Golgi	Anchor	20-42	TMH
Histoplasma capsulatum B	Secretory	Plasma	Secretory	Plasma	Anchor	20-42	TMH
Paracoccidioides brasiliensis B	Secretory	Plasma	Secretory	Plasma	Anchor	20-42	TMH
Botrytis cinerea B	Other	ER	Other	Golgi	Anchor	20-42	TMH
Sclerotinia sclerotiorum B	Other	Cyto	Secretory	Golgi	Anchor	20-42	TMH
Rhynchosporium secalis B	Secretory	Plasma	Secretory	Golgi	Anchor	20-42	TMH
Chaetomium globosum B	Other	MT	Other	Golgi	Anchor	20-41	TMH

Table 5.5 continued

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CYP51	TargetP [a]	Wolf PSORT [b]	Protein Prowler	MultiLoc [b]	SignalP	TMHMM Helix	Phobius
Neurospora crassa B	Secretory	ER	Other	Golgi	Anchor	20-41	TMH
Fusarium graminearum B	Other	Cyto	Other	Golgi	Anchor	20-42	TMH
F. oxysporum B	Other	Cyto	Other	Golgi	Anchor	20-42	TMH
F. verticillioides B	Other	Cyto	Other	Golgi	Anchor	20-42	TMH
Colletotrichum graminicola	Secretory	Extra-cellular	Other	Extra-cellular	Anchor	20-42	TMH
Colletotrichum higginsianum	Secretory	ER	Other	Extra-cellular	Anchor	20-42	TMH
Magnaporthe oryzae B	Other	ER	Other	Golgi	Anchor	15-37	TMH
Verticillium albo-atrum B	Other	ER	Other	Golgi	Anchor	24-46	TMH
V. dahliae B	Other	ER	Other	Golgi	Anchor	20-41	TMH
Pyrenophora tritici-repentis B	Other	Cyto	Other	Golgi	Anchor	20-42	TMH
Stagonospora nodorum B	Other	Plasma	Other	Golgi	Anchor	20-41	TMH
Mycosphaerella graminicola	Secretory	Cyto	Other	Extra-cellular	Anchor	20-42	TMH
Candida albicans	Secretory	Cyto-nuclear	Other	Golgi	Non-secretory	15-37	TMH
C. tropicalis	Other	Nuclear	Other	Golgi	Non-secretory	15-37	TMH
C. guilliermondii	Secretory	Golgi	Other	Golgi	Non-secretory	20-42	TMH
C. lusitaniae	Secretory	Plasma	Secretory	Golgi	Signal	15-37	TMH
Candida parapsilosis	Secretory	Mito	Secretory	Extra-cellular	Non-secretory	15-37	TMH
Lodderomyces elongisporus	Secretory	ER	Secretory	Extra-cellular	Non-secretory	15-37	TMH
Debaryomyces hansenii	Secretory	ER	Other	Extra-cellular	Non-secretory	15-37	TMH
Saccharomyces cerevisiae	Secretory	MT	Other	Golgi	Non-secretory	29-51	TMH
Schizosaccharomyces pombe	Secretory	Plasma	Secretory	Extra-cellular	Signal	5-22	Signal +TMH
Coprinopsis cinerea F1	Secretory	Plasma	Secretory	Golgi	Anchor	6-25	TMH
C. cinerea CC1G_08364	Other	Plasma	Other	Golgi	Non-secretory	38-60	Signal +TMH
Cryptococcus neoformans	Secretory	Cyto	Secretory	Golgi	Non-secretory	32-54	TMH
Puccinia graminis	Secretory	Cyto	Secretory	Golgi	Anchor	20-39	TMH
Ustilago maydis	Secretory	Plasma	Other	ER	Signal	32-54	Signal +TMH
Rhizopus oryzae F1	Secretory	ER	Secretory	Extra-cellular	Anchor	13-35	TMH
Rhizopus oryzae F5	Secretory	Nuclear	Secretory	Extra-cellular	Anchor	12-34	TMH
F. graminearum C	Secretory	Plasma	Secretory	Plasma	Anchor	15-34	TMH

Table 5.5 continued

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CYP51	TargetP [a]	Wolf PSORT [b]	Protein Prowler	MultiLoc [b]	SignalP	TMHM M Helix	Phobius
F. oxysporum C	Secretory	Plasma	Secretory	Plasma	Anchor	12-34	TMH
F. verticillioides C	Secretory	Plasma	Secretory	Golgi	Anchor	12-34	TMH
C. immitis A	Secretory	Plasma	Secretory	Golgi	Anchor	10-32	TMH
U. reesii A	Secretory	Plasma	Secretory	Plasma	Anchor	10-32	TMH
P. tritici-repentis A	Secretory	Extra-cellular	Secretory	Golgi	Signal	5-24	Signal
F. graminearum A	Secretory	Extra-cellular	Secretory	Golgi	Signal	7-29	TMH
F. oxysporum A	Secretory	Plasma	Secretory	Golgi	Signal	4-25	TMH
F. verticillioides A	Secretory	Plasma	Secretory	Golgi	Signal	4-25	TMH
C. higginsianum A	Secretory	Plasma	Secretory	Extra-cellular	Anchor	10-32	TMH
M. oryzae A	Secretory	Plasma	Secretory	Golgi	Signal	10-32	TMH
R. secalis A	Secretory	Plasma	Secretory	ER	Signal	13-35	TMH
A. flavus A1	Secretory	Plasma	Secretory	Golgi	Signal	7-28	TMH
A. oryzae A1	Secretory	Plasma	Secretory	Golgi	Signal	7-28	TMH
H. capsulatum A	Secretory	Plasma	Secretory	Plasma	Signal	7-24	TMH
A. clavatus A	Secretory	Plasma	Secretory	Plasma	Signal	7-29	TMH
A. fumigatus A	Secretory	Extra-cellular	Secretory	Golgi	Signal	7-29	TMH
A. nidulans A	Secretory	Plasma	Secretory	Golgi	Signal	7-29	TMH
A. flavus A2	Secretory	Plasma	Secretory	Golgi	Signal	5-24	TMH
A. oryzae A2	Secretory	Plasma	Secretory	Golgi	Signal	5-24	TMH
A. niger A	Secretory	Plasma	Secretory	Golgi	Signal	5-24	Signal
A. terreus A	Secretory	Plasma	Secretory	Golgi	Signal	4-23	TMH
N. fischeri A	Secretory	Extra-cellular	Secretory	Extra-cellular	Signal	7-29	TMH
B. dermatitidis A	Secretory	Mito	Secretory	Peroxi-somal	Signal	5-24	TMH
T.rubrum A	Secretory	Plasma	Secretory	Peroxi-somal	Signal	5-27	TMH
M. canis A	Secretory	Plasma	Secretory	Peroxi-somal	Signal	5-27	TMH
M.gypseum A	Secretory	Plasma	Secretory	Extra-cellular	Signal	5-27	TMH
T.equinum A	Secretory	Plasma	Secretory	Extra-cellular	Signal	5-27	TMH
T. tonsurans A	Secretory	Plasma	Secretory	Extra-cellular	Signal	5-27	TMH
T. verrucosum A	Secretory	Plasma	Secretory	Extra-cellular	Signal	5-27	TMH
A.benhamiae A	Secretory	Plasma	Secretory	Extra-cellular	Signal	2-24	TMH

[a] Shaded cells indicate locations in the secretory pathway. [b] ER: Endoplasmic reticulum; Plasma: Plasma membrane; Golgi: Golgi apparatus, MT: Mitochondrial, Cyto: Cytoplasmic. [c] TMH: Transmembrane helix
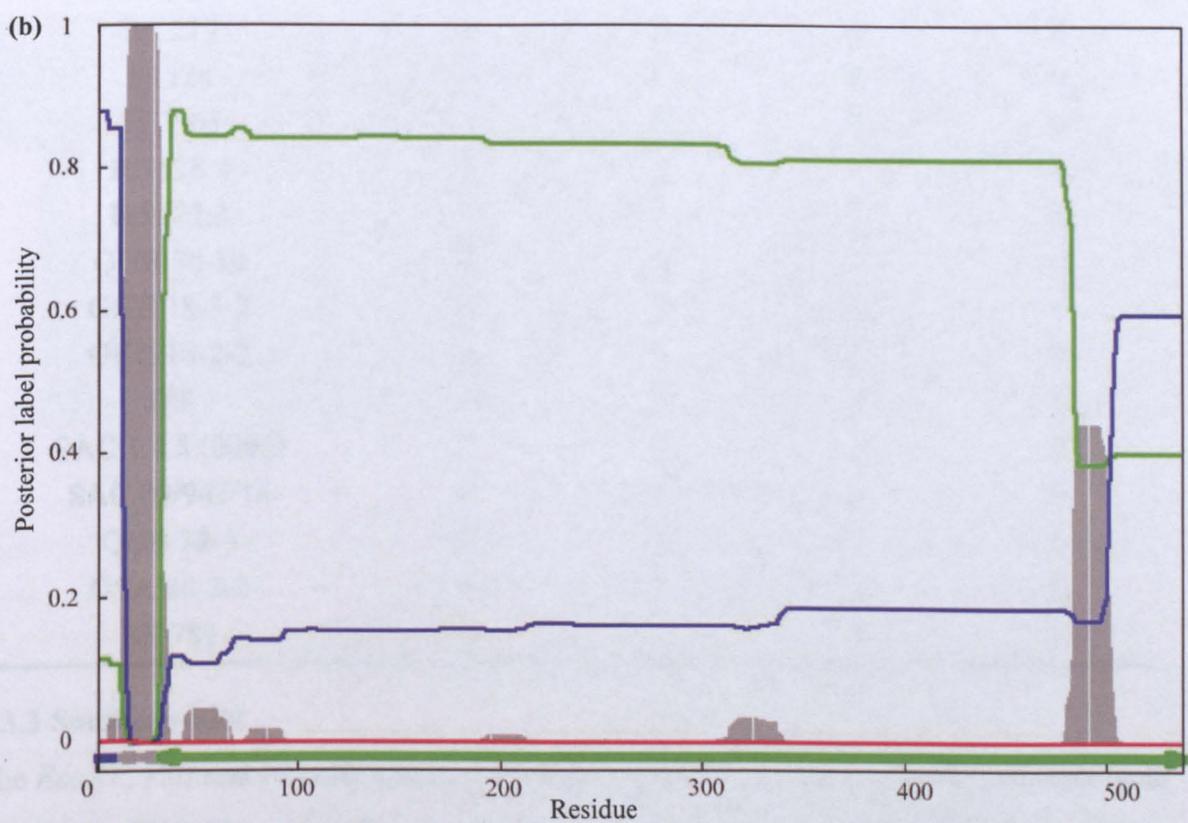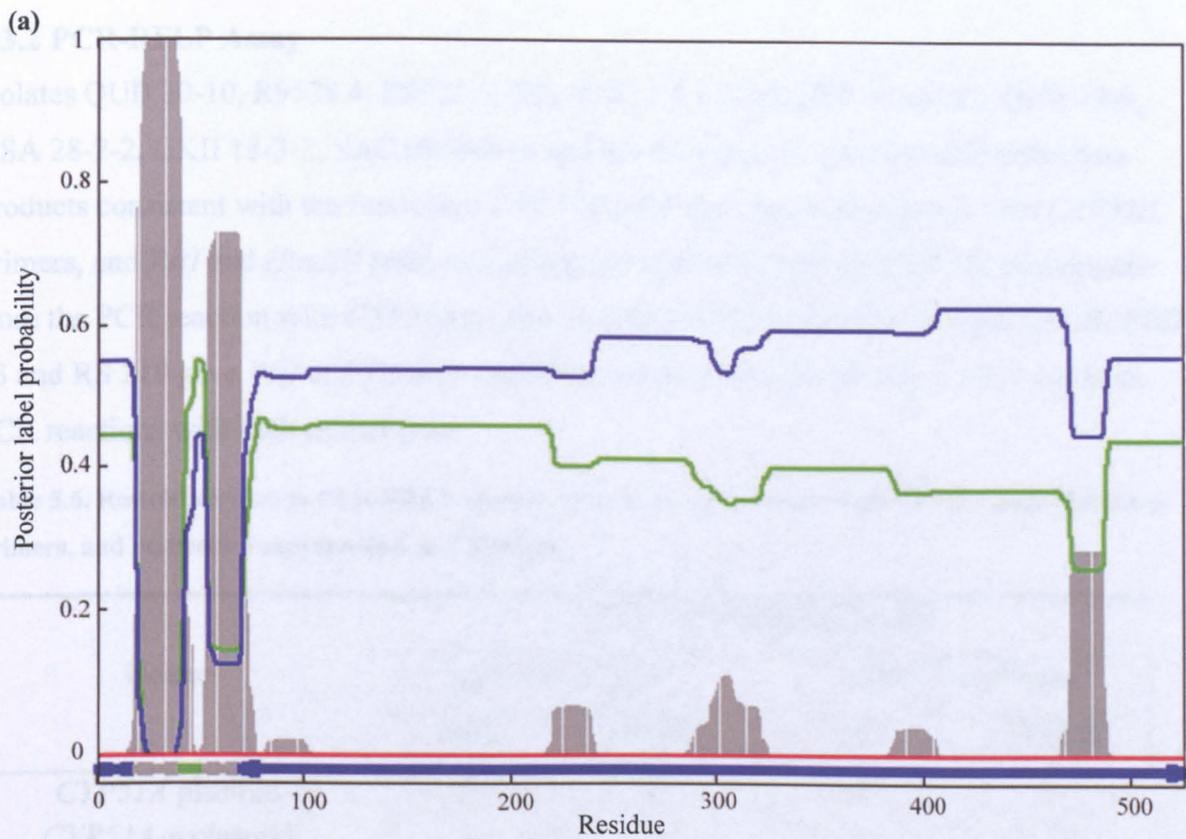
Figure 5.5. Phobius (Kall *et al.* 2004) output plots for (a) *R. secalis* CYP51A and (b) *R. secalis* CYP51B. Red: Signal peptide; Grey: Transmembrane helices; Green: Cytoplasmic; Blue: Non-cytoplasmic.

5.3.2 PCR-RFLP Assay

Isolates QUB 30-10, R9528.4, R9522.3, 788, SAC 1.4.8 (0003), GKII 18-2-3, QUB 12-3, OSA 28-2-2, GKII 18-3-2, SAC 09/943/14 and RS 783 gave *PstI* and *HindIII* restriction products consistent with the functional *CYP51A* gene from the PCR reaction with *CYP51A* primers, and *PstI* and *HindIII* restriction products consistent with the *CYP51A* pseudogene from the PCR reaction with *CYP51A-p* primers (Figure 5.6; Table 5.6). Isolates K1124, FI12-63 and RS 219 gave *PstI* and *HindIII* restriction products consistent with *CYP51A-p* from PCR reactions with both primer pairs.

Table 5.6. Restriction sites in PCR-RFLP analysis of 14 *R. secalis* isolates, with *CYP51A* and *CYP51A-p* primers, and restriction enzymes *PstI* and *HindIII*.

Isolate	PCR-RFLP restriction sites			
	CYP51A primers		*CYP51A-p* primers	
	PstI	*HindIII*	*PstI*	*HindIII*
CYP51A plasmid	0	2	0	2
CYP51A-p plasmid	1	0	1	0
RS 219	1	0	1	0
K1124	1	0	1	0
FI12-63	1	0	1	0
R 9528.4	0	2	1	0
R 9522.3	0	2	1	0
QUB 30-10	0	2	1	0
GKII 18-3-2	0	2	1	0
GKII 18-2-3	0	2	1	0
788	0	2	1	0
SAC 1.4.8 (0003)	0	2	1	0
SAC 09/943/14	0	2	1	0
QUB 12-3	0	2	1	0
OSA 28-2-2	0	2	1	0
RS 783	0	2	1	0

5.3.3 Southern Blot

The *EcoRV*, *PstI* and *HindIII* digests produced two bands for isolate 788 but only one band for isolates FI12-63 and K1124. The *EcoRI* digest is less clear for isolate 788. The same bands are visible with both probes (Figure 5.7).
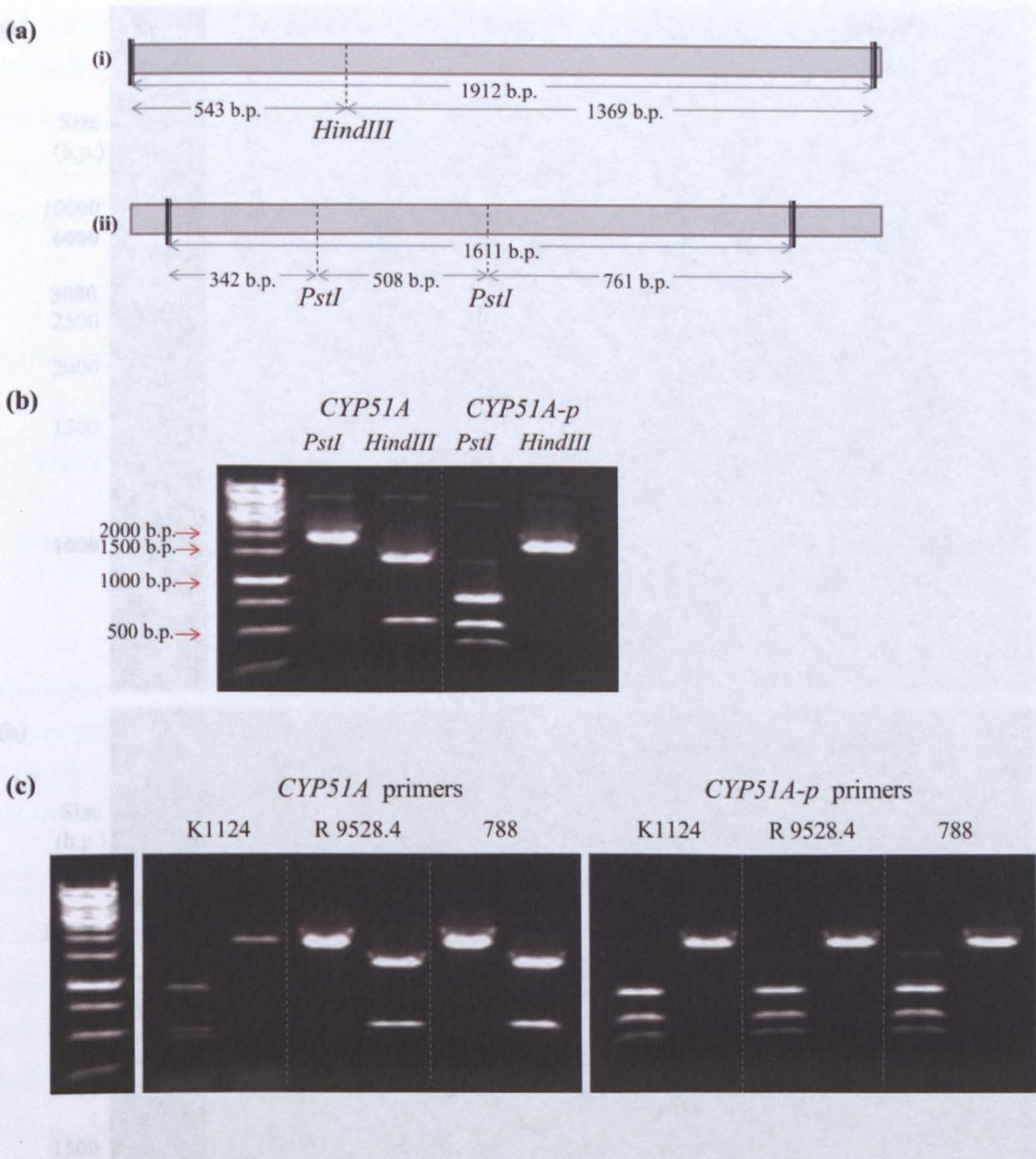
Figure 5.6. (a) Expected PCR-RFLP product lengths for *R. secalis CYP51A* genes. Solid lines indicate primer binding sites, dotted lines indicate restriction sites, arrows indicate amplicon and restriction fragment lengths. (i) *CYP51A*; (ii) *CYP51-p*

(b) Gel obtained from PCR-RFLP analysis of *R. secalis CYP51A* and *CYP51A-p* plasmids (Section 4.2.4), digested with *PstI* and *HindIII*. Ladder is GeneRuler 1kb (Fermentas).

(c) Gel obtained from PCR-RFLP analysis of *R. secalis* isolates K1124, R 9528.4 and 788, amplified with *CYP51A* and *CYP51A-p* primers, digested with (left to right for each isolate) *PstI* and *HindIII*. Ladder is GeneRuler 1kb (Fermentas).
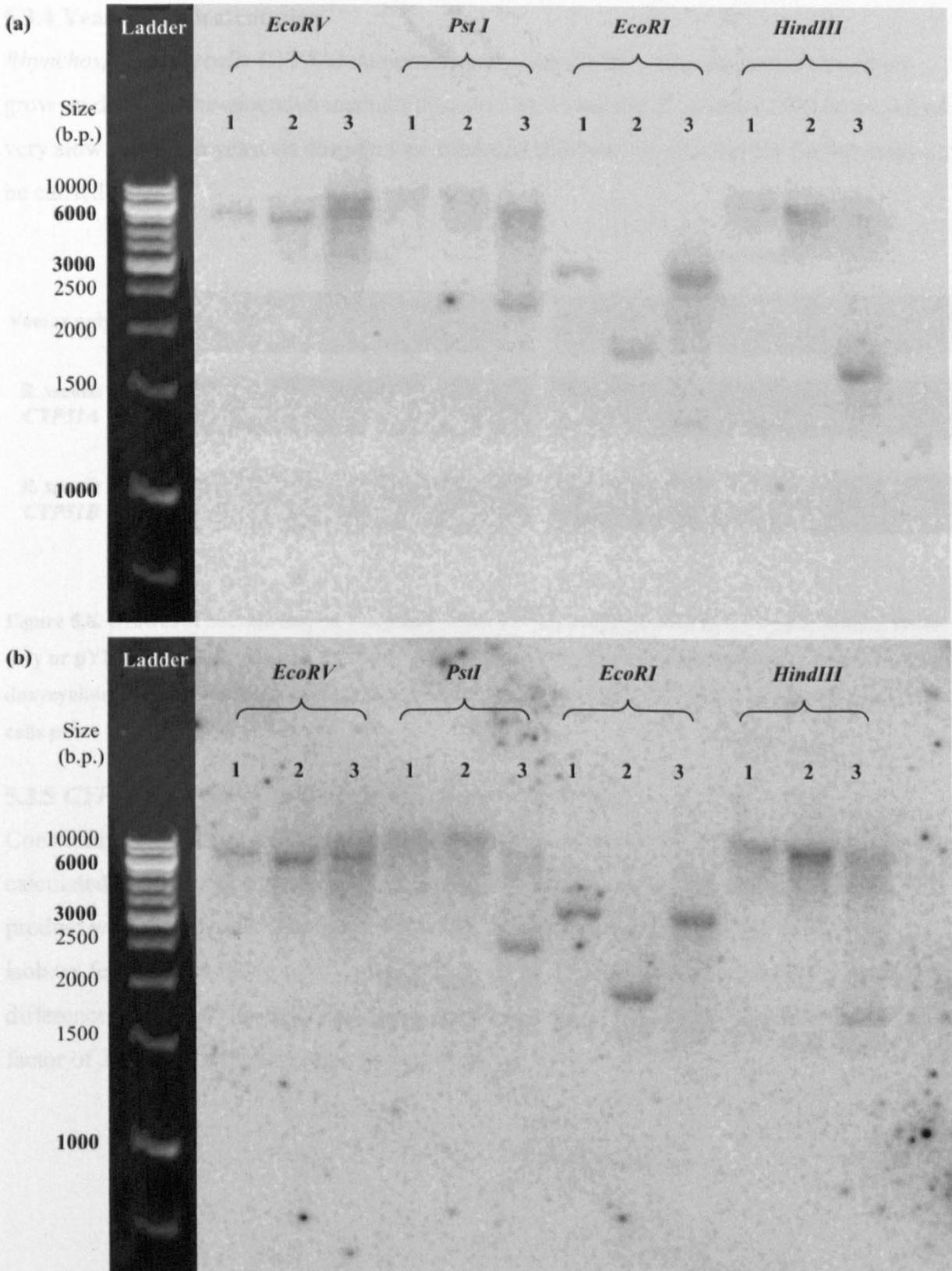
Figure 5.7. Southern blot of *R. secalis* **genomic DNA digested with restriction enzymes** *EcoRV*, *PstI*, *EcoRI* **and** *HindIII*. **(a)** *CYP51A* **probe; (b)** *CYP51A-P* **probe. Isolate 1: FI12-63; 2: K1124; 3: 788.**
Ladder (visualised on agarose gel before transfer to membrane) is GeneRuler 1kb (Fermentas).

5.3.4 Yeast Complementation

Rhynchosporium secalis CYP51A complemented yeast *CYP51*, allowing transformants to grow on doxycycline-amended medium (Figure 5.8). However, *R. secalis CYP51B* produced very slow growth in yeast on doxycycline-amended medium, insufficient for further assays to be carried out.
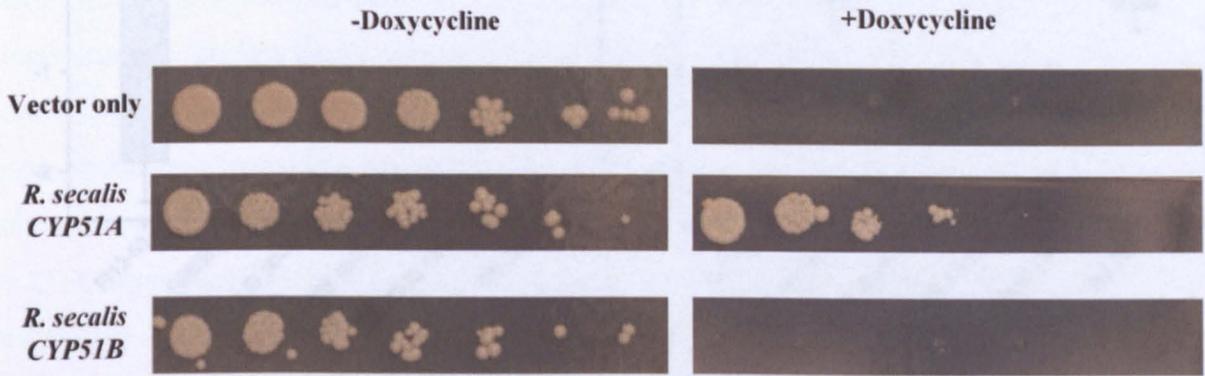
Figure 5.8. Growth of *Saccharomyces cerevisiae* strain YUG37:*erg11*transformed with pYES2-CT vector only or pYES-CT with *R. secalis CYP51A* or *CYP51B* on SD +GAL+RAF agar with and without 3 μg ml^{-1} doxycycline, with six inoculum concentrations of 1.25×10^6, 2.5×10^5, 5×10^4, 1×10^4, 2×10^3 and 4×10^2 cells μl^{-1}.

5.3.5 *CYP51* Expression Analysis: Constitutive Expression

Constitutive expression levels are shown in Figure 5.9. Isolate FI12-63 lacks *CYP51A*, so the calculated relative expression was very low, and the dissociation curve showed that the product was non-specific (Figure 5.9c). *CYP51A* relative expression levels for all other isolates fell within a factor of ten, and differences in expression level do not correlate to differences in sensitivity. For *CYP51B*, relative expression levels for all isolates fell within a factor of 3, with most differences not significant at 95%.
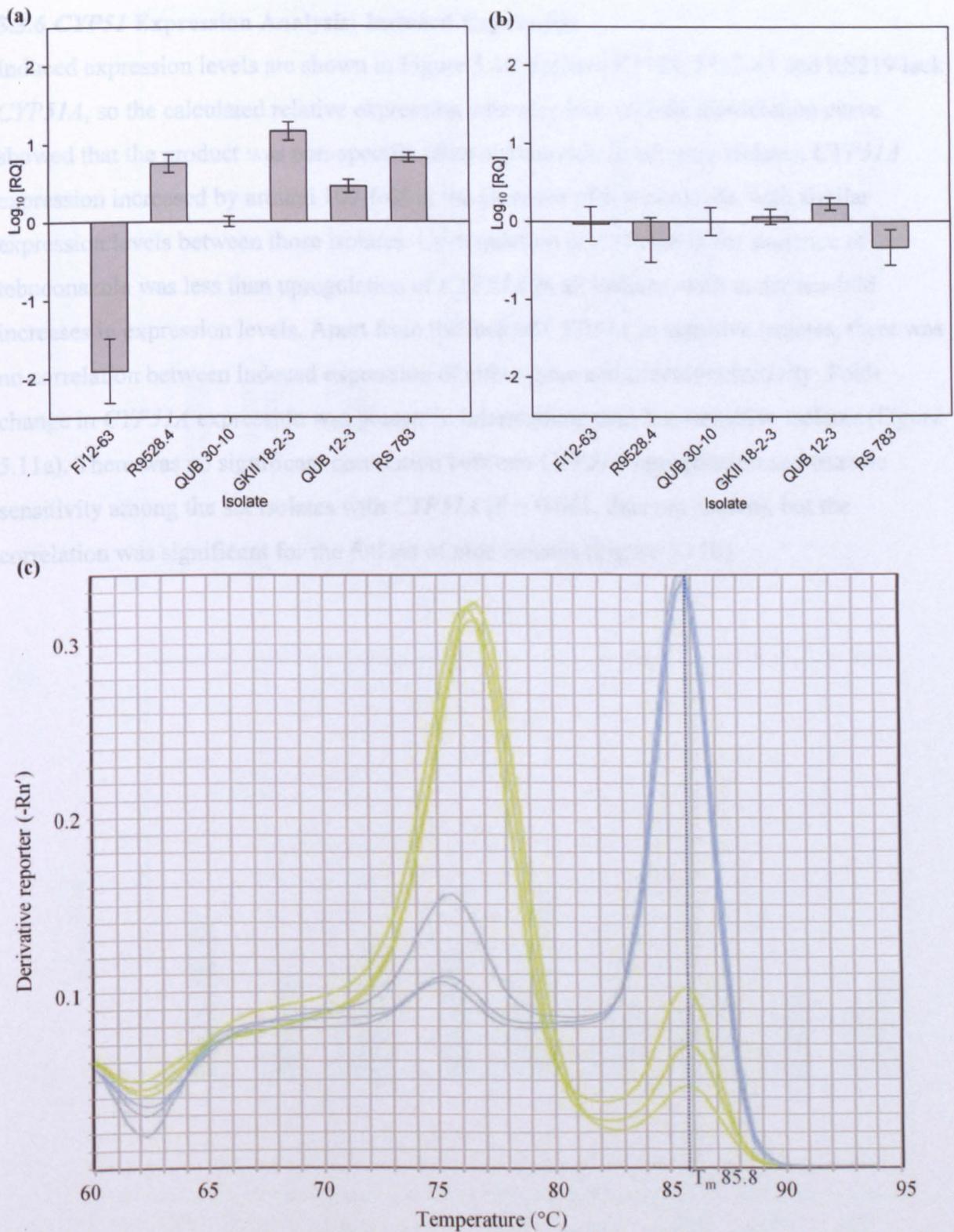
Figure 5.9. Constitutive expression of (a) *CYP51A* **and (b)** *CYP51B* **for six** *R. secalis* **isolates, as relative quantification calibrated to isolate QUB 30-10 with** *β-tubulin* **as endogenous control. Error bars show 95% confidence intervals based on the student's T distribution. (c)** *CYP51A* **qPCR dissociation curve for isolates QUB 30-10 (blue) and FI12-63 (green).**

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5.3.6 *CYP51* Expression Analysis: Induced Expression

Induced expression levels are shown in Figure 5.10. Isolates K1124, FI12-63 and RS219 lack *CYP51A*, so the calculated relative expression was very low, and the dissociation curve showed that the product was non-specific (data not shown). In all other isolates, *CYP51A* expression increased by around 100-fold in the presence of tebuconazole, with similar expression levels between those isolates. Up-regulation of *CYP51B* in the presence of tebuconazole was less than upregulation of *CYP51A* in all isolates, with under ten-fold increases in expression levels. Apart from the lack of *CYP51A* in sensitive isolates, there was no correlation between induced expression of either gene and triazole sensitivity. Fold-change in *CYP51A* expression was greater in intermediate than less-sensitive isolates (Figure 5.11a). There was no significant correlation between *CYP51B* upregulation and triazole sensitivity among the six isolates with *CYP51A* (P = 0.061, data not shown), but the correlation was significant for the full set of nine isolates (Figure 5.11b).
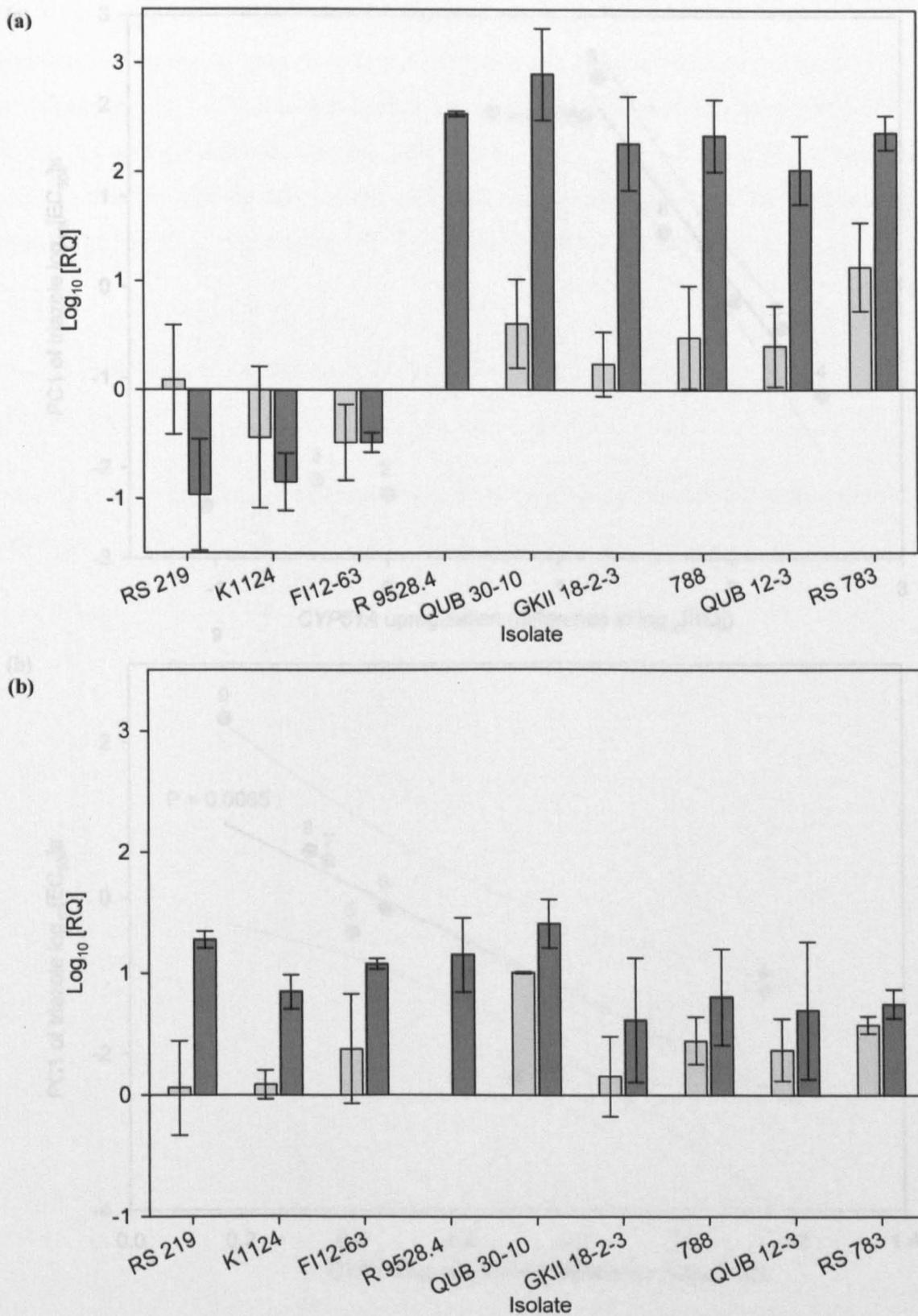
Figure 5.10. Graphs showing induced expression of (a) *CYP51A* and (b) *CYP51B*, as relative quantification, relative to isolate R 9528.4 constitutive expression, with *β-tubulin* as endogenous control. Light grey: constitutive expression, without fungicide; dark grey: induced expression, following the addition of 1 μg ml^{-1} tebuconazole. Error bars indicate standard error of biological replicates.

(a)

PC1 of triazole log$_{10}$[EC$_{50}$]s

$P = 0.0009$

CYP51A upregulation (difference in log$_{10}$[RQ])

(b)

PC1 of triazole log$_{10}$[EC$_{50}$]s

$P = 0.0085$

CYP51B upregulation (difference in log$_{10}$[RQ])
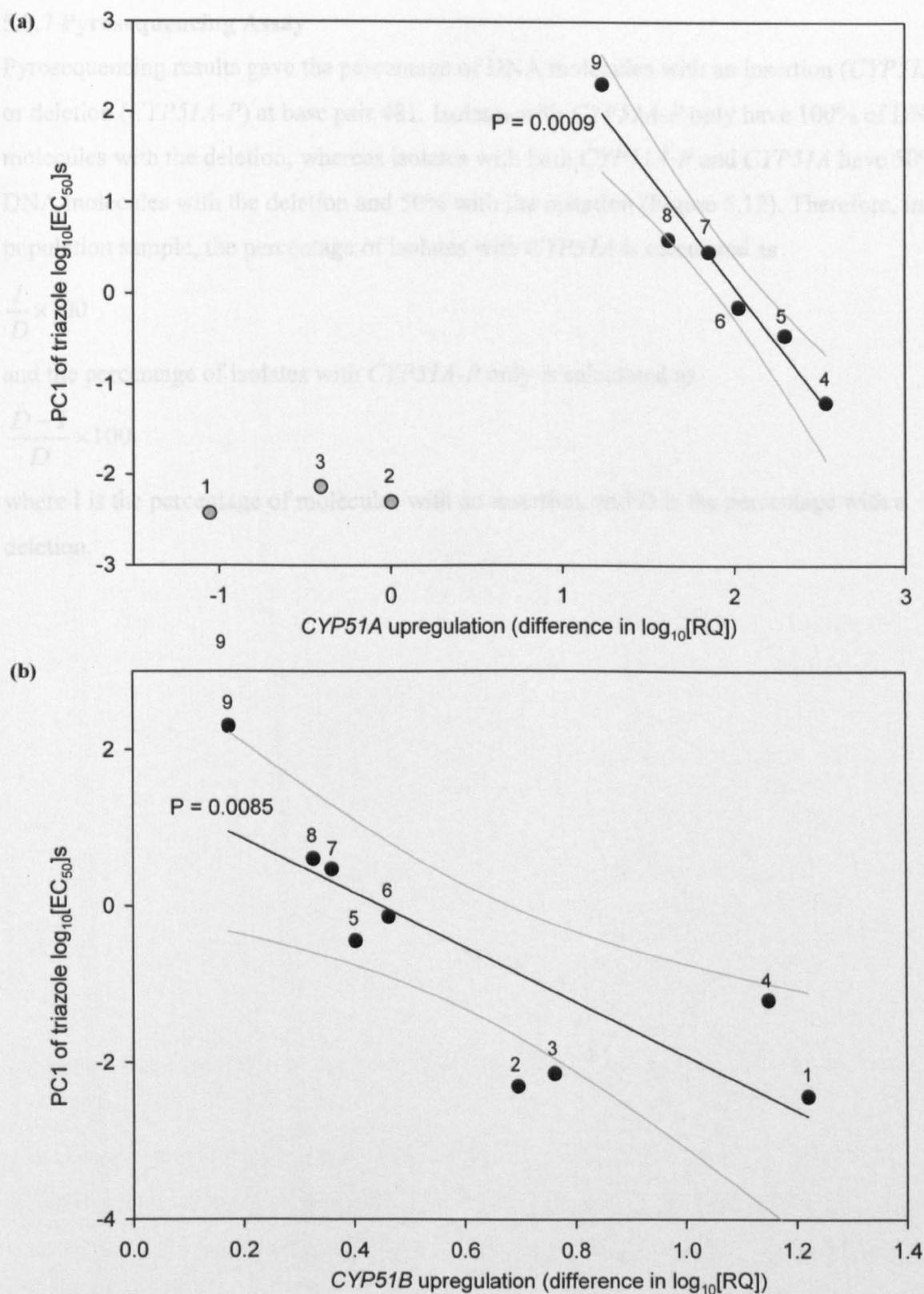
Figure 5.11. Triazole sensitivity, represented by PC1 as calculated in section 4.3.1, plotted against *CYP51* upregulation, calculated as the difference between log$_{10}$[RQ] with 1 µg ml^{-1} tebuconazole and log$_{10}$[RQ] with 1 µg ml^{-1} tebuconazole, for *R. secalis* isolates. (a) *CYP51A*: Grey circles: isolates without *CYP51A*; black circles: isolates with *CYP51A*; regression lines and P-values for isolates with *CYP51A*. (b) *CYP51B*, with regression line and P-value for all isolates. Grey lines indicate 95% confidence limits. Isolate 1: RS 219; 2: FI12-63; 3: K1124; 4: R 9528.4; 5: QUB 30-10; 6: GKII 18-2-3; 7: 788; 8: QUB 12-3; 9: RS 783.

5.3.7 Pyrosequencing Assay

Pyrosequencing results gave the percentage of DNA molecules with an insertion (*CYP51A*) or deletion (*CYP51A-P*) at base pair 481. Isolates with *CYP51A-P* only have 100% of DNA molecules with the deletion, whereas isolates with both *CYP51A-P* and *CYP51A* have 50% of DNA molecules with the deletion and 50% with the insertion (Figure 5.12). Therefore, in a population sample, the percentage of isolates with *CYP51A* is calculated as

$$\frac{I}{D} \times 100$$

and the percentage of isolates with *CYP51A-P* only is calculated as

$$\frac{D-I}{D} \times 100$$

where I is the percentage of molecules with an insertion, and D is the percentage with a deletion.
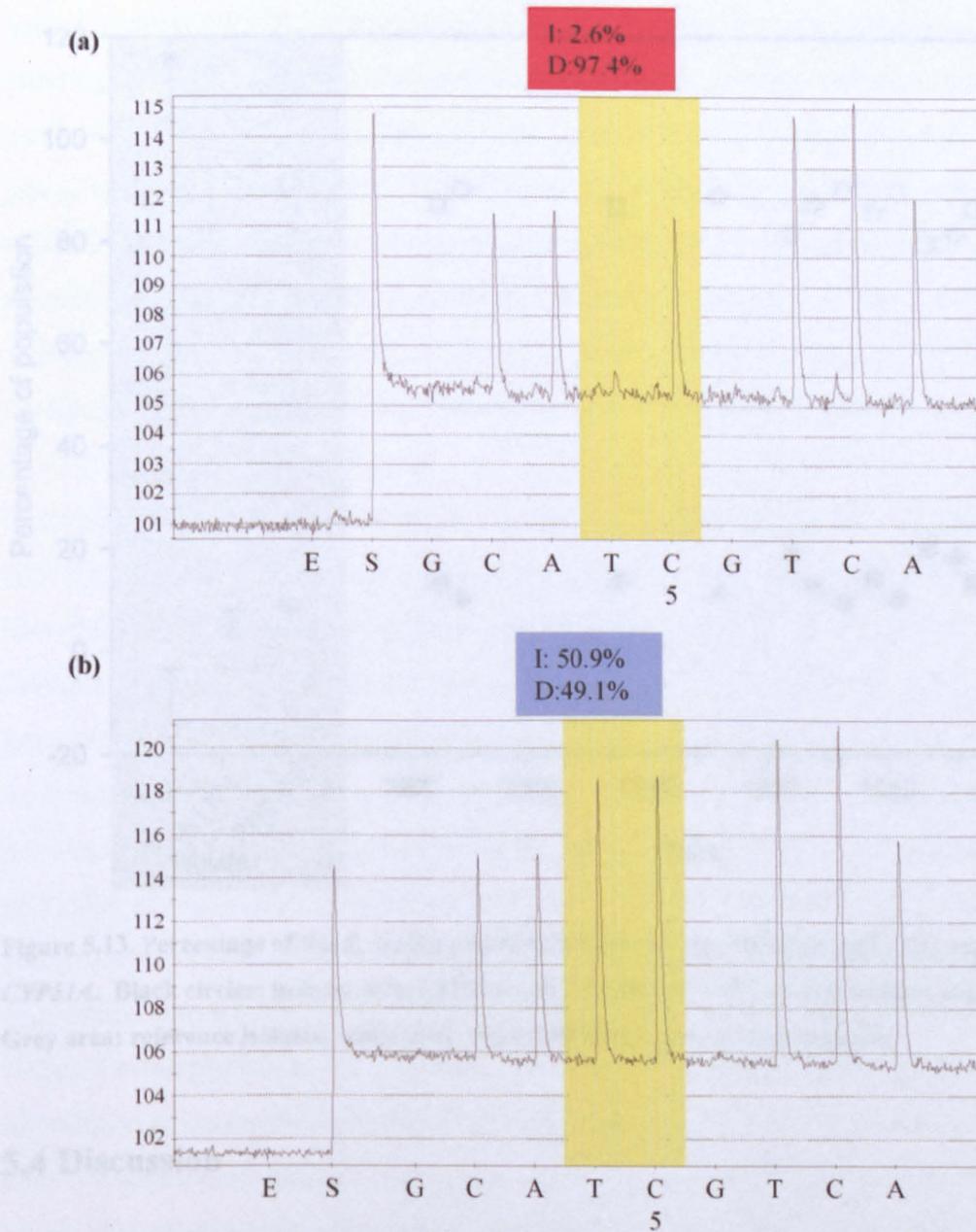
Figure 5.12. Single isolate Pyrogram traces for (a) Isolate FI12-63, with *CYP51A-P* only; (b) Isolate 788, with *CYP51A* and *CYP51A-P*.

The percentage of isolates containing *CYP51A-P* only, or *CYP51A* and *CYP51A-P*, for Hoosfield samples is shown in Figure 5.13. The proportion of isolates with *CYP51A* is low until 1998, after which it increases rapidly, with the majority of the population possessing *CYP51A* from 2002.
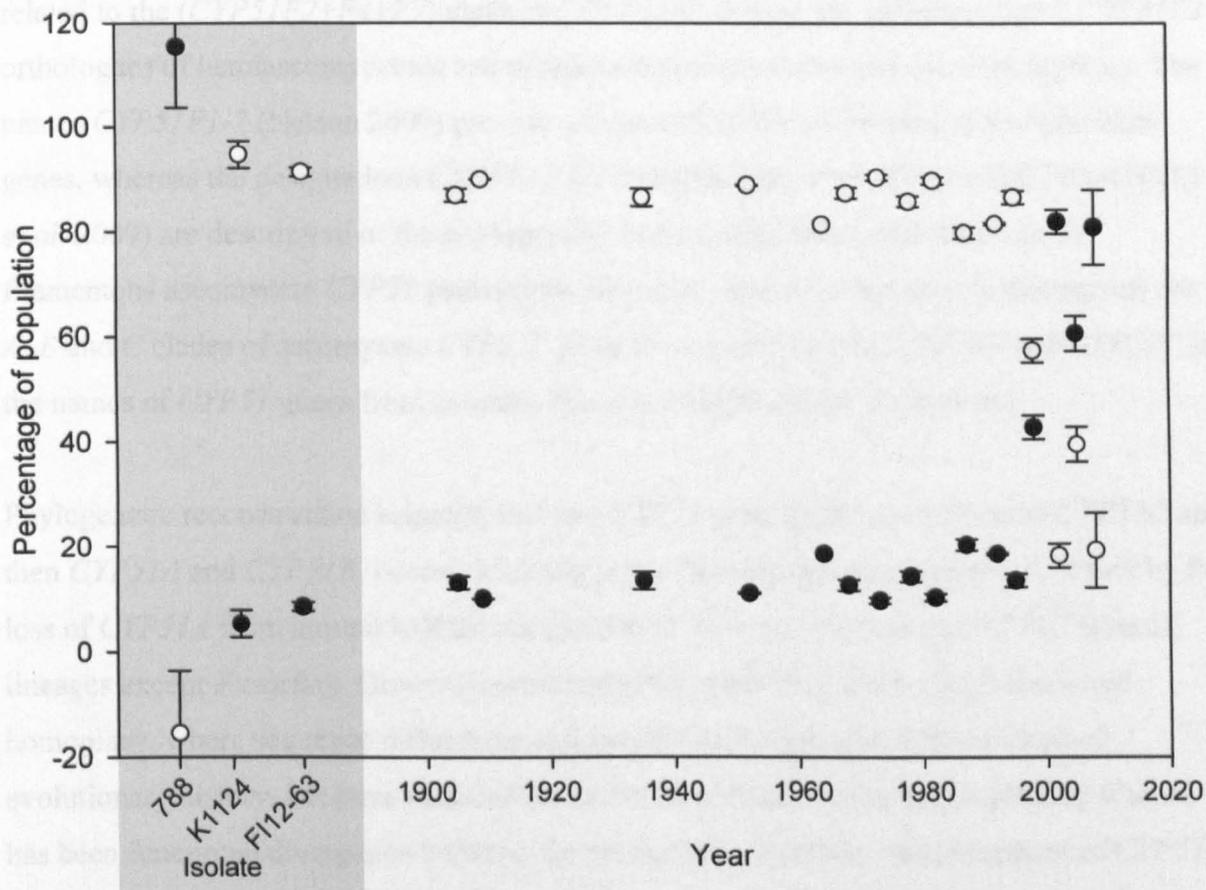
Figure 5.13. Percentage of the *R. secalis* population from the Hoosfield spring barley experiment with *CYP51A*. Black circles: isolates with *CYP51A* and *CYP51A-P*; white circles: isolates with *CYP51A-P* only. Grey area: reference isolates; white area: Hoosfield archive population samples.

5.4 Discussion

5.4.1 *CYP51* paralogues in fungal genomes

Ascomycete *CYP51* paralogues fall into three main clades. Filamentous ascomycete *CYP51F1* and *CYP51F1-p* form the *CYP51B* group; *CYP51F2*, *CYP51F4* (from *A. terreus* and *A. oryzae*) and *CYP51F7* (from *Uncinocarpus reesii* and *Coccidioides immitis*) form the *CYP51A* group, and *CYP51F3* (from *Fusarium* spp.) form the *CYP51C* group. This is consistent with the trees presented by Liu *et al.* (2011) and Becher *et al.* (2011). The second paralogue in *C. cinerea*, currently the only known basidiomycete with multiple *CYP51*s, does not fall into groups F1-7, and so a new name should be assigned, such as *CYP51F8*. It should be noted that the division into *CYP51A*, *B* and *C* is limited to the filamentous ascomycetes, with the single yeast *CYP51* orthologues forming a sister group to the *A+B+C* clade. As such, *CYP51F1* is paraphyletic, with the *CYP51F1* of filamentous ascomycetes more closely

related to the (*CYP51F2+F4+F7*) clade and *CYP51F3* than to the undifferentiated *CYP51F1* orthologues of hemiascomycetous and archiascomycetous yeasts and non-ascomycetes. The names *CYP51F1-7* (Nelson 2009) provide unique and stable nomenclature for individual genes, whereas the designations *CYP51A*, *CYP51B* (Mellado *et al.* 2001) and *CYP51C* (Yin *et al.* 2009) are descriptive of the phylogenetic and possibly functional divisions of filamentous ascomycete *CYP51* paralogues. However, care must be taken to distinguish the *A*, *B* and *C* clades of ascomycete *CYP51F* from the use of *CYP51A*, *CYP51B* and *CYP51C* in the names of *CYP51* genes from animals, bacteria and chromista, respectively.

Phylogenetic reconstruction suggests that two *CYP51* gene duplications, to give *CYP51C* and then *CYP51A* and *CYP51B*, occurred basally to the filamentous ascomycetes, followed by the loss of *CYP51A* from around half the species shown here and the loss of *CYP51C* from all lineages except *Fusarium*. However, protein-coding genes may carry a high chance of homoplasy, where sequence differences and similarities do not give a true picture of evolutionary history, but have been influenced by functional constraints, especially if there has been functional divergence between the paralogues. Therefore, the placement of *CYP51C* as a sister group to (*CYP51A* + *CYP51B*) may be due to functional divergence in *CYP51C* and greater sequence conservation in *CYP51A* and *CYP51B*. However, the pattern of intron occurrence across ascomycete *CYP51*s is also consistent with a basal split into *A*, *B* and *C* clades. On the phylogeny here, a most parsimonious reconstruction of intron presence does not require any independent gains of introns at the same position in multiple lineages, whereas a later duplication of *Fusarium CYP51*s would require an independent gain of an intron after alignment position 1946. There was also no evidence of recombination in fungal *CYP51* genes.

There have also been some further duplications within the *A* group, giving rise to *CYP51F4* in *A. flavus* and *A. oryzae*, a duplication within the *B* group resulting in a *CYP51B* pseudogene in *A. terreus*, and one gene duplication within a Basidiomycete, *C. cinerea*. However, some of these may fall within more widespread patterns of gene duplication (Machida *et al.* 2005), so the inclusion of a *CYP51* amongst the duplicated genes may not always have functional significance. *Uncinocarpus reesii CYP51F7* and *C. immitis* CIMG_00573 are in the *CYP51A* clade, but they fall at the base of the clade, rather than with the other eurotiomycetes as for *CYP51B*. This may also be down to long-branch attraction,

whereby more divergent sequences are erroneously placed outside a group of otherwise conserved sequences.

The split between *CYP51A* and *CYP51B* was better-supported on trees reconstructed from the full sequence alignment, including ambiguously-aligned regions. This suggests that consistent differences between *CYP51A* and *CYP51B* include length variation, since ambiguously-aligned regions tend to be those containing indels. These regions include the N-terminal region, which protein structure models have identified as a membrane-binding helix (Xiao *et al.* 2004; Canas-Gutierrez *et al.* 2009). Otherwise the trees reconstructed by different methods are largely congruent.

As paralogues differ in the N-terminal region, which may contain a signal protein or membrane anchor, subcellular localisation predictions were carried out. Protein location predictions predominantly identified the fungal CYP51 paralogues tested as belonging to the secretory pathway, which includes plasma membrane proteins, although predictions of location within the secretory pathway were not consistent and are generally not reliable as signals are poorly characterised (Emanuelsson *et al.* 2007). However, the N-terminal regions identified by TargetP (Emanuelsson *et al.* 2000) as secretory signals were identified by TMHHMM (Krogh *et al.* 2001) and Phobius (Kall *et al.* 2004) as membrane-binding regions, as previously reported in homology-based protein structure modelling studies (Xiao *et al.* 2004). These results are consistent with all ascomycete CYP51 paralogues being plasma-membrane bound proteins. Predicted cytoplasmic and non-cytoplasmic regions are not reliable for proteins with a single membrane-spanning region (Kall *et al.* 2004), and the second transmembrane helix identified in some peptides corresponds to the first P450 structural helix, αA' (Xiao *et al.* 2004; Canas-Gutierrez *et al.* 2009). One consistent difference between paralogous groups was observed at the extreme N-terminus. In CYP51B, the predicted transmembrane helices started at residues 19-21, with only two exceptions; in CYP51A, the helices started at or before residue 10, with a single exception starting at residue 13; and in CYP51C, the transmembrane helices started at residue 12 or 15. However, those 10-20 N-terminal residues were not recognised as signal peptides, so no differences in subcellular localisation of the three CYP51 paralogues are predicted at present, although *in silico* protein localisation tools only provide predictions, which may be disproved by experimental testing.

In addition to the N-terminal region, there is length variation between paralogues between regions αD and β3-1, including a unique insertion of around 20 residues in *R. secalis* CYP51A; between αG and αH, with insertions in some CYP51As; between αJ and αJ', with insertions in CYP51As and yeast CYP51s; between β2-1 and β2-2, with insertions in basidiomycete CYP51s; between αK'' and αL, with insertions in basidiomycete CYP51s and some CYP51Bs; and some length variability across all sequences between β3-3 and β4-1, and between β3-2 and the C-terminal. The lack of substantial length variation within any of the conserved structural motifs or substrate binding sites suggests overall structural conservation across fungal CYP51s. Furthermore, the lack of support for the CYP51A/CYP51B split in phylogenies reconstructed from only unambiguously-aligned regions indicates a lack of consistent sequence differences between CYP51A and CYP51B within the core structural motifs.

At present, the closest known relatives to *R. secalis* with *CYP51A* are sordariomycetes including *M. oryzae*, as leotiomycetes *B. cinerea* and *S. sclerotiorum* lack *CYP51A*. A wider search of other leotiomycetes for *CYP51A* may identify a closer orthologue of *R. secalis* *CYP51A*, which would shed more light on the evolution of the currently unique intron structure and the αD - β3-1 loop insert.

CYP51C is consistently recovered as a separate clade, even in phylogenies based on unambiguously-aligned regions, reflecting sequence differences in regions of the gene without length variation. Examination of amino acid sequences shows that this includes sequence variation within the substrate recognition sites (Podust *et al.* 2001), including some residues previously identified as conserved across all CYP51s or phylum-specific and conserved within fungal CYP51s. Variation is seen in SRS (Substrate Recognition Site) 1 and the following α-helix C, and in SRS4 (Podust *et al.* 2001), generally the most conserved regions of CYP51s (Lepesheva and Waterman 2007). In particular, S312 (*C. albicans* residue number) in SRS4 has been identified as part of the fully conserved eukaryotic CYP51 signature (Lepesheva and Waterman 2007; Lepesheva and Waterman 2011), but CYP51C amino acids have a threonine in place of a serine. This suggests functional conservation between CYP51A and CYP51B, and possible functional divergence of CYP51C.

Selection testing using the codon-based Z-test found no evidence of positive selection except in some comparisons with *R. oryzae CYP51F1-p*. This is a pseudogene in a basal fungal

lineage, so it is likely to be evolving neutrally but with an excess of non-synonymous mutations as dS is approaching saturation. The majority of pairwise comparisons of fungal *CYP51* sequences across all sites showed evidence of purifying selection. This is to be expected for functional protein-coding genes, with the majority of sites conserved to retain a functional protein and any positive selection associated with changes in function restricted to a few sites (Yang 2007). Diverge (Gu and Vander Velden 2002) tests for Type I functional divergence, whereby some residues are less conserved in one clade than another following gene duplication. CodeML (Yang 2007) tests for Type II functional divergence, whereby some codons experience positive selection at the point of gene duplication (Gu 1999). Diverge identified strong evidence for Type I functional divergence between all pairs of ascomycete *CYP51* clades. The greatest coefficient of functional divergence is between yeast *CYP51*s and *CYP51C*; the lowest is between *CYP51A* and *CYP51B*. In each comparison, many sites of apparent functional divergence, spread throughout the gene, were identified. Selection testing using the branch-site model in CodeML showed strong evidence of positive selection at some sites on the branch leading to *CYP51A*, *CYP51B* and *CYP51C*, with a higher proportion of sites under positive selection on the branch leading to *CYP51C*. Twenty-nine sites with significant probability of being under positive selection on the branch leading to *CYP51C* were identified by Bayes Empirical Bayes analysis, but this method works best for few sites under strong selective pressure, rather than selection across multiple sites (Zhang *et al.* 2005). Therefore there is strong evidence of Type II functional divergence of *CYP51C* at some codons but those codons cannot be reliably identified by this method. Overall, there is strong evidence of both Type I and Type II functional divergence between *CYP51* paralogues: some sites have undergone positive selection when the paralogues diverged, and some sites are more constrained in some clades than others following duplication. This functional divergence appears to involve many sites spread through the gene, making it difficult to identify the precise sites involved, so the use of covariotide models (Huelsenbeck 2002) may prove informative in future analyses.

5.4.2 *CYP51* paralogues and azole sensitivity in *R. secalis*

CYP51A and *CYP51B* genes were amplified from *R. secalis* isolates (Chapter 4). Functional *CYP51A* genes were only obtained from isolates with intermediate and reduced sensitivity to triazole fungicides, whereas a *CYP51A* pseudogene was obtained from sensitive isolates (Section 4.3.3). A PCR-RFLP assay and a Southern blot were carried out to establish whether

the pseudogene was found only in the sensitive isolates instead of the functional gene, or whether it was also found in less sensitive isolates in addition to the functional gene.

In the PCR-RFLP assay, the three fully-sensitive isolates only produced PCR products with restriction patterns corresponding to the *CYP51A* pseudogene, even with primers designed to preferentially amplify the functional *CYP51A* gene. The other isolates gave PCR products with restriction patterns corresponding to both *CYP51A* and the *CYP51A* pseudogene, with the respective primer pairs. This suggests that the functional *CYP51A* gene is absent from the fully-sensitive isolates, whereas the pseudogene is present in all isolates. Sequencing the genes amplified with the pseudogene-specific primers confirmed that *CYP51A-p* is non-functional in all isolates (Section 4.3.3). There were some differences in the pseudogene sequence from isolate K1124, including an *EcoRI* restriction site, but the four-base-pair deletion and resulting premature stop codon that make *CYP51A-p* non-functional were still present. Furthermore, alignment of *CYP51* sequences reveals that the splicing of the third intron in *CYP51A-p* removes part of helices C and D. Therefore the protein would probably be non-functional with or without the premature stop codon. The Southern blot confirmed that isolates K1124 and FI12-63 contain only one *CYP51A*, whereas isolate 788 contains two, although the gene and pseudogene are too similar to be distinguished by probes of the length required for the labelling kit used here.

Therefore *CYP51A-p* is present in all isolates, at a separate locus from *CYP51A*, which is present in isolates with intermediate and reduced sensitivity to triazole fungicides, and absent in sensitive isolates. It is not clear how large a region is absent in sensitive isolates. If *CYP51A* is located on a chromosome that is absent from sensitive isolates, this could be established by using *CYP51A* probes in a Southern hybridisation of a PFGE gel (von Felten *et al.* 2011). Alternatively, if a relatively short region is absent, it may be possible to design PCR primers for the flanking regions. Furthermore, next-generation sequencing of several isolates of *Rhynchosporium* spp. is currently underway. If this includes isolates with and without *CYP51A*, comparing the genomic sequences will reveal the portion of the genome that is absent in isolates lacking *CYP51A*.

Constitutive expression of *CYP51B* varied little between isolates, with no variation correlating with sensitivity differences. When measuring *CYP51A* constitutive expression, amplification from isolate FI12-63 cDNA was very poor. This isolate lacks *CYP51A*, so this

apparent low level of expression probably results from primer-dimer formation or non-specific amplification of the non-functional *CYP51A*. For the other isolates, *CYP51A* constitutive expression levels were slightly more variable than for *CYP51B*, with around a ten-fold range, but again no variation correlated with sensitivity differences. Therefore constitutive target-site over-expression, as found in *V. inaequalis* (Schnabel and Jones 2001) or *P. digitatum* (Hamamoto *et al.* 2000) does not contribute to reduced azole sensitivity in current isolates of *R. secalis*.

Differences in induced expression of *CYP51A* and *CYP51B* between isolates did not correlate with reduced triazole sensitivity, apart from the low values obtained for *CYP51A* expression in isolates without *CYP51A*. However, in those isolates possessing a functional *CYP51A* gene, this gene was up-regulated around 100-fold in the presence of tebuconazole, in contrast to the far lesser (around five-fold) up-regulation of *CYP51B* in all isolates. The accuracy of the absolute degrees of upregulation calculated here may be affected by the use of a single endogenous control gene, although comparisons between *CYP51A* and *CYP51B* would be unaffected so long as the same controls are used for both. This provides further evidence that the presence of a functional *CYP51A* may be related to reduced triazole sensitivity in the intermediate and less-sensitive isolates. The fold-change in *CYP51A* expression in isolates possessing *CYP51A* was negatively correlated with PC1, a composite term calculated from $\log_{10}$ [EC$_{50}$]s of the four triazoles tested (section 4.3.1). The suggests that sensitivity differences among isolates with *CYP51A* are due to a mechanism independent of *CYP51* upregulation, and the fungicide concentration used had a greater effect on more sensitive isolates, resulting in a greater change in gene expression. Therefore, *CYP51A* upregulation occurs as a quantitative response to the effects of tebuconazole, such as ergosterol depletion or accumulation of sterol intermediates, in all isolates with *CYP51A*. A weaker correlation was seen with the fold-change in *CYP51B* expression, providing further evidence that *CYP51B* expression is less responsive to the effects of triazole fungicides.

Heterologous expression in yeast demonstrated that *R. secalis CYP51A* is able to complement yeast *CYP51*. Therefore *R. secalis* CYP51A is a functional sterol 14α-demethylase, despite sequence differences including a 20-residue insertion between regions αD and β3-1 and a unique intron pattern. Yeast transformants expressing *R. secalis CYP51B* grew poorly. However, sensitive isolates of *R. secalis* can survive with CYP51B and not CYP51A. Therefore, *R. secalis* CYP51B is able to function as sole CYP51 and the poor growth of yeast

transformants is probably due to issues associated with heterologous expression, such as different codon use preferences or post-translational processing. This adds to the evidence from the phylogenetic analyses and selection testing across fungal CYP51s suggesting a lack of functional divergence between CYP51A and CYP51B in terms of protein structure and substrate, so any functional divergence is likely to be based on different transcriptional regulation as seen in the induced expression experiment. Divergence in regulatory control may be a frequent occurrence among duplicated genes in fungi (Wapinski *et al.* 2007), and differences in transcriptional control have been used to infer neo- or sub-functionalisation in other fungal gene families (Skamnioti *et al.* 2008). Therefore it appears that CYP51A and CYP51B can act upon the same substrate, but *CYP51B* is expressed at more constant levels whereas *CYP51A* is more inducible in response to the effects of DMIs. In *F. graminearum*, Becher *et al.* (2011) report around a 128-fold increase in *CYP51A* expression, compared to around an 8-fold increase in *CYP51B* expression and 3-4-fold increase in *CYP51C* expression, on exposure to tebuconazole, whereas *CYP51B* and *CYP51C* expression levels were around ten times higher than *CYP51A* without fungicide.

Therefore, the presence of *CYP51A* in some *R. secalis* isolates enables greater *CYP51* expression in the presence of triazole fungicides, conferring reduced triazole sensitivity. The presence of *CYP51A* has previously been linked to inter-specific differences in intrinsic triazole sensitivity. In *A. fumigatus*, knocking out the *CYP51A* gene resulted in increased triazole sensitivity in sensitive isolates with wild-type *CYP51A* as well as in less-sensitive isolates with *CYP51A* mutations, suggesting that the presence of *CYP51A* confers lower intrinsic azole sensitivity (Mellado *et al.* 2005). Disruption of *CYP51A* also increased azole sensitivity in *M. oryzae* and *F. graminearum*, whereas disruption of *CYP51B* did not (Liu *et al.* 2011; Yan *et al.* 2011). However, this is the first report of intraspecific variation in the presence of *CYP51* paralogues. In *C. glabrata* (Marichal *et al.* 1997) and *C. albicans* (Selmecki et al. 2006), chromosome or chromosome-arm duplications encompassing *CYP51* confer reduced triazole sensitivity in some isolates. However, the chromosome arm also contains a gene encoding an efflux pump transcription factor, and reduced intracellular accumulation of azoles was measured in isolates with chromosomal duplications. Therefore, the resistance factors attributable to a duplicate of the existing *CYP51* orthologue are relatively low, with MIC approximately doubled by each extra copy (Selmecki *et al.* 2008). In *R. secalis*, the extra copy is a diverged paralogue under different transcriptional control,

allowing a greater transcriptional response to triazoles, conferring a resistance factor of around ten against tebuconazole and propiconazole.

When *A. fumigatus* *CYP51A* and *CYP51B* were expressed in yeast, each under the control of the same promoter, *CYP51A* transformants were 16 times less sensitive to fluconazole than *CYP51B* transformants, although sensitivity to four other clinical azoles was similar for the *CYP51A* and *CYP51B* (Mellado *et al.* 2005). Therefore it is possible that intrinsic sequence differences between *CYP51A* and *CYP51B*, as well as differences in gene expression, contribute to the reduced triazole sensitivity of *R. secalis* isolates with *CYP51A*. Since *R. secalis* *CYP51B* did not produce sufficient growth in yeast to allow fungicide sensitivity testing for comparison with *R. secalis* *CYP51A*, gene replacement and promoter swaps within *R. secalis* may be necessary in order to separate the effects of sequence differences or expression levels on triazole sensitivity.

Pyrosequencing analysis of samples from the Hoosfield archive provides further evidence for the role of *CYP51A* in reduced triazole sensitivity in *R. secalis*. Levels of *CYP51A* increase from 1998 onwards, and *CYP51A* is found in the majority of the *R. secalis* population from 2002. The first isolate found to possess *CYP51A* in the present study, isolate 788, dates from 1997, and most isolates studied from after 2000 possess *CYP51A*.

Previous surveys of fungicide sensitivity in *R. secalis* populations had reported shifts in sensitivity to triadimenol and then propiconazole in the early 1990s (Kendall *et al.* 1993), with a later shift in epoxiconazole sensitivity to a bimodal distribution by 2002 (Oxley *et al.* 2003). Only one isolate in this study dates from before 1990, so the majority of the most sensitive isolates may come from a population already selected for reduced sensitivity to triadimenol, and the selection of *CYP51A* may correspond to the bimodal shift seen in 2000. This suggested scenario is supported by the cross-resistance between propiconazole and tebuconazole in isolates with *CYP51A*, whereas Kendall *et al.* (1993) reported that isolates selected for decreased propiconazole were still controlled by tebuconazole, although that study refers to field control rather than *in vitro* sensitivity. Therefore, this explanation cannot be verified without further isolates from before 1990. It is also possible that the sensitivity shifts first reported in the early 1990s took place later at the Hoosfield site. Furthermore, *CYP51A* only correlates with the initial shift from sensitive to intermediate isolates seen here. As stated in Chapter 4, further sensitivity shifts do not correlate with any differences in

CYP51 sequences or expression levels, suggesting that a non-target-site mechanism is responsible. Knocking out *CYP51A* from intermediate and less-sensitive isolates would show whether further sensitivity shifts are independent of *CYP51A*.

CYP51A presence levels prior to 1998 were measured at 10-20% of the population. However, measurements of 5-9% were obtained for single isolates without *CYP51A*. Therefore, earlier years may be too close to the detection limit of the assay to be taken as conclusive evidence for levels of *CYP51A* in the pre-selection population. The presence of the highly similar *CYP51A-p* in all isolates limits the sensitivity with which *CYP51A* may be detected, so better evidence for levels of *CYP51A* before selection may be obtained by population genetic methods. If *CYP51A* was restricted to a small part of the *R. secalis* population, genetic diversity would be lower in isolates with *CYP51A* than in the population as a whole. Following a hard selective sweep, lower diversity would be present in isolates with *CYP51A* in the absence of recombination, or linkage disequilibrium would be present following recombination. If a wide range of haplotypes are found at near-equilibrial frequencies in isolates with *CYP51A*, this would suggest a softer selective sweep, whereby an existing allele increases in frequency in an already polymorphic population.

Rhynchosporium secalis has recently been shown to be a complex of at least three host-specialised lineages, referred to as *R. commune* on barley, *R. secalis sensu stricto* on rye and *R. agropyri* on couch and related grasses (Zaffarano *et al.* 2011). This study has shown variation in the occurrence of *CYP51A* in *R. commune*, with *CYP51A* apparently having been lost from the majority of isolates before being selected back to prevalence by triazole use. It would be interesting to investigate the occurrence of *CYP51A* and *CYP51A-p* in the other *R. secalis s.l.* lineages and sister species *R. orthosporum*. This would shed more light on the the evolution of *CYP51A* in *R. secalis*, as it has been suggested that *R. secalis s.l.* lineages went through genetic bottlenecks during host shifts (Zaffarano *et al.* 2008), so if *CYP51A* was already in a minority of isolates at that point it may not have made it through the bottleneck in all lineages, or it may have been subsequently lost in some lineages but not others due to differing selective pressures or chance effects of genetic drift. Alternatively, if all other lineages possess a functional *CYP51A*, the near-loss from the barley lineage must be a very recent event, as the lineages were estimated as having diverged 1200-3600 years ago (Zaffarano *et al.* 2008). This would also have practical implications for the use of triazoles on rye, turf and forage grasses. Genome sequencing of isolates from each lineage is currently

underway, so when released the genomes can be searched for *CYP51A*, although it should be borne in mind that these lineages may also be polymorphic for *CYP51A* so its absence from the sequenced isolate does not necessarily indicate its absence from the lineage as a whole. If other lineages are found to be polymorphic for the presence of *CYP51A*, its presence should be monitored for those lineages growing on host plants on which triazoles are used.

Chapter 6

General discussion

6.1 Key findings

6.1.1 A high-throughput fungicide sensitivity bioassay can be used for *R. secalis* if multiple readings are taken to allow for heterogeneous growth

A high-throughput fungicide sensitivity bioassay has been developed for *R. secalis*. Growth conditions were optimised, first to produce spores for use as inoculum for the bioassay, and then to maximise total growth for the bioassay itself and to produce material for nucleic acid extraction. These different requirements for spores and biomass should be considered if further media and growth conditions are tested in future.

Rhynchosporium secalis grows heterogeneously in liquid culture: its growth is filamentous rather than yeast-like, and too slow to cover entire wells of a 96-well microtitre plate. Therefore, to quantify growth by measuring optical density, it is necessary to measure multiple points per well. This allows the use of an automatic plate reader, which is less labour-intensive than measuring colony diameter, and less subjective than judging density of growth by eye. This method may be applied to other species growing heterogeneously in liquid culture.

6.1.2 QoI sensitivity in *R. secalis* may be reduced by a target-site mutation encoding G143A, or by increased induced expression of *AOX*, but target-site resistance has not yet spread

A mutation in *cytochrome b* encoding the G143A substitution confers a hundred-fold reduction in *R. secalis* QoI sensitivity. However, to date, this has only been found at one site in France in 2008: it has not been found since, and has not been reported in the UK.

Alternative oxidase (AOX) activity results in smaller decreases in QoI sensitivity *in vitro* in the absence of AOX inhibitors such as SHAM. For most isolates, QoI sensitivity falls within a tenfold range, but a further tenfold shift was seen for one isolate. For these isolates, no target-site mutations were found, and sensitivity shifts were mostly reversed by adding the AOX inhibitor SHAM.

Expression analysis provided preliminary evidence for *AOX* upregulation following the addition of azoxystrobin, with an apparent correlation between decreased azoxystrobin sensitivity in the absence of SHAM and induced *AOX* expression. *AOX* upregulation following QoI fungicide addition has previously been reported in *M. oryzae* (Yukioka *et al.* 1998), and greater induced *AOX* expression correlated with lower intrinsic QoI sensitivity in *F. graminearum* compared to *M. nivale* (Kaneko and Ishii 2009). Here, greater induced expression of *AOX* is linked to intraspecific differences in *in vitro* QoI sensitivity for the first time.

6.1.3 Triazole sensitivity in *R. secalis* has declined over the last twenty years, but sensitivity differences are not associated with point mutations or constitutive over-expression of *CYP51*

Sensitivity of *R. secalis* to some triazoles had decreased tenfold by 2000, with a further tenfold shift after that. The partial cross-resistance between triazole fungicides is well-suited to Principal Components Analysis. PCA revealed that the initial sensitivity shift resulted in positive cross-resistance to the four triazoles tested, with tenfold shifts in propiconazole and tebuconazole sensitivity, and correlated but quantitatively smaller shifts for epoxiconazole and prothioconazole. Further sensitivity shifts affect prothioconazole differently from propiconazole and tebuconazole, with greater shifts in prothioconazole sensitivity seen in isolates with the lowest overall triazole sensitivity.

No point mutations in *CYP51* were correlated with differences in triazole sensitivity. The three CYP51 substitutions found were each limited to 1-3 isolates not corresponding to the three triazole sensitivity groups. Therefore target-site resistance, as seen in species including *M. graminicola* (Cools and Fraaije 2008), can be ruled out for the *R. secalis* isolates studied. Furthermore, expression analysis found no evidence of the constitutive *CYP51* overexpression caused by promoter changes in species such as *P. digitatum* (Hamamoto *et al.* 2000) or *V. inaequalis* (Schnabel and Jones 2001) with reduced triazole sensitivity.

A preliminary investigation into the possible role of enanced efflux in reduced triazole sensitivity did not find clear evidence of a correlation between reduced overall triazole sensitivity and potentiation by the putative efflux inhibitors tested. However, the effect on prothioconazole sensitivity should be tested for a wider range of isolates with reduced overall azole sensitivity, and other putative inhibitors should be tested or the intracellular

accumulation of radiolabelled fungicides should be measured before a wider role of efflux in sensitivity differences can be ruled out.

6.1.4 *Rhynchosporium secalis* isolates with reduced triazole sensitivity have a second *CYP51* paralogue

Two *CYP51* paralogues, *CYP51A* and *CYP51B*, and a pseudogene, *CYP51A-p*, are found in *R. secalis*. *CYP51B* and *CYP51A-p* were found in all isolates tested, but the functional *CYP51A* was absent from all tested isolates with the highest triazole sensitivity and present in all tested isolates with intermediate and reduced triazole sensitivity. Yeast complementation confirmed that *R. secalis* CYP51A is a functional sterol 14α-demethylase. Therefore the initial sensitivity shift against the four triazoles tested correlates with the presence of an additional target-site-encoding gene. Presence of *CYP51A* has been shown to result in reduced intrinsic triazole sensitivity in *A. fumigatus* (Mellado *et al.* 2005), *M. oryzae* (Yan *et al.* 2011) and *F. graminearum* (Liu *et al.* 2011), but this is the first report of intra-specific variation in the presence of *CYP51A*, linked to acquired triazole sensitivity differences in fungal populations.

Analysis of samples from the Hoosfield spring barley archive revealed that the majority of the *R. secalis* population in that field lacked *CYP51A* until 1998, at which point the proportion of the population with *CYP51A* increased, forming the majority of the population since 2002. This correlates with the collection dates of isolates with and without *CYP51A* from other sites.

6.1.5 The two *CYP51* paralogues in *R. secalis* result from a gene duplication basal to the filamentous ascomycetes

Phylogenetic analysis of the *CYP51* paralogues from *R. secalis* and homologues from sequenced fungal genomes produced trees suggesting that the *CYP51A* and *CYP51B* paralogues result from a duplication event basal to the filamentous ascomycetes, followed by multiple losses of *CYP51A* from various fungal lineages. *Rhynchosporium secalis CYP51A* and *CYP51A-p* diverged more recently, appearing as a sister branches.

6.1.6 The filamentous ascomycete *CYP51A* paralogue is upregulated in response to the effects of triazole fungicides

CYP51A is upregulated following the addition of tebuconazole in all tested isolates with *CYP51A*; that is, isolates with intermediate and reduced triazole sensitivity. *CYP51B*, present

in all isolates including those sensitive to triazoles, was upregulated to a far lesser degree. Therefore the upregulation of *CYP51A* appears to be at least partly responsible for the initial shift in triazole sensitivity in *R. secalis*. This resistance mechanism appears partly analogous to the target-site overexpression reported in species such as *P. digitatum* (Hamamoto *et al.* 2000), or the *CYP51* duplication seen in *C. albicans* (Selmecki *et al.* 2006), but all previous cases involve additional copies or increased transcription of an existing *CYP51* gene, rather than a functionally-diverged paralogue. There are also intrinsic sequence differences between *CYP51A* and *CYP51B*, so triazole binding may differ between the paralogues.

Greater upregulation of *CYP51A* than *CYP51B* on addition of triazoles has also been reported in *F. graminearum* (Becher *et al.* 2011). This suggests functional differentiation between *CYP51A* and *CYP51B* by divergence in transcriptional control.

6.2 Practical implications for *R. secalis* control and resistance management

6.2.1 QoIs

Target site resistance to QoIs was not found in any isolates from the UK, or in any of the 70 isolates tested except two collected by BASF in France in 2008. There have also been no further reported cases from industry monitoring (FRAC QoI Working Group 2010). Therefore QoIs remain effective in the field for the control of *R. secalis*.

However, the occurrence of isolates with the G143A substitution shows that it is possible for the G143A-encoding mutation in *cytochrome b* to arise in *R. secalis* without lethal impact on transcript processing or protein function. Some species contain an intron in *cytochrome b* that would not splice properly if the guanine at position 428 were replaced with a cytosine to give G143A (Grasso *et al.* 2006), but McCartney (2006) reported that this intron is not present in *R. secalis*. Deleterious effects on cytochrome *b* protein function have been proposed for some species (Fisher *et al.* 2004), but the occurrence of *R. secalis* isolates with G143A shows that any effects on cytochrome *b* function are insufficient to prevent the emergence of G143A under strong selective pressure. Therefore it must be assumed that the re-emergence and wider spread of QoI resistance remains a possibility in *R. secalis*, as was eventually seen with the MBC fungicides (Cooke and Locke 2002), and so monitoring must continue and resistance management guidelines should be followed.

AOX activity is not likely to result in control failure in the field, although further testing is needed to establish whether this mechanism results in any reduction in QoI sensitivity *in planta*. It has also been suggested that AOX activity may facilitate the emergence of target-site resistance (Avila-Adame and Köller 2003a), and further investigation of the mechanisms responsible for the increased upregulation of *AOX* in some *R. secalis* isolates may reveal whether G143A arose in a genetic background capable of increased *AOX* expression. However, in practical terms, it should be assumed that QoIs are a high resistance risk group of fungicides regardless of whether AOX can facilitate the emergence of resistance.

6.2.2 Triazoles

The sensitivity shifts observed for triazoles are broadly in line with previous reports (Cooke *et al.* 2004), and support current advice to UK farmers from the HGCA (Blake *et al.* 2011) and SAC (Oxley and Burnett 2010), that the newer triazoles epoxiconazole and prothioconazole still provide disease control, but shifts in sensitivity mean higher doses may be required. The range of prothioconazole sensitivities among isolates with the lowest general triazole sensitivity shows potential for further selection for reduced prothioconazole sensitivity as reported by Oxley *et al.* (2008), so resistance management guidance should be followed and prothioconazole should not be used alone (Blake *et al.* 2011). The incomplete cross-resistance between triazoles, especially between the intermediate and less-sensitive isolates, demonstrates the importance of chemical diversity with the triazoles.

The mechanism responsible for these further sensitivity shifts among intermediate and less-sensitive isolates is still not known. Therefore, molecular diagnostics are not available and monitoring still requires sensitivity bioassays to be carried out. The high-throughput sensitivity bioassay developed in this project would be useful for such monitoring. However, it is possible that less selection for reduced triazole sensitivity has taken place in some areas outside of North-Western Europe, so isolates with *CYP51A* may not yet form the majority of the population and assays developed for the detection of *CYP51A* would be useful in sensitivity monitoring. Furthermore, triazoles can be used against *R. secalis* on rye and grasses, with propiconazole and tebuconazole currently approved for use on grasses or amenity grassland, and triazoles including propiconazole, tebuconazole, epoxiconazole and prothioconazole currently approved for use on rye, in the UK (Chemicals Regulation Directorate 2010). Therefore, the occurrence of *CYP51A* in *R. secalis* lineages infecting these

hosts should also be investigated, to assess the risk of *CYP51A*-related sensitivity shifts in *R. secalis* affecting these crops.

6.2.3 Other fungicide groups

A shift in triazole sensitivity has taken place in *R. secalis*, but more slowly than in some pathogens such as *B. graminis* (Fletcher and Wolfe 1981), whereas QoI resistance has not yet spread in *R. secalis*. Along with the previously reported relatively slow spread of MBC resistance (Taggart *et al.* 1999), this supports the designation of *R. secalis* as a medium-risk pathogen (FRAC 2005).

Of the other groups of fungicides widely used against *R. secalis*, the SDHIs are considered to be at medium to high risk of resistance development, anilinopyrimidines including cyprodinil are considered medium risk, morpholines medium to low risk and chlorothalonil low risk. Therefore, the combined pathogen and fungicide risk means resistance management is important for the SDHIs and cyprodinil, and this is reliant on the availability of suitable mixing partners including the triazoles.

6.3 Emergence and spread of mutations conferring fungicide resistance in *R. secalis*

Prior to the occurrence of G143A in *R. secalis*, there was some debate as to why target site resistance to QoIs had not yet arisen in the species, with possible explanations including low mutation rates or high fitness penalties preventing the emergence of the mutation, or the population biology of *R. secalis* limiting its spread. Now G143A has been reported once (FRAC QoI Working Group 2008), but not found since (FRAC QoI Working Group 2010), it must be concluded that emergence of G143A is possible but further selection or spread is limited.

Both MBC resistance and reduced triazole sensitivity have arisen and spread more slowly than in higher resistance risk pathogens such as *B. graminis*. Studies of MBC resistance in *R. secalis* found the spread of resistance to be "erratic" (HGCA 2000). When attempting to study the effects of fungicide treatment on levels of MBC resistance levels, treatment differences were masked by high levels of variability between sites and between growing seasons (Holloman 1997). Taggart *et al.* (1999) also tested initial frequency of resistant isolates, and found that this had a greater effect on final frequency of resistant isolates at

different sites than treatment during the growing season, although the effects of fungicide treatment were apparent within sites.

Variation in the level of resistance in the initial *R. secalis* inoculum between sites and years suggests that the main inoculum source does not comprise widely-dispersed airborne spores. This is consistent with the lack of a known ascospore-producing stage in *R. secalis*, and the lack of *R. secalis* spores found in spore trapping experiments (Fountaine *et al.* 2010). Furthermore, fluctuations between years at a single site suggest that a large proportion of the initial population is brought into the site each year, as would be the expected for a primarily seed-borne disease, rather than persisting on site between growing seasons, for example on crop debris. Therefore resistance management is especially important on barley crops to be used for seed, and resistance monitoring in seed could prove useful.

Similar fluctuations between years were observed for the frequency of *CYP51A* in the *R. secalis* populations from the Hoosfield spring barley experiment. The proportion of isolates with *CYP51A* increases between 1995 and 1998 and between 1998 and 2002, then decreases again by 2005, and increases further by 2008. This also indicates that it takes more than one growing season for the proportion of isolates with *CYP51A* to increase from pre-selection levels to the majority of the population, which could indicate lower selective pressure, smaller population size or fewer generations than in pathogen-fungicide combinations for which selection of resistant isolates has taken place more rapidly.

If the seed-borne nature of *R. secalis* is a major factor limiting the spread of new mutations, this would be consistent with the selection of *CYP51A* from a low frequency within each local *R. secalis* population, rather than a hard selective sweep of new point mutations from a single or few origins across the entire metapopulation. Population genetic approaches would shed more light on this, by investigating the occurrence of *CYP51A* in pre-selection populations, as discussed in section 6.6.

6.4 Role of multiple *CYP51* paralogues in filamentous ascomycetes

Phylogenetic reconstruction suggests *CYP51* gene duplication took place around the origin of the filamentous ascomycetes, with subsequent losses of *CYP51A* from some lineages. This raises two questions: why the gene duplication emerged in the first place, and why it was then

lost from some lineages and retained in others. Some recent studies have investigated the purpose of multiple *CYP51*s through functional genetic approaches, such as assessing the effect of *CYP51A* deletion on pathogenicity (Mellado *et al.* 2005; Yan *et al.* 2011), but evolutionary studies may complement this functional approach.

Selection testing found evidence of functional divergence between all Pezizomycotina *CYP51* paralogues. However, the split between *CYP51A* and *CYP51B* was not supported when regions with length variation were excluded from the sequence alignment, and the substrate recognition sites are conserved in *CYP51A* and *CYP51B*. In contrast, *CYP51C* is consistently recovered as a separate clade based on sequence variation within the core structural regions, including residues within substrate recognition sites SRS1 and SRS4 which are conserved across other fungal CYP51s. Furthermore, *R. secalis CYP51A* is able to complement *S. cerevisiae CYP51*, and isolates with only *CYP51B* are viable but sensitive to triazoles, so both paralogues encode a functional sterol 14α-demethylase. However, expression analysis shows divergence in transcriptional regulation of *R. secalis CYP51A* and *CYP51B*, and similar results have been reported in *F. graminearum* (Becher *et al.* 2011). Therefore *CYP51B* and *CYP51A* both catalyse the same step in sterol biosynthesis, but *CYP51B* is present in all species and expressed at a relatively constant level, whereas *CYP51A* is present in some species as an extra *CYP51*, the expression of which is readily inducible.

This raises questions as to why additional induced *CYP51* expression may be needed. Intrinsic QoI resistance is found in species such as *S. tenacellus* and *Mycena galopoda* that produce natural strobilurins (Kraiczy *et al.* 1996). Therefore, the presence of *CYP51A*, reducing intrinsic triazole sensitivity and responding transcriptionally to the effects of triazoles, raises the possibility that *CYP51A* is an adaptation to a naturally-occurring CYP51 inhibitor, although it has been demonstrated that induction of *CYP51A* expression is proportional to the effects of the fungicide on a fungal isolate, so the transcriptional response is presumably induced by altered sterol content rather than direct detection of the inhibitor. Therefore other causes of sterol stress, such as higher requirements at particular points in the fungal life cycle, for example sporulation (Yan *et al.* 2011), could also induce *CYP51A* expression. Further studies of expression levels of other genes in the ergosterol biosynthesis pathway, and of sterol composition, would reveal whether *CYP51A* induced expression is part of a general upregulation of sterol biosynthesis, or specific to that step. Microarray studies of *F. graminearum* showed some increase in expression of most ergosterol biosynthesis genes in

response to tebuconazole treatment, but upregulation was greatest in *CYP51A* (Becher *et al.* 2011).

If *CYP51A* has evolved as an adaptation to natural *CYP51* inhibitors, these may be defence compounds produced by hosts, allelopathic compounds from competing micro-organisms or toxins produced by the fungi themselves. The gene duplication giving rise to *CYP51A* and *CYP51B* took place around the time the Pezizomycotina diverged from the Saccharomycotina. It has been estimated that the crown Pezizomycotina (Lecanoromycetes, Sordariomycetes and Eurotiomycetes) diverged 320-400 million years ago, during the late Devonian or early Carboniferous period, whereas the basal pezizomycotina (crown Pezizomycotina plus Pezizomycetes) diverged 400-520 million years ago, during the late Cambrian to early Ordovician (Lucking *et al.* 2009). These estimates may be refined in future as rate models are improved and if more calibrator fossils are identified (Berbee and Taylor 2010), but the time range for *CYP51* paralogue divergence could also be narrowed by investigating whether basal Pezizomycotina, such as Pezizomycetes and Orbiliomycetes, possess a differentiated *CYP51B* (and possibly *CYP51A*) or an undifferentiated *CYP51*. A tBLASTn search of the draft genome assemblies of the Pezizomycetes *Phymatotrichum omnivorum* (University of Oklahoma Advanced Center for Genome Technology, http://www.genome.ou.edu) and *Tuber melanosporum* (Genoscope, http://www.genoscope.cns.fr) identified fragments of a single CYP51 in each species, and preliminary phylogenetic analyses placed these amino acid sequences together on a branch between the yeast and crown Pezizomycotina CYP51s, branching off from the crown Pezizomycotina prior to CYP51 paralogue divergence, but further analyses are needed once full sequences are available. An Orbiliomycete, *Orbilia auricolor*, is currently awaiting sequencing (Joint Genome Initiative, http://www.jgi.doe.gov/). However, all estimates place the divergence of *CYP51A* and *CYP51B* long before the evolution of flowering plants or of mammals, so if the divergence was an adaptation to hosts, different host taxa should be considered. The current estimate for the divergence time of *CYP51A* and *CYP51B* encompasses the origin of land plants, from liverworts through to the establishment of fern and lycophyte forests, forming soils and altering atmospheric composition (Kenrick and Crane 1997). It also encompasses the origin and diversification of fish from the first vertebrates to the origin of amphibians (Rowe 2004), and the early origins of terrestrial arthropods including insects (Wheeler *et al.* 2004). These changes enabled fungal diversification into a wide range of niches, and it would be easy to be drawn into story-telling

as to why one of these niches required additional *CYP51*s, but such suggestions would only be testable based on the biology of extant species.

CYP51A was subsequently lost from several lineages, but retained in others. It could be hypothesised that some lineages experienced continued exposure to natural CYP51 inhibitors or other sterol stress and other lineages did not. This could be investigated by analysis of correlations between current fungal metabolites, hosts or environmental factors and presence of *CYP51A*. Any such studies should use phylogenetic comparative methods, rather than species-by-species comparisons, to correct for phylogenetic autocorrelation. For example, all *Aspergillus* species possess *CYP51A*, and share many other features due to being closely related. Therefore, species-by-species comparison may conclude that these features are correlated with presence of *CYP51A*, whereas phylogenetic comparative methods correct for shared features of species within a single lineage.

However, even with a phylogenetic comparative approach, there is an assumption that the pattern of loss or retention of *CYP51A* is predominantly adaptive, rather than being affected by chance process such as genetic drift. Furthermore, current knowledge of which species have retained *CYP51A* is based largely on the single isolates used for genome sequencing, with a few exceptions such as *A. fumigatus* for which *CYP51A* has been sequenced from many isolates to study target site mutations (Diaz-Guerra *et al.* 2003). It has been demonstrated in the current study that presence of *CYP51A* can vary within species. Whole genome resequencing projects currently underway or planned for some fungal species, using second generation sequencing methods, will give a fuller picture of intraspecific variation.

6.5 Constraints and contingency in molecular evolution

An ongoing debate in evolutionary biology concerns the extent to which evolution is predictable. For crop protection, attempts to predict resistance have focussed on how quickly resistance is likely to emerge and how this may be managed (Brent and Hollomon 2007b). However, when evolutionary biologists discuss predictability, they are generally considering which adaptations will emerge: whether functional and developmental constraints result in convergent evolution of a limited number of adaptive solutions, or whether chance events and historical contingency make evolution inherently unpredictable. The evolution of fungicide resistance may provide some valuable insights into this debate, since similar selective

pressures (i.e. fungicide application) have been applied repeatedly to different populations and species. Therefore, repeated emergence of the same mechanism would be indicative of constrained, predictable evolution, whereas the occurrence of disparate adaptations to the same selective pressure would suggest unpredictability due to chance events or historical contingency.

6.5.1 Predictability in molecular evolution: the debate so far

While examples of morphological convergence have long been known, in recent years increasing attention has been paid to constraints and convergence in molecular evolution, such as the constraints on β-lactamase substitutions in the evolution of antibiotic resistance (Weinreich *et al.* 2006), the concentration of mutations in restricted regions of genes and genomes due to constraints on other regions (Stern and Orgogozo 2009), or convergent recruitment of the same genes during the multiple origins of C4 photosynthesis (Christin *et al.* 2010). These examples have been countered by claims of contingency in molecular evolution. The evolution of citrate utilisation in one population of *E. coli* in the Long-Term Evolutionary Experiment (LTEE) was found to be dependent upon an earlier enabling mutation, and therefore described as historically contingent (Blount *et al.* 2008). While this is true for a literal definition of historical contingency, it must be presumed until shown otherwise that the rare potentiating mutation could still arise in other populations, after which citrate metabolism could also evolve: it is still a question of when or whether an adaptation might emerge, rather than alternate adaptive paths being followed. Clearer evidence of alternative adaptive paths was obtained when experimental evolution was carried out with fluctuating environmental conditions, with different populations showing different fitness responses interpreted as "distinct adaptive peaks", although the molecular adaptations responsible have not yet been reported (Cooper and Lenski 2010).

One possible molecular mechanism for path-dependent historicity (Desjardins 2011) is sign epistasis. Sign epistasis is an interaction between mutations whereby a mutation is either beneficial or deleterious depending on the presence of another mutation, whereas negative or antagonistic epistasis means one mutation has a quantitatively smaller effect in the presence of another mutation (Khan *et al.* 2011).Sign epistasis has been reported between two mutations in an experimentally evolving yeast population (Kvitek and Sherlock 2011). Both mutations evolved multiple times, but were never found together, and testing in near-isogenic mutants confirmed that either mutation alone was beneficial but the combination was

deleterious. Therefore each mutation represented a separate adaptive peak, and mutants that first gained the less fit of the two mutations were confined to a local optimum. Another example of sign epistasis resulting in path-dependent evolution, contingent upon which mutation arises first, has recently been reported in the experimental evolution of cefotaxime resistance in *E. coli* (Salverda *et al.* 2011). Most lines evolved three mutations conferring the greatest reduction in cefoxatime sensitivity, but two lines each gained a different initial mutation, resulting in reduced cefoxatime sensitivity but sign epistasis with mutations from the global optimum set. Therefore these lines only evolved a smaller reduction in cefoxatime sensitivity, have become trapped at a local adaptive peak.

It should be noted that in both of these cases, evolution was constrained, not to a single solution but a more complex set of possible solutions. These varying, interacting constraints resulted in a rugged fitness landscape, with the possibility of lineages being trapped by a local adaptive peak (Kvitek and Sherlock 2011). Therefore the question is not whether evolutionary pathways depend upon constraints or contingency, but whether the constraints are universal or dependent on genetic background; that is, whether the constraints are contingent.

Apart from the level of contingency, the predictability of evolution also depends upon the role of chance. In large populations, with high mutation rates or common mutations, a particular mutation is almost certain to arise, and evolution is effectively deterministic. Smaller populations or rare mutations result in stochastic sensitivity. In a simple constraint dependent pathway, evolution is predictable, but where there is stochastic sensitivity this can only be a probabilistic rather than a deterministic prediction. In a more contingent pathway, evolution may still be predictable, if each step is likely to occur (Dick *et al.* 2009), and if the nature of the contingency is known. The greatest intrinsic unpredictability would result from a combination of stochasticity and contingency, whereby the pathway is dependent on chance events at preceding steps. However, even a deterministic, simply constrained pathway may be unpredictable in practise if the constraints are not known: for example, UV mutagenesis studies to identify mutations that could result in fungicide resistance often generate a wider range of mutants than are subsequently found in the field, due to *in planta* fitness costs associated with some mutations.

6.5.2 Predictability in evolution of fungicide resistance

The evolution of QoI resistance in cytochrome *b* is a relatively simple constraint-dominated pathway. In most cases, the G143A substitution gives optimum fitness under selection by QoI fungicides, whereas F129L confers a lower reduction in QoI sensitivity. However, there is sign epistasis between the G143A mutation and the intron at codon 143 in some fungi. Therefore, constraints on cytochrome *b* are contingent on the loss or gain of this intron, but as this contingent relationship is known, the evolution of QoI resistance is still predictable for a given lineage subject to knowing whether the intron is present.

In the case of triazole resistance, the situation is more complicated. Mutations in *CYP51* have different effects on fitness depending on the compounds used, and epistatic interactions with other *CYP51* mutations (Cools *et al.* 2010). Therefore it would be difficult to predict which adaptations to selection by azole use will emerge in any given species without detailed knowledge of CYP51 structure and the impact of each possible mutation. Additionally, in some species, mutations and overexpression of *CYP51A* confer reduced triazole sensitivity (Diaz-Guerra *et al.* 2003), whereas in species without *CYP51A* this is not possible and only *CYP51B* and non-target-site mutations can occur, and so the mutations available are contingent upon whether *CYP51A* has been lost from that lineage. Furthermore, epistasis between mutations, coupled with selection of different mutations by different azoles, means the range of mutations that can emerge at a given time may be dependent on the triazoles used in the past. For example, in *M. graminicola*, the S524T substitution in a wild-type *CYP51* background appears advantageous under selection by prothioconazole, but it is not found in a wild-type background as other mutations had been selected by previous triazole use (Cools *et al.* 2011). The re-emergence of *CYP51A* in *R. secalis*, demonstrated in the present study, provides a further example of historical contingency. Phylogenetic reconstruction suggests a single origin of *CYP51A* followed by multiple losses, and it appears that *R. secalis CYP51A* was almost lost. However, before it was lost completely, a change in selective pressure due to azole use appears to have resulted in the re-emergence of *CYP51A*. Had it been lost completely, that particular adaptive pathway would not have been available in *R. secalis*. Therefore the adaptive pathway taken by *R. secalis* in response to selection by triazoles was contingent on chance events.

6.6 Future research directions

The *R. secalis* genome has been sequenced, and assembly and annotation are currently underway before public release. Two isolates from barley, and one from each of the other two *R. secalis* host-specialised lineages and *R. orthosporum*, were selected for sequencing (Navarro-Quezada *et al.* 2011).

Of the barley isolates being sequenced, one was collected before 1997 and the other is more recent. It is likely that the more recent isolate contains *CYP51A* and the older isolate does not. If this is the case, analysis of the sequenced genomes will reveal the size of the genomic region missing in isolates without *CYP51A*, whether it is a single gene, a region of genes or an entire chromosome. Whether the other lineages contain *CYP51A* could reveal how recently *R. secalis* ancestors started losing that paralogue. There is evidence that host shifts resulted in genetic bottlenecks (Zaffarano *et al.* 2008), so if *CYP51A* was already being lost from the ancestral species as bottlenecks occurred, it may be absent from some lineages. Presence of *CYP51A* in other lineages would also have implications for triazole resistance risk of *Rhynchsporium* spp. on grasses and rye. However, these lineages may also be polymorphic for the presence of *CYP51A*, so the presence or absence in the sequenced isolate cannot be assumed to mean universal presence or absence across the lineage.

Availability of full genome sequences, including the promoter regions of genes of interest, will also allow the generation of gene knockouts or reporter lines. For example, *AOX* promoter-reporter lines would allow induced expression to be monitored over a greater number of time points, to avoid missing maximum expression. *CYP51A* knockouts would confirm the impact of *CYP51A* on triazole sensitivity in a uniform genetic background, and gene replacement with *CYP51B*, or recombinant constructs containing the coding sequence of one paralogue with the promoter of the other, would enable further investigation of the contribution of sequence differences and overexpression to the reduced triazole sensitivity conferred by *CYP51A*.

Genome sequences may also provide potential markers for population genetic studies. This would be especially useful for assessing pre-selection levels of *CYP51A*, since the accuracy of direct measurement from pre-selection samples is limited by the presence of the highly similar and more abundant *CYP51A-p*. Diversity of genes located close to *CYP51A* could be

compared to diversity in unlinked genes, to infer whether a hard or a softer selective sweep has taken place.

In addition to further scientific developments, future research directions may result from evolutionary developments in *R. secalis*. This may include re-emergence of G143A, sensitivity shifts against new fungicide groups such as SDHIs, or new mechanisms resulting in further shifts in triazole sensitivity.

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Appendices

Appendix 1. Azoxystrobin EC₅₀s of *R. secalis* isolates.

Isolate	Year of collection	Location	EC$_{50}$ Azoxystrobin (μg ml^{-1}) Mean (Standard error)	
			0 μg ml^{-1} SHAM	40 μg ml^{-1} SHAM
K1124	Pre-1994	Long Ashton Research Station, UK	0.095 (0.030)	0.036 (0.007)
SK7	1984	Long Ashton Research Station, UK	0.338 (0.140)	0.083 (0.021)
NKT12	1996	Norway	0.139 (0.022)	0.043 (0.001)
FI12-63	1996	Finland	0.014 (0.001)	0.014 (0.001)
			0.159 (0.004)	0.039 (0.006)
			0.0378	0.0228
R157 (1)	1997	USA	0.061 (0.001)	0.048 (0.001)
R157 (2)	1997	USA	0.099 (0.006)	0.044 (0.001)
788	1997	France	0.199 (0.034)	0.031 (0.005)
			0.028 (0.009)	0.028 (0.003)
			0.032 (0.007)	0.033 (0.001)
			0.022 (0.003)	0.022 (0.004)
			0.127 (0.005)	0.033 (0.002)
SAC 0003 1-4-2 30	2000	Scottish Agricultural College, UK	0.040 (0.002)	0.095 (0.001)
SAC 1-4-8 (0003)	2000	Scottish Agricultural College, UK	0.014	0.004 (0.000)
			0.053 (0.003)	0.016 (0.001)
SAC 8-3-8 (0003)	2000	Scottish Agricultural College, UK	0.043 (0.004)	0.098 (0.017)
			0.078 (0.033)	0.070 (0.016)
SAC 0003 8.1.8 00 (0.123)	2000	Scottish Agricultural College, UK	0.052 (0.007)	0.038 (0.000)
SAC 0004 1.2.4 00	2000	Scottish Agricultural College, UK	0.172 (0.058)	0.063 (0.008)
QUB 9-10	2001	Northern Ireland	0.042 (0.004)	0.061 (0.007)
QUB 30-10	2001	Northern Ireland	0.051 (0.001)	0.033 (0.003)
QUB 18-2	2001	Northern Ireland	0.038 (0.000)	0.021 (0.001)
QUB 30-13	2001	Northern Ireland	0.068 (0.002)	0.040 (0.003)
QUB 12-3	2001	Northern Ireland	0.120 (0.018)	0.094 (0.010)
QUB 18-9	2001	Northern Ireland	0.129 (0.019)	0.082 (0.021)
			0.239 (0.029)	0.156 (0.021)
R 9517.1	2001	ARINI, Northern Ireland	0.130 (0.037)	0.051 (0.001)
R 9524.2	2001	ARINI, Northern Ireland	0.132 (0.026)	0.051 (0.001)

Isolate	Year of collection	Location	Log₁₀ [EC₅₀ Azoxystrobin (µg ml⁻¹)] Mean (Standard error)	
			$0\ \mu g\ ml^{-1}$ SHAM	$40\ \mu g\ ml^{-1}$ SHAM
R 9528.4	2001	ARINI, Northern Ireland	0.109 (0.007)	0.139 (0.004)
R 9516.1	2001	ARINI, Northern Ireland	0.123 (0.012)	0.058 (0.003)
R 9511.4	2001	ARINI, Northern Ireland	0.066 (0.006)	0.187 (0.075)
R 9522.3	2001	ARINI, Northern Ireland	0.082 (0.012)	0.076 (0.010)
R 9519.2	2001	ARINI, Northern Ireland	0.161 (0.021)	0.030 (0.001)
RS01ch2.126	2001	Switzerland	0.103 (0.036)	0.116 (0.004)
RS01ch2.126.5.5	2001	Switzerland	0.106 (0.005)	0.051 (0.007)
RS01ch2.306.5.5	2001	Switzerland	0.129 (0.010)	0.061 (0.023)
RS01ch01A12a5.5	2001	Switzerland	0.111 (0.043)	0.075 (0.023)
3.1 2/7	2001	Rothamsted Research, UK	0.062 (0.010)	0.107 (0.015)
3.1 4/7	2001	Rothamsted Research, UK	0.108 (0.029)	0.088 (0.005)
3.1 5/7	2001	Rothamsted Research, UK	0.104 (0.009)	0.065 (0.027)
5.2 2/7	2001	Rothamsted Research, UK	0.067 (0.017)	0.085 (0.050)
T.I. 3.1 11/7	2001	Trenthome Farm, Nottingham, UK	0.069 (0.009)	0.110 (0.012)
M.S. 1.1 13/7	2001	Trenthome Farm, Nottingham, UK	0.092 (0.005)	0.113 (0.005)
M.S. 5.1 13/7	2001	Trenthome Farm, Nottingham, UK	0.080 (0.020)	0.044 (0.004)
GKII 18-2-1	2002	Rothamsted Research, UK	0.046 (0.001)	0.045 (0.000)
GKII 18-2-2	2002	Rothamsted Research, UK	0.042 (0.001)	0.034 (0.003)
GKII 18-2-3	2002	Rothamsted Research, UK	0.094 (0.011)	0.080 (0.008)
GKII 18-3-1	2002	Rothamsted Research, UK	0.063 (0.005)	0.049 (0.008)
GKII 18-3-2	2002	Rothamsted Research, UK	0.077 (0.011)	0.111 (0.011)
GKII 18-3-3	2002	Rothamsted Research, UK	0.203 (0.020)	0.065 (0.004)
GKII 20-3-1	2002	Rothamsted Research, UK	0.025 (0.010)	0.067 (0.005)
			0.034 (0.004)	0.049 (0.006)
OSA 28-2-2	2002	Rothamsted Research, UK	0.016 (0.002)	0.048 (0.001)
			0.125 (0.000)	0.055 (0.005)
OSA 10-4-1	2002	Rothamsted Research, UK	0.118 (0.016)	0.072 (0.038)
OSA 10-4-28	2002	Rothamsted Research, UK	0.029 (0.003)	0.028 (0.002)
OSB 28-2-2	2002	Rothamsted Research, UK	0.032 (0.007)	0.019 (0.003)
			0.194 (0.038)	0.294 (0.040)

Isolate	Year of collection	Location	Log₁₀ [EC₅₀ Azoxystrobin ($\mu g\ ml^{-1}$)] Mean (Standard error)	
			0 $\mu g\ ml^{-1}$ SHAM	40 $\mu g\ ml^{-1}$ SHAM
OSB 24-4-1	2002	Rothamsted Research, UK	0.087 (0.001)	0.036 (0.016)
OSB 24-4-21	2002	Rothamsted Research, UK	0.122 (0.000)	0.050 (0.000)
OSB 24-4-47	2002	Rothamsted Research, UK	0.073 (0.002)	0.095 (0.028)
LARS 12-4-2	2002	Long Ashton Research Station, UK	0.073 (0.002)	0.025 (0.001)
LARS 12-4-3	2002	Long Ashton Research Station, UK	0.063 (0.000)	
LARS 8-4-2.5	2002	Long Ashton Research Station, UK	0.074 (0.011)	0.048 (0.021)
Sheringham 1	2002	Sheringham, Norfolk, UK	0.105 (0.019)	0.069 (0.005)
Sheringham 2	2002	Sheringham, Norfolk, UK	0.097 (0.005)	0.063 (0.004)
XNC 2000 3-2 T1 B	2002	Northern Ireland	0.186 (0.023)	0.150 (0.006)
XNC 2000 4-2-4 T1	2002	Northern Ireland	0.178 (0.045)	
SAC 09/943/186	2007	Scottish Agricultural College, UK	0.032 (0.001)	0.016 (0.001)
SAC 09/943/62	2007	Scottish Agricultural College, UK	0.046 (0.000)	0.029 (0.001)
SAC 09/943/73	2007	Scottish Agricultural College, UK	0.030 (0.001)	0.010 (0.005)
SAC 09/943/13	2007	Scottish Agricultural College, UK	0.035 (0.002)	0.042 (0.007)
SAC 09/943/115	2007	Scottish Agricultural College, UK	0.042 (0.002)	0.039 (0.002)
SAC 09/943/131	2007	Scottish Agricultural College, UK	0.028 (0.004)	0.013 (0.001)
SAC 09/943/14	2007	Scottish Agricultural College, UK	0.054 (0.004)	0.060 (0.008)
	2007	Scottish Agricultural College, UK	0.052 (0.005)	0.093 (0.004)
SAC 09/943/132	2007	Scottish Agricultural College, UK	0.307 (0.053)	0.037 (0.024)
SAC 09/943/178	2007	Scottish Agricultural College, UK	0.041 (0.003)	0.055 (0.008)
RS 219	2004	UK (Syngenta)	0.031 (0.002)	0.028 (0.004)
RS 683	2004	UK (Syngenta)	0.064 (0.021)	0.052 (0.001)
			0.139 (0.014)	0.097 (0.000)
RS 783	2004	UK (Syngenta)	2.26 (0.523)	0.095 (0.012)
R.s. 2310 4.2	2008	France (BASF QoI monitoring)	0.023 (0.004)	0.017 (0.002)
R.s. 2313 4.2	2008	France (BASF QoI monitoring)	> 100	> 100
R.s. 2314 4.2	2008	France (BASF QoI monitoring)	> 100	> 100
R.s. 2318 4.2	2008	France (BASF QoI monitoring)	0.083 (0.026)	0.039 (0.007)

Appendix 2. cytochrome *b* sequences from *R. secalis* isolates.

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                   1                                                                              85
Sy1-14     (1)   MRIFKSHPLLKLVNSYIIDSPQPSNLSYLWNFGSLLAVCLAIQIVTGVTLAMHYNPSILEAFNSIEHIMRDVNNGWLIRYLHSNT
K1124      (1)   -----------------DSPQPSNLSYLWNFGSLLAVCLAIQIVTGVTLAMHYNPSILEAFNSIEHIMRDVNNGWLIRYLHSNT
788        (1)   -----------------SPQPSNLSYLWNFGSLLAVCLAIQIVTGVTLAMHYNPSILEAFNSIEHIMRDVNNGWLIRYLHSNT
QUB 30-10  (1)   -----------------DSPQPSNLSYLWNFGSLLAVCLAIQIVTGVTLAMHYNPSILEAFNSIEHIMRDVNNGWLIRYLHSNT
R 9528.4   (1)   -----------------DSPQPSNLRYLWNFGSLLAVCLAIQIVTGVTLAMHYNPSILEAFNSIEHIMRDVNNGWLIRYLHSNT
GKII 18-2-3(1)   -----------------DSPQPSNLXYLWNFGSLLAVCLAIQIVTGVTLAMHYNPSILEAFNSIEHIMRDVNNGWLIRYLHSNT
RS 219     (1)   -----------------DSPQPSNLSYLWNFGSLLAVCLAIQIVTGVTLAMHYNPSILEAFNSIEHIMRDVNNGWLIRYLHSNT
RS 783     (1)   -----------------DSPQPSNLSYLWNFGSLLAVCLAIQIVTGVTLAMHYNPSILEAFNSIEHIMRDVNNGWLIRYLHSNT
Consensus  (1)   MRIFKSHPLLKLVNSYIIDSPQPSNLSYLWNFGSLLAVCLAIQIVTGVTLAMHYNPSILEAFNSIEHIMRDVNNGWLIRYLHSNT

                   86                                                                             170
Sy1-14     (86)  ASFFFFLVYLHMGRGLYYGSYRAPRTLVWTIGTFIFILMIVTAFLGYVLPYGQMSLWGATVITNLMSAIPWIGQDIVEFIWGGFS
K1124      (68)  ASFFFFLVYLHMGRGLYYGSYRAPRTLVWTIGTFIFILMIVTAFLGYVLPYGQMSLWGATVITNLMSAIPWIGQDIVEFIWGGFS
788        (67)  ASFFFFLVYLHMGRGLYYGSYRAPRTLVWTIGTFIFILMIVTAFLGYVLPYGQMSLWGATVITNLMSAIPWIGQDIVEFIWGGFS
QUB 30-10  (68)  ASFFFFLVYLHMGRGLYYGSYRAPRTLVWTIGTFIFILMIVTAFLGYVLPYGQMSLWGATVITNLMSAIPWIGQDIVEFIWGGFS
R 9528.4   (68)  ASFFFFLVYLHMGRGLYYGSYRAPRTLVWTIGTFIFILMIVTAFLGYVLPYGQMSLWGATVITNLMSAIPWIGQDIVEFIWGGFS
GKII 18-2-3(68)  ASFFFFLVYLHMGRGLYYGSYRAPRTLVWTIGTFIFILMIVTAFLGYVLPYGQMSLWGATVITNLMSAIPWIGQDIVEFIWGGFS
RS 219     (68)  ASFFFFLVYLHMGRGLYYGSYRAPRTLVWTIGTFIFILMIVTAFLGYVLPYGQMSLWGATVITNLMSAIPWIGQDIVEFIWGGFS
RS 783     (68)  ASFFFFLVYLHMGRGLYYGSYRAPRTLVWTIGTFIFILMIVTAFLGYVLPYGQMSLWGATVITNLMSAIPWIGQDIVEFIWGGFS
Consensus  (86)  ASFFFFLVYLHMGRGLYYGSYRAPRTLVWTIGTFIFILMIVTAFLGYVLPYGQMSLWGATVITNLMSAIPWIGQDIVEFIWGGFS

                   171                                                                            255
Sy1-14     (171) VNNATLNRFFALHFVLPFILAALVLMHLIALHDSAGSGNPLGVSGNYDRLPFAPYFLFKDLITIFLFIFVLSLFVFFMPNVLGDS
K1124      (153) VNNATLNRFFALHFVLPFILAALVLMHLIALHDSAGSGNPLGVSGNYDRLPFAPYFLFKDLITIFLFIFVLSLFVFFMPNVLGDS
788        (152) VNNATLNRFFALHFVLPFILAALVLMHLIALHDSAGSGNPLGVSGNYDRLPFAPYFLFKDLITIFLFIFVLSLFVFFMPNVLGDS
QUB 30-10  (153) VNNATLNRFFALHFVLPFILAALVLMHLIALHDSAGSGNPLGVSGNYDRLPFAPYFLFKDLITIFLFIFVLSLFVFFMPNVLGDS
R 9528.4   (153) VNNATLNRFFALHFVLPFILAALVLMHLIALHDSAGSGNPLGVSGNYDRLPFAPYFLFKDLITIFLFIFVLSLFVFFMPNVLGDS
GKII 18-2-3(153) VNNATLNRFFALHFVLPFILAALVLMHLIALHDSAGSGNPLGVSGNYDRLPFAPYFLFKDLITIFLFIFVLSLFVFFMPNVLGDS
RS 219     (153) VNNATLNRFFALHFVLPFILAALVLMHLIALHDSAGSGNPLGVSGNYDRLPFAPYFLFKDLITIFLFIFVLSLFVFFMPNVLGDS
RS 783     (153) VNNATLNRFFALHFVLPFILAALVLMHLIALHDSAGSGNPLGVSGNYDRLPFAPYFLFKDLITIFLFIFVLSLFVFFMPNVLGDS
Consensus  (171) VNNATLNRFFALHFVLPFILAALVLMHLIALHDSAGSGNPLGVSGNYDRLPFAPYFLFKDLITIFLFIFVLSLFVFFMPNVLGDS
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Logo₁₀ [EC₅₀(µg ml⁻¹)]

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              256                                                                         340
Sy1-14      (256) ENYVVANPMQTPPAIVPEWYLLPFYAILRSIPNKLLGVIAMLSAILVILAMPFTDLSRSRGIQFRPLSKIAFYIFVANFLILMVL
K1124       (238) ENYVVANPMQTPPAIVPEWYLLPFYAILRSIPNKLLGVIAMLSAILVILAMPFTDLSRSRGIQFRPLSKIAFYIFVANFLILMVL
788         (237) ENYVVANPMQTPPAIVPEWYLLPFYAILRSIPNKLLGVIAMLSAILVILAMPFTDLSRSRGIQFRPLSKIAFYIFVANFLILMVL
QUB 30-10   (238) ENYVVANPMQTPPAIVPEWYLLPFYAILRSIPNKLLGVIAMLSAILVILAMPFTDLSRSRGIQFRPLSKIAFYIFVANFLILMVL
R 9528.4    (238) ENYVVANPMQTPPAIVPEWYLLPFYAILRSIPNKLLGVIAMLSAILVILAMPFTDLSRSRGIQFRPLSKIAFYIFVANFLILMVL
GKII 18-2-3 (238) ENYVVANPMQTPPAIVPGWYLLPFYAILRSIPNKLLGVIAMLSAILVILAMPFTDLSRSRGIQFRPLSK
RS 219      (238) ENYVVANPMQTPPAIVPEWYLLPFYAILRSIPNKLLGVIAMLSAILVILAMPFTDLSRSRGIQFRPLSK
RS 783      (238) ENYVVANPMQTPPAIVPEWYLLPFYAILRSIPNKLLGVIAMLSAILVILAMPFTDLSRSRGIQFRPLSKIAFYIFVANFLILMVL
Consensus   (256) ENYVVANPMQTPPAIVPEWYLLPFYAILRSIPNKLLGVIAMLSAILVILAMPFTDLSRSRGIQFRPLSKIAFYIFVANFLILMVL

              341                                                      396
Sy1-14      (341) GAKHVESPFIEFGQISTVIYFSHFLIIVPLVSLIENSLIDLNTSIDYSSPSVLEKA
K1124       (323) GAKHVESPFIEFGQISTVIYFSHFLIIVPLVSLIENSLIDLNTSIDYSSPS-----
788         (322) GAKHVESPFIEFGQISTVIYFSHFLIIVPLVSLIENSLIDLNTSIDYSSPS-----
QUB 30-10   (323) GAKHVESPFIEFGQISTVIYFSHFLIIVPLVSLIENSLIDLNTSIDYSSPS-----
R 9528.4    (323) GAKHVESPFIEFGQISTVIYFSHFLIIVPLVSLIENSLIDLNTSIDYSSPS-----
GKII 18-2-3 (323) GAKHVESPFIEFGQISTVTYFSHFLIIVPLVSLIENSLIDLNTSIDYSSPS-----
RS 219      (307) -------------------------------------------------------
RS 783      (323) GAKHVESPFIEFGQISTVIYFSHFLIIVPLVSLIENSLIDLNTSIDYSSPS-----
Consensus   (341) GAKHVESPFIEFGQISTVIYFSHFLIIVPLVSLIENSLIDLNTSIDYSSPSVLEKA
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Appendix 3. Azoxystrobin EC$_{50}$s of *R. secalis* isolates.

Isolate	Year of collection	Log$_{10}$ [EC$_{50}$(µg ml^{-1})] Mean (Standard error)			
		Epoxiconazole	Prothioconazole	Tebuconazole	Propiconazole
K1124	Pre-1994	0.040 (0.001)	0.210 (0.033)	0.055 (0.006)	0.020 (0.004)
SK7	1984	0.050 (0.013)	0.139 (0.020)	0.030 (0.001)	0.015 (0.001)
NKT12	1996	0.023 (0.000)	0.207 (0.048)	0.044 (0.014)	0.013 (0.004)
FI12-63	1996	0.043 (0.005)	0.164 (0.002)	0.040 (0.004)	0.005 (0.001)
R157 (1)	1997	0.042 (0.014)	0.222 (0.021)	0.073 (0.023)	0.054 (0.005)
R157 (2)	1997	0.035 (0.001)	0.156 (0.025)	0.028 (0.001)	0.011 (0.002)
788	1997	0.570 (0.004)	1.40 (0.335)	5.88 (0.840)	11.1 (0.785)
	1997	0.520 (0.053)	1.27 (0.165)	5.31 (0.215)	9.52 (1.38)
SAC 0003 1-4-2 30	2000	0.877 (0.217)	0.506 (0.173)	17.90 (1.70)	13.4 (1.75)
SAC 1-4-8 (0003)	2000	0.417 (0.040)	1.30 (0.165)	5.15 (0.190)	12.9 (3.10)
SAC 8-3-8 (0003)	2000	0.084 (0.001)	0.463 (0.023)	0.101 (0.004)	0.104 (0.029)
SAC 0003 8.1.8 00 (0.123)	2000	0.035 (0.002)	0.423 (0.122)	0.099 (0.063)	0.020 (0.002)
SAC 0004 1.2.4 00	2000	0.489 (0.058)	0.523 (0.050)	6.82 (0.095)	20.8 (1.95)
QUB 9-10	2001	0.376 (0.062)	0.627 (0.029)	5.15 (0.265)	18.7 (0.850)
QUB 30-10	2001	0.128 (0.006)	1.08 (0.030)	1.14 (0.020)	0.278 (0.058)
QUB 18-2	2001	0.555 (0.071)	1.25 (0.040)	4.77 (0.925)	1.20 (0.000)
QUB 30-13	2001	0.077 (0.033)	0.265 (0.026)	1.88 (0.789)	0.189 (0.032)
QUB 12-3	2001	1.58 (0.535)	7.48 (0.460)	29.0 (3.80)	7.35 (0.845)
QUB 18-9	2001	0.391 (0.095)	0.425 (0.098)	10.3 (2.67)	11.8 (3.24)
R 9517.1	2001	-	0.510 (0.051)	0.876 (0.033)	0.071 (0.015)
R 9524.2	2001	0.034 ( )	0.775 (0.047)	0.815 (0.035)	0.128 (0.020)
R 9528.4	2001	0.037 (0.007)	0.686 (0.022)	1.09 (0.035)	0.436 (0.010)
R 9516.1	2001	-	0.134 (0.048)	0.349 (0.030)	0.164 (0.042)
R 9511.4	2001	0.033 (0.001)	0.929 (0.001)	1.86 (0.675)	0.515 (0.081)
R 9522.3	2001	0.052 (0.005)	0.539 (0.010)	1.32 (0.005)	1.03 (0.140)
R 9519.2	2001	-	0.192 (0.009)	0.441 (0.055)	0.142 ( )
RS01ch2.126	2001	1.70 (0.040)	0.512 (0.353)	15.3 (0.750)	4.21 (1.67)
RS01ch2.126.5.5	2001	0.408 (0.021)	0.469 (0.102)	4.09 (0.500)	3.63 (0.890)
RS01ch2.306.5.5	2001	0.499 (0.078)	0.442 (0.002)	4.30 (0.175)	4.43 (0.290)

Isolate	Year of collection	Log₁₀ [EC₅₀ (µg ml⁻¹)] Mean (Standard error)			
		Epoxiconazole	Prothioconazole	Tebuconazole	Propiconazole
RS01ch01A12a5.5	2001	0.774 (0.059)	0.997 (0.291)	10.8 (0.100)	-
3.1 2/7	2001	0.712 (0.003)	0.703 (0.029)	7.73 (0.805)	36.5 (1.90)
3.1 4/7	2001	-	0.938 (0.357)	11.6 (0.350)	0.007 (0.00)
3.1 5/7	2001	0.335 (0.154)	3.475 (0.005)	43.1(38.1)	-
5.2 2/7	2001	-	-	-	
T.I.3.1 11/7	2001		0.507 (0.056)	6.19 (2.17)	1.71 (0.980)
M.S.1.1 13/7	2001	0.255 (0.059)	1.39 (0.075)	6.02 (0.835)	2.98 (1.49)
M.S.5.1 13/7	2001	0.228 (0.019)	0.876 (0.125)	4.56 (0.170)	24.4 (3.45)
GKII 18-2-1	2002	0.098 ( )	1.97 (0.348)	11.2 (1.07)	14.9 (5.50)
GKII 18-2-2	2002	0.177 (0.031)	0.279 (0.065)	1.96 (0.260)	14.4 (1.46)
GKII 18-2-3	2002	0.263 (0.010)	0.450 (0.052)	5.08 (0.080)	6.33 (0.405)
GKII 18-3-1	2002	0.174 (0.026)	0.201 (0.034)	1.89 (0.085)	13.7 (1.65)
GKII 18-3-2	2002	0.179 (0.001)	0.052 (0.003)	4.26 (0.050)	5.12 (0.230)
GKII 18-3-3	2002	0.183 (0.052)	0.043 (0.024)	3.78 (0.395)	13.9 (0.800)
GKII 20-3-1	2002	0.302 (0.009)	0.863 (0.011)	4.17 (0.510)	8.32 (0.895)
OSA 28-2-2	2002	1.06 ( )	10.2 (0.880)	28.1 (0.650)	10.8 (3.43)
OSA 10-4-1	2002	0.320 (0.106)	0.732 (0.039)	4.41 (0.460)	12.8 (0.444)
OSA 10-4-28	2002	0.455 (0.004)	-	8.56 ( )	8.37 (1.07)
OSB 28-2-2	2002	0.410 (0.076)	0.208 (0.007)	3.77 (0.605)	13.5 (4.37)
		0.381 (0.049)	0.462 (0.022)	1.87 (0.231)	0.513 ( )
OSB 24-4-1	2002	0.264 (0.006)	0.343 (0.105)	2.85 (0.620)	1.38 (0.232)
OSB 24-4-21	2002	0.053 (0.030)	0.492 (0.061)	6.11 (0.240)	2.79 (0.902)
OSB 24-4-47	2002	0.100 (0.014)	0.606 (0.337)	7.404 (1.48)	2.24 (1.38)
LARS 12-4-3	2002	0.537 (0.137)	1.13 (0.085)	8.33 (1.70)	3.89 (1.25)
LARS 8-4-2.5	2002	0.173 (0.026)	0.266 (0.080)	3.79 (0.050)	-
Sheringham 1	2002	0.610 (0.064)	0.926 (0.070)	5.63 (0.210)	4.57 (0.237)
Sheringham 2	2002	0.510 (0.032)	1.12 (0.060)	6.01 (0.845)	20.3 (2.20)
XNC 2000 3-2 T1 B	2002	0.092 (0.003)	1.24 (0.180)	1.04 (0.097)	11.1 (0.900)
XNC 2000 4-2-4 T1	2002	0.996 (0.071)		11.0 (0.450)	0.142 (0.001)

Isolate	Year of collection	Log$_{10}$ [EC$_{50}$(µg ml^{-1})] Mean (Standard error)			
		Epoxiconazole	Prothioconazole	Tebuconazole	Propiconazole
SAC 09/943/186	2007	0.126 (0.013)	0.299 (0.037)	3.13 (0.891)	8.13 (1.92)
SAC 09/943/62	2007	-	-	2.47 (1.49)	-
SAC 09/943/73	2007	0.565 (0.006)	2.44 (0.672)	14.6 (0.374)	5.90 (2.99)
SAC 09/943/13	2007	0.172 (0.072)	0.139 (0.008)	2.69 (0.979)	10.8 ()
		0.659 (0.027)	1.17 (0.385)	7.65 (3.06)	-
SAC 09/943/115	2007	0.506 (0.114)	1.08 (0.353)	5.75 (0.575)	5.36 (0.045)
SAC 09/943/131	2007	0.228 (0.002)	0.224 (0.041)	4.19 (0.474)	50.7 (14.9)
SAC 09/943/14	2007	0.572 (0.170)	1.10 (0.196)	16.9 (0.015)	9.24 (1.93)
SAC 09/943/132	2007	0.367 (0.024)	23.0 (3.30)	7.80 (1.1)	37.9 ()
SAC 09/943/178	2007	0.300 (0.011)	0.488 (0.074)	5.71 (0.184)	
RS 219	2004	0.030 (0.001)	0.107 (0.002)	0.054 (0.001)	0.037 (0.007)
RS 683	2004	0.935 (0.043)	3.57 (0.808)	19.0 (0.53)	8.20 (3.18)
RS 783	2004	7.63 ()	6.33 (4.71)	> 100	> 100
R.s. 2310 4.2	2008	0.219 (0.027)	0.781 (0.043)	4.84 (1.071)	12.1 (10.8)
R.s. 2313 4.2	2008	0.255 (0.055)	1.39 (0.112)	10.2 (9.51)	-
R.s. 2314 4.2	2008	0.148 (0.018)	0.140 (0.019)	2.56 (0.461)	2.79 (0.157)
R.s. 2318 4.2	2008	0.336 (0.024)	1.23 (0.178)	5.16 (0.267)	3.58 ()

Appendix 4. *CYP51* sequences of *R. secalis* isolates. (a) *CYP51B* translated sequences, (b) *CYP51A* translated sequences, (c) *CYP51A-p* sequences.

```
                              1                                                          75
CYP51B              (1)   MGIFEAVTVPLAQQVSQRGLGVVIAAGFAAFLVVSVILNVLSQILFKNPNEPPIVEHFFPIIGSTVTYGIDPYKF
K1124               (1)   ------------------------------SFLVVSVILNVLSQILFKNPNEPPIVEHFFPIIGSTVTYGIDPYKF
FI12-63             (1)   ------------------------------FLVVSVILNVLSQILFKNPNEPPIVEHFFPIIGSTVSYGIDPYKF
788                 (1)   ------------------------------FLVVSVILNVLSQILFKNPNEPPIVEHFFPIIGSTVTYGIDPYKF
SAC 0003 1.4.8 00 (10) (1) ------------------------------FLVVSVILNVLSQILFKNPNEPPIVEHFFPIIGSTVTYGIDPYKF
QUB 30-10           (1)   ------------------------------SFLVVSVILNVLSQILFKNPNEPPIVEHFFPIIGSTVSYGIDPYKF
QUB 12-3            (1)   ------------------------------AFLVVSVILNVLSQILFKNPNEPPIVEHFFPIIGSTVTYGIDPYKF
R9528.4             (1)   -------------------------------LVVSVILNVLSQILFKNPNEPPIVEHFFPIIGSTVSYGIDPYKF
R 9522.3            (1)   ------------------------------SFLVVSVILNVLSQILFKNPNEPPIVEHFFPIIGSTVTYGIDPYKF
GKII 18-2-3         (1)   -------------------------------LVVSVILNVLSQILFKNPNEPPIVEHFFPIIGSTVTYGIDPYKF
GKII 18-3-2         (1)   ------------------------------FLVVSVILNVLSQILFKNPNEPPIVEHFFPIIGSTVTYGIDPYKF
OSA 28-2-2          (1)   -------------------------------LVVSVILNVLSQILFKNPNEPPIVEHFFPIIGSTVTYGIDPYKF
SAC 09/943/14       (1)   ------------------------------FLVVSVILNVLSQILFKNPNEPPIVEHFFPIIGSTVTYGIDPYKF
RS 219              (1)   --------------------------------VSVILNVLSQILFKNPNEPPIVEHFFPIIGSTVTYGIDPYKF
RS 783              (1)   ------------------------------FLVVSVILNVLSQILFKNPNEPPIVEHFFPIIGSTVTYGIDPYKF
Consensus           (1)   MGIFEAVTVPLAQQVSQRGLGVVIAAGFA FLVVSVILNVLSQILFKNPNEPPIVEHFFPIIGSTVTYGIDPYKF

                              76                                                         150
CYP51B              (76)  FFDNKAKYGEVFTFILLGKKTTVYLGTHGNEFILNGKIKDVNAEEVYTVLTTPVFGKDVVYDCPNSKLMEQKKFM
K1124               (47)  FFDNKAKYGEVFTFILLGKKTTVYLGTHGNEFILNGKIKDVNAEEVYTVLTTPVFGKDVVYDCPNSKLMEQKKFM
FI12-63             (46)  FFDNKAKYGEVFTFILLGKKTTVYLGTHGNEFILNGKIKDVNAEEVYTVLTTPVFGKDVVYDCPNSKLMEQKKFM
788                 (46)  FFDNKAKYGEVFTFILLGKKTTVYLGTHGNEFILNGKIKDVNAEEVYTVLTTPVFGKDVVYDCPNSKLMEQKKFM
SAC 0003 1.4.8 00 (10) (46) FFDNKAKYGEVFTFILLGKKTTVYLGTHGNEFILNGKIKDVNAEEVYTVLTTPVFGKDVVYDCPNSKLMEQKKFM
QUB 30-10           (47)  FFDNKAKYGEVFTFILLGKKTTVYLGTHGNEFILNGKIKDVNAEEVYTVLTTPVFGKDVVYDCPNSKLMEQKKFM
QUB 12-3            (47)  FFDNKAKYGEVFTFILLGKKTTVYLGTHGNEFILNGKIKDVNAEEVYTVLTTPVFGKDVVYDCPNSKLMEQKKFM
R9528.4             (45)  FFDNKAKYGEVFTFILLGKKTTVYLGTHGNEFILNGKIKDVNAEEVYTVLTTPVFGKDVVYDCPNSKLMEQKKFM
R 9522.3            (47)  FFDNKAKYGEVFTFILLGKKTTVYLGTHGNEFILNGKIKDVNAEEVYTVLTTPVFGKDVVYDCPNSKLMEQKKFM
GKII 18-2-3         (45)  FFDNKAKYGEVFTFILLGKKTTVYLGTHGNEFILNGKIKDVNAEEVYTVLTTPVFGKDVVYDCPNSKLMEQKKFM
GKII 18-3-2         (46)  FFDNKAKYGEVFTFILLGKKTTVYLGTHGNEFILNGKIKDVNAEEVYTVLTTPVFGKDVVYDCPNSKLMEQKKFM
OSA 28-2-2          (45)  FFDNKAKYGEVFTFILLGKKTTVYLGTHGNEFILNGKIKDVNAEEVYTVLTTPVFGKDVVYDCPNSKLMEQKKFM
SAC 09/943/14       (46)  FFDNKAKYGEVFTFILLGKKTTVYLGTHGNEFILNGKIKDVNAEEVYTVLTTPVFGKDVVYDCPNSKLMEQKKFM
RS 219              (43)  FFDNKAKYGEVFTFILLGKKTTVYLGTHGNEFILNGKIKDVNAEEVYTVLTTPVFGKDVVYDCPNSKLMEQKKFM
RS 783              (46)  FFDNKAKYGEVFTFILLGKKTTVYLGTHGNEFILNGKIKDVNAEEVYTVLTTPVFGKDVVYDCPNSKLMEQKKFM
Consensus           (76)  FFDNKAKYGEVFTFILLGKKTTVYLGTHGNEFILNGKIKDVNAEEVYTVLTTPVFGKDVVYDCPNSKLMEQKKFM
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244

151

Label	Pos	Sequence
CYP51B	(151)	KIGLTTEAFKTYVPIIQDEVETFIKGSAAFKGHKGTVNIPAQMAEITIYTASHALQGKDCRDKFDHSFAELYHAL
K1124	(122)	KIGLTTEAFKTYVPIIQDEVETFIKGSAAFKGHKGTVNIPAQMAEITIYTASHALQGKDCRDKFDHSFAELYHAL
FI12-63	(121)	KIGLTTEAFKTYVPIIQDEVETFIKGSAAFKGHKGTVNIPAQMAEITIYTASHALQGKDCRDKFDHSFAELYHAL
788	(121)	KIGLTTEAFKTYVPIIQDEVETFIKGSAAFKGHKGTVNIPAQMAEITIYTASHALQGKDCRDKFDHSFAELYHAL
SAC 0003 1.4.8 00 (10)	(121)	KIGLTTEAFKTYVPIIQDEVETFIKGSAAFKGHKGTVNIPAQMAEITIYTASHALQGKDCRDKFDHSFAELYHAL
QUB 30-10	(121)	KIGLTTEAFKTYVPIIQDEVETFIKGSAAFKGHKGTVNIPAQMAEITIYTASHALQGKDCRDKFDHSFAELYHAL
QUB 12-3	(122)	KIGLTTEAFKTYVPIIQDEVETFIKGSAAFKGHKGTVNIPAQMAEITIYTASHALQGKDCRDKFDHSFAELYHAL
R9528.4	(122)	KIGLTTEAFKTYVPIIQDEVETFIKGSAAFKGHKGTVNIPAQMAEITIYTASHALQGKDCRDKFDHSFAELYHAL
R 9522.3	(120)	KIGLTTEAFKTYVPIIQDEVETFIKGSAAFKGHKGTVNIPAQMAEITIYTASHALQGKDCRDKFDHSFAELYHAL
GKII 18-2-3	(122)	KIGLTTEAFKTYVPIIQDEVETFIKGSAAFKGHKGTVNIPAQMAEITIYTASHALQGKDCRDKFDHSFAELYHAL
GKII 18-3-2	(120)	KIGLTTEAFKTYVPIIQDEVETFIKGSAAFKGHKGTVNIPAQMAEITIYTASHALQGKDCRDKFDHSFAELYHAL
OSA 28-2-2	(121)	KIGLTTEAFKTYVPIIQDEVETFIKGSAAFKGHKGTVNIPAQMAEITIYTASHALQGKDCRDKFDHSFAELYHAL
SAC 09/943/14	(120)	KIGLTTEAFKTYVPIIQDEVETFIKGSAAFKGHKGTVNIPAQMAEITIYTASHALQGKDCRDKFDHSFAELYHAL
RS 219	(121)	KIGLTTEAFKTYVPIIQDEVETFIKGSAAFKGHKGTVNIPAQMAEITIYTASHALQGKDCRDKFDHSFAELYHAL
RS 783	(118)	KIGLTTEAFKTYVPIIQDEVETFIKGSAAFKGHKGTVNIPAQMAEITIYTASHALQGKDCRDKFDHSFAELYHAL
Consensus	(151)	KIGLTTEAFKTYVPIIQDEVETFIKGSAAFKGHKGTVNIPAQMAEITIYTASHALQGKDCRDKFDHSFAELYHAL

225

226

Label	Pos	Sequence
CYP51B	(226)	DMGFSPINEMLHWAPLPHNRARDHAQRTVAKAYMEIMETRRKDKKSLDNMDIMSQLMRSTYKNGVPVPDMEIAHM
K1124	(197)	DMGFSPINEMLHWAPLPHNRARDHAQRTVAKAYMEIMETRRKDKKSLDNMDIMSQLMRSTYKNGVPVPDMEIAHM
FI12-63	(196)	DMGFSPINEMLHWAPLPHNRARDHAQRTVAKAYMEIMETRRKDKKSLDNMDIMSQLMRSTYKNGVPVPDMEIAHM
788	(196)	DMGFSPINEMLHWAPLPHNRARDHAQRTVAKAYMEIMETRRKDKKSLDNMDIMSQLMRSTYKNGVPVPDMEIAHM
SAC 0003 1.4.8 00 (10)	(196)	DMGFSPINEMLHWAPLPHNRARDHAQRTVAKAYMEIMETRRKDKKSLDNMDIMSQLMRSTYKNGVPVPDMEIAHM
QUB 30-10	(197)	DMGFSPINEMLHWAPLPHNRARDHAQRTVAKAYMEIMETRRKDKKSLDNMDIMSQLMRSTYKNGVPVPDMEIAHM
QUB 12-3	(197)	DMGFSPINEMLHWAPLPHNRARDHAQRTVAKAYMEIMETRRKDKKSLDNMDIMSQLMRSTYKNGVPVPDMEIAHM
R9528.4	(195)	DMGFSPINEMLHWAPLPHNRARDHAQRTVAKAYMEIMETRRKDKKSLDNMDIMSQLMRSTYKNGVPVPDMEIAHM
R 9522.3	(197)	DMGFSPINEMLHWAPLPHNRARDHAQRTVAKAYMEIMETRRKDKKSLDNMDIMSQLMRSTYKNGVPVPDMEIAHM
GKII 18-2-3	(195)	DMGFSPINEMLHWAPLPHNRARDHAQRTVAKAYMEIMETRRKDKKSLDNMDIMSQLMRSTYKNGVPVPDMEIAHM
GKII 18-3-2	(196)	DMGFSPINEMLHWAPLPHNRARDHAQRTVAKAYMEIMETRRKDKKSLDNMDIMSQLMRSTYKNGVPVPDMEIAHM
OSA 28-2-2	(195)	DMGFSPINEMLHWAPLPHNRARDHAQRTVAKAYMEIMETRRKDKKSLDNMDIMSQLMRSTYKNGVPVPDMEIAHM
SAC 09/943/14	(196)	DMGFSPINEMLHWAPLPHNRARDHAQRTVAKAYMEIMETRRKDKKSLDNMDIMSQLMRSTYKNGVPVPDMEIAHM
RS 219	(193)	DMGFSPINEMLHWAPLPHNRARDHAQRTVAKAYMEIMETRRKDKKSLDNMDIMSQLMRSTYKNGVPVPDMEIAHM
RS 783	(196)	DMGFSPINEMLHWAPLPHNRARDHAQRTVAKAYMEIMETRRKDKKSLDNMDIMSQLMRSTYKNGVPVPDMEIAHM
Consensus	(226)	DMGFSPINEMLHWAPLPHNRARDHAQRTVAKAYMEIMETRRKDKKSLDNMDIMSQLMRSTYKNGVPVPDMEIAHM

300

```
                                  301                                                                          375
        CYP51B (301) MIALLMAGQHSSSSSSSTWIILRLAARPDILEELYQEQLEVLGSDLPALQYEDLAKLTKHQNVLKEVLRLHTPIHS
         K1124 (272) MIALLMAGQHSSSSSSSTWIILRLAARPDILEELYQEQLEVLGSDLPALQYEDLAKLTKHQNVLKEVLRLHTPIHS
       FI12-63 (271) MIALLMAGQHSSSSSSSTWIILRLAARPDILEELYQEQLEVLGSDLPALQYEDLAKLTKHQNVLKEVLRLHTPIHS
           788 (271) MIALLMAGQHSSSSSSSTWIILRLAARPDILEELYQEQLEVLGSDLPALQYEDLAKLTKHQNVLKEVLRLHTPIHS
SAC 0003 1.4.8 00 (10) (271) MIALLMAGQHSSSSSSSTWIILRLAARPDILEELYQEQLEVLGSDLPALQYEDLAKLTKHQNVLKEVLRLHTPIHS
       QUB 30-10 (272) MIALLMAGQHSSSSSSSTWIILRLAARPDILEELYQEQLEVLGSDLPALQYEDLAKLTKHQNVLKEVLRLHTPIHS
        QUB 12-3 (272) MIALLMAGQHSSSSSSSTWIILRLAARPDILEELYQEQLEVLGSDLPALQYEDLAKLTKHQNVLKEVLRLHTPIHS
        R9528.4 (270) MIALLMAGQHSSSSSSSTWIILRLAARPDILEELYQEQLEVLGSDLPALQYEDLAKLTKHQNVLKEVLRLHTPIHS
       R 9522.3 (272) MIALLMAGQHSSSSSSSTWIILRLAARPDILEELYQEQLEVLGSDLPALQYEDLAKLTKHQNVLKEVLRLHTPIHS
      GKII 18-2-3 (270) MIALLMAGQHSSSSSSSTWIILRLAARPDILEELYQEQLEVLGSDLPALQYEDLAKLTKHQNVLKEVLRLHTPIHS
      GKII 18-3-2 (271) MIALLMAGQHSSSSSSSTWIILRLAARPDILEELYQEQLEVLGSDLPALQYEDLAKLTKHQNVLKEVLRLHTPIHS
       OSA 28-2-2 (270) MIALLMAGQHSSSSSSSTWIILRLAARPDILEELYQEQLEVLGSDLPALQYEDLAKLTKHQNVLKEVLRLHTPIHS
   SAC 09/943/14 (271) MIALLMAGQHSSSSSSSTWIILRLAARPDILEELYQEQLEVLGSDLPALQYEDLAKLTKHQNVLKEVLRLHTPIHS
         RS 219 (268) MIALLMAGQHSSSSSSSTWIILRLAARPDILEELYQEQLEVLGSDLPALQYEDLAKLTKHQNVLKEVLRLHTPIHS
         RS 783 (271) MIALLMAGQHSSSSSSSTWIILRLAARPDILEELYQEQLEVLGSDLPALQYEDLAKLTKHQNVLKEVLRLHTPIHS
      Consensus (301) MIALLMAGQHSSSSSSSTWIILRLAARPDILEELYQEQLEVLGSDLPALQYEDLAKLTKHQNVLKEVLRLHTPIHS


                                  376                                                                          450
        CYP51B (376) IMRKVKSPMPIAGTKFVIPTSHVLLASPGFSSREATYFPDPLKWEPHRWDPESGGVLGTEVEEESYDYGYGLISK
         K1124 (347) IMRKVKSPMPIAGTKFVIPTSHVLLASPGFSSREATYFPDPLKWEPHRWDPESGGVLGTEVEEESYDYGYGLISK
       FI12-63 (346) IMRKVKSPMPIAGTKFVIPTSHVLLASPGFSSREATYFPDPLKWEPHRWDPESGGVLGTEVEEESYDYGYGLISK
           788 (346) IMRKVKSPMPIAGTKFVIPTSHVLLASPGFSSREATYFPDPLKWEPHRWDPESGGVLGTEVEEESYDYGYGLISK
SAC 0003 1.4.8 00 (10) (346) IMRKVKSPMPIAGTKFVIPTSHVLLASPGFSSREATYFPDPLKWEPHRWDPESGGVLGTEVEEESYDYGYGLISK
       QUB 30-10 (347) IMRKVKSPMPIAGTKFVIPTSHVLLASPGFSSREATYFPDPLKWEPHRWDPESGGVLGTEVEEESYDYGYGLISK
        QUB 12-3 (347) IMRKVKSPMPIAGTKFVIPTSHVLLASPGFSSREATYFPDPLKWEPHRWDPESGGVLGTEVEEESYDYGYGLISK
        R9528.4 (345) IMRKVKSPMPIAGTKFVIPTSHVLLASPGFSSREATYFPDPLKWEPHRWDPESGGVLGTEVEEESYDYGYGLISK
       R 9522.3 (347) IMRKVKSPMPIAGTKFVIPTSHVLLASPGFSSREATYFPDPLKWEPHRWDPESGGVLGTEVEEESYDYGYGLISK
      GKII 18-2-3 (345) IMRKVKSPMPIAGTKFVIPTSHVLLASPGFSSREATYFPDPLKWEPHRWDPESGGVLGTEVEEESYDYGYGLISK
      GKII 18-3-2 (346) IMRKVKSPMPIAGTKFVIPTSHVLLASPGFSSREATYFPDPLKWEPHRWDPESGGVLGTEVEEESYDYGYGLISK
       OSA 28-2-2 (345) IMRKVKSPMPIAGTKFVIPTSHVLLASPGFSSREATYFPDPLKWEPHRWDPESGGVLGTEVEEESYDYGYGLISK
   SAC 09/943/14 (346) IMRKVKSPMPIAGTKFVIPTSHVLLASPGFSSREATYFPDPLKWEPHRWDPESGGVLGTEVEEESYDYGYGLISK
         RS 219 (343) IMRKVKSPMPIAGTKFVIPTSHVLLASPGFSSREATYFPDPLKWEPHRWDPESGGVLGTEVEEESYDYGYGLISK
         RS 783 (346) IMRKVKSPMPIAGTKFVIPTSHVLLASPGFSSREATYFPDPLKWEPHRWDPESGGVLGTEVEEESYDYGYGLISK
      Consensus (376) IMRKVKSPMPIAGTKFVIPTSHVLLASPGFSSREATYFPDPLKWEPHRWDPESGGVLGTEVEEESYDYGYGLISK
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                               451                                                        526
CYP51B             (451)  GAKSPYLPFGAGRHRCIGEQFANVQLITITAVMVRYFKFRNLDGSSKVVETDYTSLFSRPLAPAVVEWEKREKVKV
K1124              (422)  GAKSPYLPFGAGRHRCIGEQFANVQLITITAVMVRYFKFRT------------------------------------
FI12-63            (421)  GAKSPYLPFGAGRHRCIGEQFANVQLITITAVMVRYFKFRNL----------------------------------
788               (421)  GAKSPYLPFGAGRHRCIGEQFANVQLITITAVMVRYFKFRNLDGSSKVVETDYTSLFSRPLAPAVVEW---------
SAC 0003 1.4.8 00 (10) (421)  GAKSPYLPFGAGRHRCIGEQFANVQLITITAVMVRYFKFRNLDGSSKLLRLIIRV-----------------
QUB 30-10          (422)  GAKSPYLPFGAGRHRCIGEQFANVQLITITAVMVRYSKFRT------------------------------------
QUB 12-3           (422)  GAKSPYLPFGAGRHRCIGEQFANVQLITITAVMVRYFKFRT------------------------------------
R9528.4            (420)  GAKSPYLPFGAGRHRCIGEQFANVQLITITAVMVRYFKFMN------------------------------------
R 9522.3           (422)  GAKSPYLPFGAGRHRCIGEQFANVQLITITAVMVRYFKFRTLDGSSK-----------------------------
GKII 18-2-3        (420)  GAKSPYLPFGAGRHRCIGEQFANVQLITITAVMVRYFKFTN------------------------------------
GKII 18-3-2        (421)  GAKSPYLPFGAGRHRCIGEQFANVQLITITAVMVRYFKFKN------------------------------------
OSA 28-2-2         (420)  GAKSPYLPFGAGRHRCIGEQFANVQLITITAVMVRYFKFRT------------------------------------
SAC 09/943/14      (421)  GAKSPYLPFGAGRHRCIGEQFANVQLITITAVMVRYFKFRTSMEARSC----------------------------
RS 219             (418)  GAKSPYLPFGAGRHRCIGEQFANVQLITITAVMVRYFKFRTS-----------------------------------
RS 783             (421)  GAKSPYLPFGAGRHRCIGEQFANVQLITITAVMVRYFKFRT------------------------------------
Consensus          (451)  GAKSPYLPFGAGRHRCIGEQFANVQLITITAVMVRYFKFRT
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Appendix 4 (b)

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                              1                                                            75
       CYP51A         (1)  MLGIESVLVSSIRYGFYTLTLLIVTVLLNALHQLLPKKKSEPPLVWHWIPFIGNAIEYGTDPLAFYTKCQRQHGP
       K1124          (1)  ----------------------------PPHALHQILPKKKSEPPLVWHWIPFIGNAIEYGTDPLAFYTKCQRHHGP
       FI12-63        (1)  -------------------------SILLNALHQILQKKKSEPPLVWHWIPFIGNAIEYGTDPLAFYTKCQRHHGP
       788            (1)  -------------------------YSPAHALHQLLPKKKSEPPLVWHWIPFIGNAIEYGTDPLAFYTKCQRQHGP
SAC 0003 1.4.8 00 (10) (1)  -------------------------SVPAHALHQLLPKKKSEPPLVWHWIPFIGNAIEYGTDPLAFYTKCQRQHGP
       QUB 30-10      (1)  --------------------------GPAHALHQLLPKKKSEPPLVWHWIPFIGNAIEYGTDPLAFYTKCQRQHGP
       QUB 12-3       (1)  ----------------------------LLNALHQLLPKKKSEPPLVWHWIPFIGNAIEYGTDPLAFYTKCQRQHGP
       R 9528.4       (1)  ----------------------------PAHALHQLLPKKKSEPPLVWHWIPFIGNAIEYGTDPLAFYTKCQRQHGP
       R 9522.3       (1)  ----------------------------LAHALHQLLPKKKSEPPLVWHWIPFIGNAIEYGTDPLAFYTKCQRQHGP
       GKII 18-2-3    (1)  -----------------------------LHALHQLLPKKKSEPPLVWHWIPFIGNAIEYGTDPLAFYTKCQRQHGP
       GKII 18-3-2    (1)  ----------------------------SPAHALHQLLPKKKSEPPLVWHWIPFIGNAIEYGTDPLAFYTKCQRQHGP
       OSA 28-2-2     (1)  -------------------------SVPAHALHQLLPKKKSEPPLVWHWIPFIGNAIEYGTDPLAFYTKCQRQHGP
       SAC 09/943/14  (1)  ----------------------------LLNALHQLLPKKKSEPPLVWHWIPFIGNAIEYGTDPLAFYTKCQRHHGP
       RS 219         (1)  -------------------------QVLLNALHQLLPKKKSEPPLVWHWIPFIGNAIEYGTDPLAFYTKCQRQHGP
       RS 783         (1)
       Consensus      (1)                       HALHQLLPKKKSEPPLVWHWIPFIGNAIEYGTDPLAFYTKCQRQHGP


                              76                                                           150
       CYP51A        (76)  IFTYILFGKKMTVYLGLEGNEFILNGRLQDVNAEDIY----------GPLTTPVFGPNVIYDCPNSKLMEQKKFVKFGLTQR
       K1124         (50)  IFTYILFGRKMTVYLGLEVN--LSSMVDCRMSMQKIFMVH--------
       FI12-63       (52)  IFTHILFGRKMTVYLGLEVN--LSSMVDCRMSMQKIFMVH--------
       788           (52)  IFTYILFGKKMTVYLGLEGNEFILNGRLQDVNAEDIY----------GPLTTPVFGPNVIYDCPNSKLMEQKKFVKFGLTQR
SAC 0003 1.4.8 00 (10)(52) IFTYILFGKKMTVYLGLEGNEFILNGRLQDVNAEDIY----------GPLTTPVFGPNVIYDCPNSKLMEQKKFVKFGLTQR
       QUB 30-10     (51)  IFTYILFGKKMTVYLGLEGNEFILNGRLQDVNAEDIY----------GPLTTPVFGPNVIYDCPNSKLMEQKKFVKFGLTQR
       QUB 12-3      (50)  IFTYILFGKKMTVYLGLEGNEFILNGRLQDVNAEDIY----------GPLTTPVFGPNVIYDCPNSKLMEQKKFVKFGLTQR
       R 9528.4      (50)  IFTYILFGKKMTVYLGLEGNEFILNGRLQDVNAEDIY----------GPLTTPVFGPNVIYDCPNSKLMEQKKFVKFGLTQR
       R 9522.3      (50)  IFTYILFGKKMTVYLGLEGNEFILNGRLQDVNAEDIY----------GPLTTPVFGPNVIYDCPNSKLMEQKKFVKFGLTQR
       GKII 18-2-3   (49)  IFTYILFGKKMTVYLGLEGNEFILNGRLQDVNAEDIY----------GPLTTPVFGPNVIYDCPNSKLMEQKKFVKFGLTQR
       GKII 18-3-2   (51)  IFTYILFGKKMTVYLGLEGNEFILNGRLQDVNAEDIY----------GPLTTPVFGPNVIYDCPNSKLMEQKKFVKFGLTQR
       OSA 28-2-2    (52)  IFTYILFGKKMTVYLGLEGNEFILNGRLQDVNAEDIY----------GPLTTPVFGPNVIYDCPNSKLMEQKKFVKFGLTQR
       SAC 09/943/14 (50)  IFTYILFGRKMTVYLGLEVN--LSSMVDCRMSMQKIFMVH--------
       RS 219        (40)  IFTHILFGRKMTVYLGLEGNEFILNGRLQDVNAEDIY----------GPLTTPVFGPNVIYDCPNSKLMEQKKFVKFGLTQR
       RS 783        (52)  IFTYILFGKKMTVYLGLEGNEFILNGRLQDVNAEDIY----------GPLTTPVFGPNVIYDCPNSKLMEQKKFVKFGLTQR
       Consensus     (76)  IFTYILFGKKMTVYLGLEGNEFILNGRLQDVNAEDIYGPLTTPVFGPNVIYDCPNSKLMEQKKFVKFGLTQR
```

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                              151                                                                        225
         CYP51A  (148)        ALEQHVPLIENEVLSYMASSPSFIPQSPTSPSSSDQSTTKMEQKLQTAIIDLPHTMAQITIFTAGRALQGPEVRT
          K1124   (88)        -------------------------------------------------------------------------
        FI12-63   (90)        -------------------------------------------------------------------------
            788  (124)        ALEQHVPLIENEVLSYMASSPSFIPQSPTSPSSSDQSTTKMEQKLQTAIIDLPHTMAQITIFTAGRALQGPEVRT
SAC 0003 1.4.8 00 (10) (124)  ALEQHVPLIENEVLSYMASSPSFIPQSPTSPSSSDQSTTKMEQKLQTAIIDLPHTMAQITIFTAGRALQGPEVRT
       QUB 30-10  (123)       ALEQHVPLIENEVLSYMASSPSFIPQSPTSPSSSDQSTTKMEQKLQTAIIDLPHTMAQITIFTAGRALQGPEVRT
        QUB 12-3  (122)       ALEQHVPLIENEVLSYMASSPSFIPQSPTSPSSSDQSTTKMEQKLQTAIIDLPHTMAQITIFTAGRALQGPEVRT
        R 9528.4  (122)       ALEQHVPLIENEVLSYMASSPSFIPQSPTSPSSSDQSTTKMEQKLQTAIIDLPHTMAQITIFTAGRALQGPEVRT
        R 9522.3  (122)       ALEQHVPLIENEVLSYMASSPSFI[S]QSPTSPSSSDQSTTKMEQKLQTAIIDLPHTMAQITIFTAGRALQGPEVRT
     GKII 18-2-3  (121)       ALEQHVPLIENEVLSYMASSPSFIPQSPTSPSSSDQSTTKMEQKLQTAIIDLPHTMAQITIFTAGRALQGPEVRT
     GKII 18-3-2  (123)       ALEQHVPLIENEVLSYMASSPSFIPQSPTSPSSSDQSTTKMEQKLQTAIIDLPHTMAQITIFTAGRALQGPEVRT
      OSA 28-2-2  (124)       ALEQHVPLIENEVLSYMASSPSFIPQSPTSPSSSDQSTTKMEQKLQTAIIDLPHTMAQITIFTAGRALQGPEVRT
   SAC 09/943/14  (122)       ALEQHVPLIENEVLSYMASSPSFIPQSPTSPSSSDQSTTKMEQKLQTAIIDLPHTMAQITIFTAGRALQGPEVRT
          RS 219   (78)       -------------------------------------------------------------------------
          RS 783  (124)       ALEQHVPLIENEVLSYMASSPSFIPQSPTSPSSSDQSTTKMEQKLQTAIIDLPHTMAQITIFTAGRALQGPEVRT
       Consensus  (151)       ALEQHVPLIENEVLSYMASSPSFIPQSPTSPSSSDQSTTKMEQKLQTAIIDLPHTMAQITIFTAGRALQGPEVRT


                              226                                                                        300
         CYP51A  (223)        KLTDEFAGLYDDLDHGFRPINFLAPWLPLPQNWRRDKAHAKMMSVYMDIISRRREKSGGGVVGRGMEREEIDMID
          K1124   (88)        -------------------------------------------------------------------------
        FI12-63   (90)        -------------------------------------------------------------------------
            788  (199)        KLTDEFAGLYDDLDHGFRPINFLAPWLPLPQNWRRDKAHAKMMSVYMDIISRRREKSGGGVVGRGMEREEIDMID
SAC 0003 1.4.8 00 (10) (199)  KLTDEFAGLYDDLDHGFRPINFLAPWLPLPQNWRRDKAHAKMMSVYMDIISRRREKSGGGVVGRGMEREEIDMID
       QUB 30-10  (198)       KLTDEFAGLYDDLDHGFRPINFLAPWLPLPQNWRRDKAHAKMMSVYMDIISRRREKSGGGVVGRGMEREEIDMID
        QUB 12-3  (197)       KLTDEFAGLYDDLDHGFRPINFLAPWLPLPQNWRRDKAHAKMMSVYMDIISRRREKSGGGVVGRGMEREEIDMID
        R 9528.4  (197)       KLTDEFAGLYDDLDHGFRPINFLAPWLPLPQNWRRDKAHAKMMSVYMDIISRRREKSGGGVVGRGMEREEIDMID
        R 9522.3  (197)       KLTDEFAGLYDDLDHGFRPINFLAPWLPLPQNWRRDKAHAKMMSVYMDIISRRREKSGGGVVGRGMEREEIDMID
     GKII 18-2-3  (196)       KLTDEFAGLYDDLDHGFRPINFLAPWLPLPQNWRRDKAHAKMMSVYMDIISRRREKSGGGVVGRGMEREEIDMID
     GKII 18-3-2  (198)       KLTDEFAGLYDDLDHGFRPINFLAPWLPLPQNWRRDKAHAKMMSVYMDIISRRREKSGGGVVGRGMEREEIDMID
      OSA 28-2-2  (199)       KLTDEFAGLYDDLDHGFRPINFLAPWLPLPQNWRRDKAHAKMMSVYMDIISRRREKSGGGVVGRGMEREEIDMID
   SAC 09/943/14  (197)       KLTDEFAGLYDDLDHGFRPINFLAPWLPLPQNWRRDKAHAKMMSVYMDIISRRREKSGGGVVGRGMEREEIDMID
          RS 219   (78)       -------------------------------------------------------------------------
          RS 783  (199)       KLTDEFAGLYDDLDHGFRPINFLAPWLPLPQNWRRDKAHAKMMSVYMDIISRRREKSGGGVVGRGMEREEIDMID
       Consensus  (226)       KLTDEFAGLYDDLDHGFRPINFLAPWLPLPQNWRRDKAHAKMMSVYMDIISRRREKSGGGVVGRGMEREEIDMID
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375

CYP51A	(298)	NLMTCVYKSGETIPDSEIACMMITILMAGQHSSSSSSCWIMLHLASRPDLQEELYREQQDANLYLAGNRGLQYQD
K1124	(88)	---
FI12-63	(90)	---
SAC 0003 1.4.8 00 (10) 788	(274)	NLMTCVYKSGETIPDSEIACMMITILMAGQHSSSSSSCWIMLHLASRPDLQEELYREQQDANLYLAGNRGLQYQD
QUB 30-10	(274)	NLMTCVYKSGETIPDSEIACMMITILMAGQHSSSSSSCWIMLHLASRPDLQEELYREQQDANLYLAGNRGLQYQD
QUB 12-3	(273)	NLMTCVYKSGETIPDSEIACMMITILMAGQHSSSSSSCWIMLHLASRPDLQEELYREQQDANLYLAGNRGLQYQD
R 9528.4	(272)	NLMTCVYKSGETIPDSEIACMMITILMAGQHSSSSSSCWIMLHLASRPDLQEELYREQQDANLYLAGNRGLQYQD
R 9522.3	(272)	NLMTCVYKSGETIPDSEIACMMITILMAGQHSSSSSSCWIMLHLASRPDLQEELYREQQDANLYLAGNRGLQYQD
GKII 18-2-3	(271)	NLMTCVYKSGETIPDSEIACMMITILMAGQHSSSSSSCWIMLHLASRPDLQEELYREQQDANLYLAGNRGLQYQD
GKII 18-3-2	(273)	NLMTCVYKSGETIPDSEIACMMITILMAGQHSSSSSSCWIMLHLASRPDLQEELYREQQDANLYLAGNRGLQYQD
OSA 28-2-2	(274)	NLMTCVYKSGETIPDSEIACMMITILMAGQHSSSSSSCWIMLHLASRPDLQEELYREQQDANLYLAGNRGLQYQD
SAC 09/943/14	(272)	NLMTCVYKSGETIPDSEIACMMITILMAGQHSSSSSSCWIMLHLASRPDLQEELYREQQDANLYLAGNRGLQYQD
RS 219	(78)	---
RS 783	(274)	NLMTCVYKSGETIPDSEIACMMITILMAGQHSSSSSSCWIMLHLASRPDLQEELYREQQDANLYLAGNRGLQYQD
Consensus	(301)	NLMTCVYKSGETIPDSEIACMMITILMAGQHSSSSSSCWIMLHLASRPDLQEELYREQQDANLYLAGNRGLQYQD

450

CYP51A	(373)	LEKLKLLSDVVKETLRLHSSIHSVMRAVKNDLPIPGTPYVVTTDKVLIASPLVTHLSPEHFSDPEEWDPYRWATP
K1124	(88)	---
FI12-63	(90)	---
SAC 0003 1.4.8 00 (10) 788	(349)	LEKLKLLSDVVKETLRLHSSIHSVMRAVKNDLPIPGTPYVVTTDKVLIASPLVTHLSPEHFSDPEEWDPYRWATP
QUB 30-10	(349)	LEKLKLLSDVVKETLRLHSSIHSVMRAVKNDLPIPGTPYVVTTDKVLIASPLVTHLSPEHFSDPEEWDPYRWATP
QUB 12-3	(348)	LEKLKLLSDVVKETLRLHSSIHSVMRAVKNDLPIPGTPYVVTTDKVLIASPLVTHLSPEHFSDPEEWDPYRWATP
R 9528.4	(347)	LEKLKLLSDVVKETLRLHSSIHSVMRAVKNDLPIPGTPYVVTTDKVLIASPLVTHLSPEHFSDPEEWDPYRWATP
R 9522.3	(347)	LEKLKLLSDVVKETLRLHSSIHSVMRAVKNDLPIPGTPYVVTTDKVLIASPLVTHLSPEHFSDPEEWDPYRWATP
GKII 18-2-3	(346)	LEKLKLLSDVVKETLRLHSSIHSVMRAVKNDLPIPGTPYVVTTDKVLIASPLVTHLSPEHFSDPEEWDPYRWATP
GKII 18-3-2	(348)	LEKLKLLSDVVKETLRLHSSIHSVMRAVKNDLPIPGTPYVVTTDKVLIASPLVTHLSPEHFSDPEEWDPYRWATP
OSA 28-2-2	(349)	LEKLKLLSDVVKETLRLHSSIHSVMRAVKNDLPIPGTPYVVTTDKVLIASPLVTHLSPEHFSDPEEWDPYRWATP
SAC 09/943/14	(347)	LEKLKLLSDVVKETLRLHSSIHSVMRAVKNDLPIPGTPYVVTTDKVLIASPLVTHLSPEHFSDPEEWDPYRWATP
RS 219	(78)	---
RS 783	(349)	LEKLKLLSDVVKETLRLHSSIHSVMRAVKNDLPIPGTPYVVTTDKVLIASPLVTHLSPEHFSDPEEWDPYRWATP
Consensus	(376)	LEKLKLLSDVVKETLRLHSSIHSVMRAVKNDLPIPGTPYVVTTDKVLIASPLVTHLSPEHFSDPEEWDPYRWATP

376

301

250

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                                451                                                          525
          CYP51A      (448)     GDIEKDEEVDYGYGVISKGTRSPYLPFGAGRHRCIGEKFAFVNLLTIIATLVRGYKWSLVPGGEEGGQIPKTDYS
           K1124       (88)     ---------------------------------------------------------------------------
         FI12-63       (90)     ---------------------------------------------------------------------------
             788               ----------------------------------------------------------------------------
SAC 0003 1.4.8 00 (10)  (424)    GDIEKDEEVDYGYGVISKGTRSPYLPFGAGRHRCIGEKFAFVNLLTIIATLVRGYK-------------------
        QUB 30-10      (424)     GDIEKDEEVDYGYGVISKGTRSPYLPFGAGRHRCIGEKFAFVNLLTIIATLVRGYKWS------------------
         QUB 12-3      (423)     GDIEKDEEVDYGYGVISKGTRSPYLPFGAGRHRCIGEKFAFVNLLTIIATLVRGYK-------------------
         R 9528.4      (422)     GDIEKDEEVDYGYGVISKGTRSPYLPFGAGRHRCIGEKFAFVNLLTIIATLVRGYKWSL-----------------
         R 9522.3      (422)     GDIEKDEEVDYGYGVISKGTRSPYLPFGAGRHRCIGEKFAFVNLLTIIATLVRGYKWS------------------
      GKII 18-2-3      (421)     GDIEKDEEVDYGYGVISKGTRSPYLPFGAGRHRCIGEKFAFVNLLTIIATLVRGYKWS------------------
      GKII 18-3-2      (423)     GDIEKDEEVDYGYGVISKGTRSPYLPFGAGRHRCIGEKFAFVNLLTIIATLVRGYKWSL-----------------
       OSA 28-2-2      (424)     GDIEKDEEVDYGYGVISKGTRSPYLPFGAGRHRCIGEKFAFVNLLTIIATLVRGYKWS------------------
    SAC 09/943/14      (422)     GDIEKDEEVDYGYGVISKGTRSPYLPFGAGRHRCIGEKFAFVNLLTIIATLVRGYKWS------------------
           RS 219       (78)     ---------------------------------------------------------------------------
           RS 783      (424)     GDIEKDEEVDYGYGVISKGTRSPYLPFGAGRHRCIGEKFAFVNLLTIIATLVRGYKWS------------------
        Consensus      (451)     GDIEKDEEVDYGYGVISKGTRSPYLPFGAGRHRCIGEKFAFVNLLTIIATLVRGYKWS


                                526       543
          CYP51A      (523)     SMVSRPMPGSRVRWEKRV
           K1124       (88)     ------------------
         FI12-63       (90)     ------------------
             788      (480)     ------------------
SAC 0003 1.4.8 00 (10)  (482)    ------------------
        QUB 30-10      (479)     ------------------
         QUB 12-3      (481)     ------------------
         R 9528.4      (480)     ------------------
         R 9522.3      (480)     ------------------
      GKII 18-2-3      (480)     ------------------
      GKII 18-3-2      (481)     ------------------
       OSA 28-2-2      (482)     ------------------
    SAC 09/943/14      (480)     ------------------
           RS 219       (78)     ------------------
           RS 783      (482)     ------------------
        Consensus      (526)
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                            1                                                                           92
CYP51A           (1)   ATGCTGGGTATCTTCTCGGTGCTAGTTTCTAGCATCCGCTATGGATTCTACACCCTCACTCTCCTTATTGTGCAGTCCTGCTCAACGCTCT
CYP51A-p: K1124  (1)   ------------------------------------------------------------------------CCTCCTC-ACGCTCT
FI12-63          (1)   ------------------------------------------------------------------------TCAATCCTCCTCAACGCTCT
788              (1)   -------------------------
QUB 30-10        (1)   -------------------------
QUB 12-3         (1)   ----------AGGGCGGATTGGGCCGACGTCGCATGCTCCGGCCGCCATGGC
R 9528.4         (1)   -------------------------
GKII 18-2-3      (1)   -------------------------
RS 219           (1)   -------------------------
RS 783           (1)   -------------------------
Consensus        (1)   -------------------------


                            93                                                                          184
CYP51A           (93)  ACACCAACTCCTCCCAAAGAAGAAGTCCGAACCGCCTTGGTATGGCATTGATGGATCCCATTTATCGGAATGCCATCGGAATGTGAATCTCCCT
K1124            (15)  ACACCAAATCCTCCCAAAGAAGAAGTCCGAACCGCCTTGGTATGGCATTGATGGATCCCCTTTATTGGGAATGCCATCGAATGTGAATCTCCCT
FI12-63          (21)  ACACCAAATCCTCCCAAAGAAGAAGTCCGAACCGCCTTGGTATGGCATTGATGGATCCCCTTTATTGGGAATGCCATCGAATGTGAATCTCCCT
788              (1)   -------TAAAAGAAGAGAGTCCGAACCGCCTTGGTATGGCATTGATGGATCCCCTTTATTGGGAATGCCATCGAATGTGAATCTCCCT
QUB 30-10        (1)   -------TAAAAGAAGAGAGTCCGAACCGCCTTGGTATGGCATTGATGGATCCCCTTTATTGGGAATGCCATCGAATGTGAATCTCCCT
QUB 12-3         (44)  GGCCGCGGGAATTCGATTAAAGAAGTCCGAACCGCCTTGGTATGGCATTGATGGATCCCCTTTATTGGGAATGCCATCGAATGTGAATCTCCCT
R 9528.4         (1)   -------TAAAGAAGAAGTCCGAACCGCCTTGGTATGGCATTGATGGATCCCCTTTATTGGGAATGCCATCGAATGTGAATCTCCCT
GKII 18-2-3      (1)   ----------TAGTCCGAACCGCCTTGGTATGGCATTGATGGATCCCCTTTATTGGGAATGCCATCGAATGTGAATCTCCCT
RS 219           (1)   -------TAAAAGAAGAAGTCCGAACCGCCTTGGTATGGCATTGATGGATCCCCTTTATTGGGAATGCCATCGAATGTGAATCTCCCT
RS 783           (1)   -------TAAAAGAAGAAGTCCGAACCGCCTTGGTATGGCATTGATGGATCCCCTTTATTGGGAATGCCATCGAATGTGAATCTCCCT
Consensus        (93)  AAAAGAAGAAGTCCGAACCGCCTTGGTATGGCATTGATGGATCCCCTTATTGGGAATGCCATCGAATGTGAATCTCCCT


                            185                                                                         276
CYP51A           (185) TTTCCTCCTTCCTAACCCTCTTGATCTTTCTTTCTCACCCTCCTCCTTCCTCACCCTATTATCACCCCCTCC-ATTATCACATTCAGTAG
K1124            (107) TTTACTCCTTCCTAACCCTCTTGATCTTTCTTTCTCACCCTCCTCCTTCCTCACCCCCC-CATTATCAACACCCCC-CATTACATTCAGTAG
FI12-63          (113) TTTACTCCTTCCTAACCCTCTTGATCTTTCTTTCTCACCCTCCTCCTTCCTCACCCCCCCCATTATCAACATCCCCCCATTACATTCAGTAG
788              (81)  TTTACTCCTTCCTAACCCTCTTGATCTTTCTTTCTCACCCTCCTCCTTCCTCACCCCCCCCATTATCAACATCCCCCCATTACATTCAGTAG
QUB 30-10        (81)  TTTACTCCTTCCTAACCCTCTTGATCTTTCTTTCTCACCCTCCTCCTTCCTCACCCCCCCCATTATCAACATCCCCCCATTACATTCAGTAG
QUB 12-3         (136) TTTACTCCTTCCTAACCCTCTTGATCTTTCTTTCTCACCCTCCTCCTTCCTCACCCCCCCCATTATCAACACCCCCCCATTACATTCAGTAG
R 9528.4         (80)  TTTACTCCTTCCTAACCCTCTTGATCTTTCTTTCTCACCCTCCTCCTTCCTCACCCCCCCCATTATCAACATCACCCCCATTACATTCAGTAG
GKII 18-2-3      (71)  TTTACTCCTTCCTAACCCTCTTGATCTTTCTTTCTCACCCTCCTCCTTCCTCACCCCCCCCATTATCAACATCACCCCCATTACATTCAGTAG
RS 219           (80)  TTTACTCCTTCCTAACCCTCTTGATCTTTCTTTCTCACCCTCCTCCTTCCTCACCCCCCCCATTATCAACATCACCCCCATTACATTCAGTAG
RS 783           (79)  TTTACTCCTTCCTAACCCTCTTGATCTTTCTTTCTCACCCTCCTCCTTCCTCACCCCCCCCATTATCAACATCACCCCCATTATCACATTCAGTAG
Consensus        (185) TTTACTCCTTCCTAACCCTCTTGATCTTTCTTTCTCACCCTCCTCCTTCCTCACCCCCCCCATTATCAACATTCAACATTCAGTAG
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DNA sequence alignment (page 253)

Block 1 — positions 277–368

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                  277                                                                           368
CYP51A      (276) AGCAAAGATACTGACATGCAACCATTTTCTAGATGGCACCGATCCTCTAGCTTTCTACACAAAATGTCAACGGCACCATGGCCGATCTT
K1124       (198) AGCAAAAGTTACTGACATGCAACCGTTTCTCTAAATGGCACCGATCCTCTAGCTTTCTACACAAAATGTCAACGGCACCATGGCCGATCTT
FI12-63     (205) AGCAAAAGTTACTGACATGCAACCGTTTCTCTAGATGGCACCGATCCTCTAGCTTTCTACACAAAATGTCAACGGCACCATGGCCGATCTT
788         (173) AGCAAAAGTTACTGACATGCAACCGTTTCTCTAGATGGCACCGATCCTCTAGCTTTCTACACAAAATGTCAACGGCACCATGGCCGATCTT
QUB 30-10   (173) AGCAAAAGTTACTGACATGCAACCGTTTCTCTAGATGGCACCGATCCTCTAGCTTTCTACACAAAATGTCAACGGCACCATGGCCGATCTT
QUB 12-3    (228) AGCAAAAGTTACTGACATGCAACCGTTTCTCTAGATGGCACCGATCCTCTAGCTTTCTACACAAAATGTCAACGGCACCATGGCCGATCTT
R 9528.4    (172) AGCAAAAGTTACTGACATGCAACCGTTTCTCTAGATGGCACCGATCCTCTAGCTTTCTACACAAAATGTCAACGGCACCATGGCCGATCTT
GKII 18-2-3 (163) AGCAAAAGTTACTGACATGCAACCGTTTCTCTAGATGGCACCGATCCTCTAGCTTTCTACACAAAATGTCAACGGCACCATGGCCGATCTT
RS 219      (172) AGCAAAAGTTACTGACATGCAACCGTTTCTCTAGATGGCACCGATCCTCTAGCTTTCTACACAAAATGTCAACGGCACCATGGCCGATCTT
RS 783      (171) AGCAAAAGTTACTGACATGCAACCGTTTCTCTAGATGGCACCGATCCTCTAGCTTTCTACACAAAATGTCAACGGCACCATGGCCGATCTT
Consensus   (277) AGCAAAAGTTACTGACATGCAACCGTTTCTCTAGATGGCACCGATCCTCTAGCTTCTACACAAAATGTCAACGGCACCATGGCCGATCTT
```

Block 2 — positions 369–460

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                  369                                                                    460
CYP51A      (368) CACTTATATTCTTTTCGGCAAAAAAATGACTGTTTATCTTGGGCTGGAAGGAAAGTTTATCCTCAATGATGAGAGATGTCAACG
K1124       (290) CACTTATATTCTTTTCGGCAAAAAAATGACTGTTTATCTTGGGCTGGAAG---------TGAATTTATCCTCAATGGTGAGAGATGTCAATG
FI12-63     (297) CACTCATATTCTTTTCGGCAAAAAAATGACTGTTTATCTTGGGCTGGAAG---------TGAATTTATCCTCAATGGTGAGAGATGTCAATG
788         (265) CACTCATATTCTTTTCGGCAAAAAAATGACTGTTTATCTTGGGCTGGAAG---------TGAATTTATCCTCAATGGTGAGAGATGTCAATG
QUB 30-10   (265) CACTCATATTCTTTTCGGCAAAAAAATGACTGTTTATCTTGGGCTGGAAG---------TGAATTTATCCTCAATGGTGAGAGATGTCAATG
QUB 12-3    (320) CACTCATATTCTTTTCGGCAAAAAAATGACTGTTTATCTTGGGCTGGAAG---------TGAATTTATCCTCAATGGTGAGAGATGTCAATG
R 9528.4    (264) CACTCATATTCTTTTCGGCAAAAAAATGACTGTTTATCTTGGGCTGGAAG---------TGAATTTATCCTCAATGGTGAGAGATGTCAATG
GKII 18-2-3 (255) CACTCATATTCTTTTCGGCAAAAAAATGACTGTTTATCTTGGGCTGGAAG---------TGAATTTATCCTCAATGGTGAGAGATGTCAATG
RS 219      (264) CACTCATATTCTTTTCGGCAAAAAAATGACTGTTTATCTTGGGCTGGAAG---------TGAATTTATCCTCAATGGTGAGAGATGTCAATG
RS 783      (263) CACTCATATTCTTTTCGGCAAAAAAATGACTGTTTATCTTGGGCTGGAAG---------TGAATTTATCCTCAATGGTGAGAGATGTCAATG
Consensus   (369) CACTCATATTCTTTTCGGCAGAAAAATGACTGTTTATCTTGGGCTGGAAG         TGAATTTATCCTCAATGGTGAGAGATGTCAATG
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Block 3 — positions 461–552

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                  461                                                                     552
CYP51A      (460) CGGAAGATATTTATGGTCCACTGAGTGTGTTCTCCCCTATCCTCCACTCTCTGAAATCGCTCAGCCTTCTA
K1124       (378) CGGAAGATATTTATGGTCCACTGAGTGTGTAATTCCCCTTCCTCCACATCCAGTCTCCTTGAGATCGCTAAGCTCCA
FI12-63     (385) CAGAAGATATTTATGGTCCACTGAGTGTGTAATTCCCCTTCCTCCACATCCAGTCTCCTTGAGATCGCTAAGCTCCA
788         (353) CAGAAGATATTTATGGTCCACTGAGTGTGTAATTCCCCTTCCTCCACATCCAGTCTCCTTGAGATCGCTAAGCTCCA
QUB 30-10   (353) CAGAAGATATTTATGGTCCACTGAGTGTGTAATTCCCCTTCCTCCACATCCAGTCTCCTTGAGATCGCTAAGCTCCA
QUB 12-3    (408) CAGAAGATATTTATGGTCCACTGAGTGTGTAATTCCCCTTCCTCCACATCCAGTCTCCTTGAGATCGCTAAGCTCCA
R 9528.4    (352) CAGAAGATATTTATGGTCCACTGAGTGTGTAATTCCCCTTCCTCCACATCCAGTCTCCTTGAGATCGCTAAGCTCCA
GKII 18-2-3 (343) CAGAAGATATTTATGGTCCACTGAGTGTGTAATTCCCCTTCCTCCACATCCAGTCTCCTTGAGATCGCTAAGCTCCA
RS 219      (352) CAGAAGATATTTATGGTCCACTGAGTGTGTAATTCCCCTTCCTCCACATCCAGTCTCCTTGAGATCGCTAAGCTCCA
RS 783      (351) CAGAAGATATTTATGGTCCACTGAGTGTGTAATTCCCCTTCCTCCACATCCAGTCTCCTTCACATCAGTCTCCTTGAGATCGCTAAGCTCCA
Consensus   (461) CAGAAGATATTTATGGTCCACTGAGTGTGTAATTCCCCTTCCTCCACATCCAGTCTCTTGAGATGCTCAAGCTCTCCA
```

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Block 1 (positions 553–644):

Label	(start)
CYP51A	(552)
K1124	(470)
FI12-63	(477)
788	(445)
QUB 30-10	(445)
QUB 12-3	(500)
R 9528.4	(444)
GKII 18-2-3	(435)
RS 219	(444)
RS 783	(443)
Consensus	(553)

Block 2 (positions 645–736):

Label	(start)
CYP51A	(644)
K1124	(562)
FI12-63	(569)
788	(537)
QUB 30-10	(537)
QUB 12-3	(592)
R 9528.4	(536)
GKII 18-2-3	(527)
RS 219	(536)
RS 783	(535)
Consensus	(645)

Block 3 (positions 737–828):

Label	(start)
CYP51A	(736)
K1124	(654)
FI12-63	(661)
788	(629)
QUB 30-10	(629)
QUB 12-3	(684)
R 9528.4	(628)
GKII 18-2-3	(619)
RS 219	(628)
RS 783	(627)
Consensus	(737)

Sequence alignment (positions 828–920, 920–1012, 1012–1104)

Block 1 (positions 829–920)

Label	Pos	Sequence
CYP51A	(828)	CTTCCTCCGACCAAGCACAACCAAATGGAACAGCTCAAACAGGCCATAATAGACTGGCTCACACCATGGCTCAGATCACAATCTTC
K1124	(746)	CTTGCTCTAACCAAGGCATAACCAAAACGGAACAAAAACTTCAAACAGCCATAGTAGACCTTCCCATACCATGTCTCAGATCACAATCTTC
FI12-63	(753)	CTTGCTCTAACCAAGGCATAACCAAAACGGAACAAAAACTTCAAACAGCCATAGTAGACCTTCCCATACCATGTCTCAGATCACAATCTTC
788	(721)	CTTGCTCTAACCAAGGCATAACCAAAACGGAACAAAAACTTCAAACAGCCATAGTAGACCTTCCCATACCATGTCTCAGATCACAATCTTC
QUB 30-10	(721)	CTTGCTCTAACCAAGGCATAACCAAAACGGAACAAAAACTTCAAACAGCCATAGTAGACCTTCCCATACCATGTCTCAGATCACAATCTTC
QUB 12-3	(776)	CTTGCTCTAACCAAGGCATAACCAAAACGGAACAAAAACTTCAAACAGCCATAGTAGACCTTCCCATACCATGTCTCAGATCACAATCTTC
R 9528.4	(720)	CTTGCTCTAACCAAGGCATAACCAAAACGGAACAAAAACTTCAAACAGCCATAGTAGACCTTCCCATACCATGTCTCAGATCACAATCTTC
GKII 18-2-3	(711)	CTTGCTCTAACCAAGGCATAACCAAAACGGAACAAAAACTTCAAACAGCCATAGTAGACCTTCCCATACCATGTCTCAGATCACAATCTTC
RS 219	(720)	CTTGCTCTAACCAAGGCATAACCAAAACGGAACAAAAACTTCAAACAGCCATAGTAGACCTTCCCATACCATGTCTCAGATCACAATCTTC
RS 783	(719)	CTTGCTCTAACCAAGGCATAACCAAAACGGAACAAAAACTTCAAACAGCCATAGTAGACCTTCCCATACCATGTCTCAGATCACAATCTTC
Consensus	(829)	CTTGCTCTAACCAAGGCATAACCAAAACGGAACAAAAACTTCAAACAGCCATAGTAGACCTTCCCATACCATGTCTCAGATCACAATCTTC

Block 2 (positions 921–1012)

Label	Pos	Sequence
CYP51A	(920)	ACAGCCGGTCGCGCTCTTCAAGGCCCGGAGTAAACTTCACAGACGAATTCGCGATTTGTATGATGATCTCGACCACGGTTCCG
K1124	(838)	ACAGCTGGTCGCGCTCTTCAAGGCCCGGAGTAAACTCACAGAGCAATTCGGATTTGTATGATGATCTCGACCACGGTTCG
FI12-63	(845)	ACAGCTGGTCGCGCTCTTCAAGGCCCGGAGAGACTCACAGAGCAATTCGGATTTGTATGATGATCTCGACCACGGTTCG
788	(813)	ACAGCTGGTCGCGCTCTTCAAGGCCCGGAGAGACTCACAGAGCAATTCGGATTTGTATGATGATCTCGACCACGGTTCG
QUB 30-10	(813)	ACAGCTGGTCGCGCTCTTCAAGGCCCGGAGAGACTCACAGAGCAATTCGGATTTGTATGATGATCTCGACCACGGTTCG
QUB 12-3	(868)	ACAGCTGGTCGCGCTCTTCAAGGCCCGGAGAGACTCACAGAGCAATTCGGATTTGTATGATGATCTCGACCACGGTTTCG
R 9528.4	(812)	ACAGCTGGTCGCGCTCTTCAAGGCCCGGAGAGACTCACAGAGCAATTCGGATTTGTATGATGATCTCGACCACGGTTCG
GKII 18-2-3	(803)	ACAGCTGGTCGCGCTCTTCAAGGCCCGGAGAGACTCACAGACTAATTCGGATTTGTATGATGATCTCGACCACGGTTCG
RS 219	(812)	ACAGCTGGTCGCGCTCTTCAAGGCCCGGAGAGACTCACAGACTAATTCGGATTTGTATGATGATCTCGACCACGGTTCG
RS 783	(811)	ACAGCTGGTCGCGCTCTTCAAGGCCCGGAGAGACTCACAGACTAATTCGGATTTGTATGATGATCTCGACCACGGTTCG
Consensus	(921)	ACAGCTGGTCGCGCTCTTCAAGGCCCGGAGAGACTCACAGACTAATTCGCGGATTTGTATGATGATCTCGACCACGGTTCG

Block 3 (positions 1013–1104)

Label	Pos	Sequence
CYP51A	(1012)	GCCAATCAACTTCCTGCTCCGTGGTTACCACTGCCTCAGAACTGGAGAAGGGATAAAGCACATGCAAAGATGAGTGTATATGGACA
K1124	(930)	GCCAATCAACTTCCTGCTCCGTGCTACCGCCTCAGAACTGGAGAAGAGATAAAGCACATGCAAAGATGAGTGTATATGGACA
FI12-63	(937)	GCCAATCAACTTCCTGCTCCGTGCGTACCGCCTCAGAACTGGAGAAGAGATAAAGCACATGCAAAGATGAGTGTATATGGACA
788	(905)	GCCAATCAACTTCCTGCTCCGTGCGTACCGCCTCAGAACTGGAGAAGAGATAAAGCACATGCAAAGATGAGTGTATATGGACA
QUB 30-10	(905)	GCCAATCAACTTCCTGCTCCGTGCCTACCGCCTCAGAACTGGAGAAGAGATAAAGCACATGCAAAGATGAGTGTATATGGACA
QUB 12-3	(960)	GCCAATCAACTTCCTGCTCCGTGCCTACCGCCTCAGAACTGGAGAAGAGATAAAGCACATGCAAAGATGAGTGTATATGGACA
R 9528.4	(904)	GCCAATCAACTTCCTGCTCCGTGCCTACCGCCTCAGAACTGGAGAAGAGATAAAGCACATGCAAAGATGAGTGTATATGGACA
GKII 18-2-3	(895)	GCCAATCAACTTCCTGCTCCGTGCCTACCGCCTCAGAACTGGAGAAGAGATAAAGCACATGCAAAGATGAGTGTATATGGACA
RS 219	(904)	GCCAATCAACTTCCTGCTCCGTGCCTACCGCCTCAGAACTGGAGAAGAGATAAAGCACATGCAAAGATGAGTGTATATGGACA
RS 783	(903)	GCCAATCAACTTCCCGCTCCGTGCCTACCGCCTCAGAACTGGAGAAGAGATAAAGCACATGCAAAGATGAGTGTATATGGACA
Consensus	(1013)	GCCAATCAACTTCCGCTCCGTGGCTACCGCAGAACTGGAGAAGAGATAAAGCACATGCAAAGATGAGTGTATATGGACA

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Sequence alignment (positions 1105–1380)

Block 1 (top: 1196; start: 1105)

```
CYP51A      (1104)  TCATATCACGACGAGCGTGAAAAGAGTGGTGGTGGGGAGGAGAATGGATAATCTCATGACT
K1124       (1022)  TCATATCACGACGATGCGGAAAAGAGTGGTGGTGGGGAGGAGAATGGAGAATCTCATGACT
FI12-63     (1029)  TCATATCACGACGATGCGGAAAAGAGTGGTGGTGGGGAGGAGAATTGACAATCTCATGACT
788         (997)   TCATATCACGACGATGCGGAAAAGAGTGGTGGTGGGGAGGAGAATTGACAATCTCATGACT
QUB 30-10   (997)   TCATATCACGACGATGCGGAAAAGAGTGGTGGTGGGGAGGAGAATTGACAATCTCATGACT
QUB 12-3    (1052)  TCATATCACGACGATGGGGAAAAGAGTGGTGGTGGGGAGGAGAATTGACAATCTCATGACT
R 9528.4    (996)   TCATATCACGACGATGGGGAAAAGAGTGGTGGTGGGGAGGAGAATTGACAATCTCATGACT
GKII 18-2-3 (987)   TCATATCACGACGATGGGGAAAAGAGTGGTGGTGGGGAGGAGAATTGACAATCTCATGACT
RS 219      (996)   TCATATCACGACGATGGGGAAAAGAGTGGTGGTGGGGAGGAGAATTGACAATCTCATGACT
RS 783      (995)   TCATATCACGACGATGGGGAAAAGAGTGGTGGTGGGGAGGAGAATTGACAATCTCATGACT
Consensus   (1105)  TCATATCACGACGATGGGAAAAGAGTGGTGGTGGGGAGGAGAATTGACATGATTGACAATCTCATGACT
```

Block 2 (top: 1288; start: 1197)

```
CYP51A      (1196)  TGTGTCTATAAATCCGGAGAAACGATTCCAGAGCAGCGAGATTGCGTGTATGATGATCACGATCCTTATGGCGGGACAACATTCGTCGAGC
K1124       (1114)  TGTGTCTATAAATCCGGAGAAACGATCCCAGAGCGGCGAAATTGCGTGTATGATGATCTGATCCTTATGGCGGGACAAAAACACTCGTCCAGC
FI12-63     (1121)  TGTGTCTATAAATCCGGAGAAACGATCCCAGAGCATGCGAAATTGCGTGTATGATGATCTGATCCTTATGGCGGGACAAAAACACTCGTCCAGC
788         (1089)  TGTGTCTATAAATCCGGAGAAACGATCCCAGAGCATGCGAAATTGCGTGTATGATGATCTGATCCTTATGGCGGGACAA--ACTCGTCCAGC
QUB 30-10   (1089)  TGTGTCTATAAATCCGGAGAAACGATCCCAGAGCATGCGAAATTGCGTGTATGATGATCTGATCCTTATGGCGGGACAA--ACTCGTCCAGC
QUB 12-3    (1144)  TGTGTCTATAAATCCGGAGAAACGATCCCAGAGCATGCGAAATTGCGTGTATGATGATCTGATCCTTATGGCGGGACAA--ACTCGTCCAGC
R 9528.4    (1088)  TGTGTCTATAAATCCGGAGAAACGATCCCAGAGCATGCGAAATTGCGTGTATGATGATCTGATCCTTATGGCGGGACAA--ACTCGTCCAGC
GKII 18-2-3 (1079)  TGTGTCTATAAATCCGGAGAAACGATCCCAGAGCATGCGAAATTGCGTGTATGATGATCTGATCCTTATGGCGGGACAA--ACTCGTCCAGC
RS 219      (1088)  TGTGTCTATAAATCCGGAGAAACGATCCCAGAGCATGCGAAATTGCGTGTATGATGATCTGATCCTTATGGCGGGACAA--ACTCGTCCAGC
RS 783      (1087)  TGTGTCTATAAATCCGGAGAAACGATCCCAGAGCATGCGAAATTGCGTGTATGATGATCTGATCCTTATGGCGGGACAA--ACTCGTCCAGC
Consensus   (1197)  TGTGTCTATAAATCCGGAGAAACGATCCCAGAGCGAAATTGCCGTGTATGATGATCTGATCCTTATGGCGGGACAACACTCGTCCAGC
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Block 3 (top: 1380; start: 1289)

```
CYP51A      (1286)  AGTTCCTGCTGCTGGATTATGCTCATCTGCCCAGATCTTCAGGAGGAATTGTATCGCGAGCAACAGGATGCGAATCTCTACT
K1124       (1206)  AGTTCCTCGAGCTGGATTATGCTGCATCTAGCCTCTGCGCCCAGATCTTCAGGAGGAATTGTATCGCGAGCAACAGGATGCGAATCCTTATCT
FI12-63     (1213)  AGTTCCTCGAGCTGGATTATGCTGCATCTAGCCTCTGCGCCCAGATCTTCAGGAGGAATTGTATCGCGAGCAACAGGATGCGAATCCTTATCT
788         (1179)  AGTTCCTCGAGCTGGATTATGCTGCATCTAGCCTCTGCGCCCAGATCTTCAGGAGGAATTGTATCGCGAGCAACAGGATGCGAATCCTTATCT
QUB 30-10   (1179)  AGTTCCTCGAGCTGGATTATGCTGCATCTAGCCTCTGCGCCCAGATCTTCAGGAGGAATTGTATCGCGAGCAACAGGATGCGAATCCTTATCT
QUB 12-3    (1234)  AGTTCCTCGAGCTGGATTATGCTGCATCTAGCCTCTGCGCCCAGATCTTCAGGAGGAATTGTATCGCGAGCAACAGGATGCGAATCCTTATCT
R 9528.4    (1178)  AGTTCCTCGAGCTGGATTATGCTGCATCCAGCTCTGCGCCCAGATCTTCAGGAGGAATTGTATCGCGAGCAACAGGATGCGAATCCTTATCT
GKII 18-2-3 (1169)  AGTTCCTCGAGCTGGATTATGCTGCATCTAGCCTCTGCGCCCAGATCTTCAGGAGGAATTGTATCGCGAGCAACAGGATGCGAATCCTTATCT
RS 219      (1178)  AGTTCCTCGAGCTGGATTATGCTGCATCTAGCCTCTGCGCCCAGATCTTCAGGAGGAATTGTATCGCGAGCAACAGGATGCGAATCCTTATCT
RS 783      (1177)  AGTTCCTCGAGCTGGATTATGCTGCATCTAGCCTCTGCGCCCAGATCTTCAGGAGGAATTGTATCGCGAGCAACAGGATGCGAATCCTTATCT
Consensus   (1289)  AGTTCCTCGAGCTGGATTATGCTCATCTAGCCTCTGCGCCCAGATCTTCAGGAGGAATTGTATCGCGAGCAACAGGATGCGAATCCTTATCT
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DNA sequence alignment (nucleotide positions 1381–1656)

Block 1 — positions 1381 to 1472

Sample	Start	End
CYP51A	(1378)	1472
K1124	(1298)	
FI12-63	(1305)	
788	(1271)	
QUB 30-10	(1271)	
QUB 12-3	(1326)	
R 9528.4	(1270)	
GKII 18-2-3	(1261)	
RS 219	(1270)	
RS 783	(1269)	
Consensus	(1381)	

Block 2 — positions 1473 to 1564

Sample	Start	End
CYP51A	(1470)	1564
K1124	(1390)	
FI12-63	(1397)	
788	(1363)	
QUB 30-10	(1363)	
QUB 12-3	(1418)	
R 9528.4	(1362)	
GKII 18-2-3	(1353)	
RS 219	(1362)	
RS 783	(1361)	
Consensus	(1473)	

Block 3 — positions 1565 to 1656

Sample	Start	End
CYP51A	(1562)	1656
K1124	(1482)	
FI12-63	(1489)	
788	(1455)	
QUB 30-10	(1455)	
QUB 12-3	(1510)	
R 9528.4	(1454)	
GKII 18-2-3	(1445)	
RS 219	(1454)	
RS 783	(1453)	
Consensus	(1565)	

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                    1657                                                                         1748
CYP51A      (1654)  AGAGGTGGATTATGGATATGGTGTGATTAGCAAGGGACTAGGAGTCCGTACTTACCGTTTGGTGCGGGCAGAGACATCGATGCATTGGGGAGA
K1124       (1574)  AGAGGCGGGTTATGGATATGGTGTGATTAGTAAGGAGATTAGGAGTTCCTTATTTACCGTTTGGTGA-CGGGCAGAGACATCGGTGCATTGGGGAGA
FI12-63     (1581)  AGAGGCGGGTTATGGATATGGTGTGATTAGTAAGGAGATTAGGAGTTCCTTATTTACCGTTTGGTGA-CGGGCAGAGACATCGGTGCATTGGGGAGA
788         (1546)  AGAGGTGGGTTATGGATATGGTGTGATTAGTAAGGAGATTAGGAGTTCCTTATTTACCGTTTGGTGAA--------------------------
QUB 30-10   (1546)  AGAGGTGGGTTATGGATATGGTGTGATTAGTAAGGAGATTAGGAGTTCCTTATTTACCGTTTGGTGA---AATCACTAGTGAATTCGCGCGCC
QUB 12-3    (1601)  AGAGGTGGGTTATGGATATGGTGTGATTAGTAAGGAGATTAGGAGTTCCTTATTTACCGTTTGGTG----AATCACTAGTGAATTCGCGCGCC
R 9528.4    (1545)  AGAGGTGGGTTATGGATATGGTGTGATTAGTAAGGAGATTAGGAGTTCCTTATTTACCGTTTGGTGA
GKII 18-2-3 (1536)  AGAGGTGGGTTATGGATATGGTGTGATTAGTAAGGAGATTAGGAGTTCCTTATTTACCGTTTGGTGA
RS 219      (1545)  AGAGGTGGGTTATGGATATGGTGTGATTAGTAAGGAGATTAGGAGTTCCTTATTTACCGTTTGGTGAA
RS 783      (1544)  AGAGGTGGGTTATGGATATGGTGTGATTAGTAAGGAGATTAGGAGTTCCTTATTTACCGTTTGGTGA
Consensus   (1657)  AGAGGTGGGTTATGGATATGGTGTGATTAGTAAGGAGATTAGGAGATTCCTTATTTACCGTTTGGTGA

                    1749                                                                         1840
CYP51A      (1746)  AGTTTGCATTTGTCAATTTATTGACGATTATAGCTACGCTGGTGAGGGGATATAAGTGAGTCTTGTCCCTGGGGAGGAGGAGGAGGACAG
K1124       (1640)  --------------------------------------------------------------------------------------------
FI12-63     (1673)  AGTTTGCATTCGTCAATTCATTGACGATTATAGCCACGCTTGTGAGGGGATATAGGTGAGTCT----------------------------
788         (1613)  --------------------------------------------------------------------------------------------
QUB 30-10   (1612)  --------------------------------------------------------------------------------------------
QUB 12-3    (1688)  GCCTGCAGGTCGACCATATGGGAGAGCTCCCAACGCGTTGGATGCATAGCTTGAGTATTCTATAGGTCACC----------------------
R 9528.4    (1611)  --------------------------------------------------------------------------------------------
GKII 18-2-3 (1602)  --------------------------------------------------------------------------------------------
RS 219      (1612)  --------------------------------------------------------------------------------------------
RS 783      (1610)  --------------------------------------------------------------------------------------------
Consensus   (1749)  

                    1841                                     1918
CYP51A      (1838)  ATCCCAAAGACGGATTATTCGAGTATGGTCTCTAGACCTATGCCGGGCTCGAGAGTTAGATGGGAAAAGAGGGTCTAG
K1124       (1640)  ----------------------------------------------------------------------------------
FI12-63     (1737)  ----------------------------------------------------------------------------------
788         (1613)  ----------------------------------------------------------------------------------
QUB 30-10   (1612)  ----------------------------------------------------------------------------------
QUB 12-3    (1759)  ----------------------------------------------------------------------------------
R 9528.4    (1611)  ----------------------------------------------------------------------------------
GKII 18-2-3 (1602)  ----------------------------------------------------------------------------------
RS 219      (1612)  ----------------------------------------------------------------------------------
RS 783      (1610)  ----------------------------------------------------------------------------------
Consensus   (1841)  
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                                            1                                                           100
H. sapiens CYP7           (1)  MTTSLIW-------G-IAI--AAC-------CC-------LW---LIL-G------IR-R--RQT-GEPPLENGLIPYLG
M. tuberculosis CYP51B1   (1)  ------------------------------------------------------M-SAVALPRVSGGHDEHG
T. cruzi CYP51E1          (1)  --------------------------M--F-IEAIVLALTALILYSVYSVKSF-N-TTR-P-TDPPVYPTVPFLG
A. thaliana CYP51G1       (1)  ------------------MELDS-EN-KLL-KTGL-VIVATLVIAKLIFSFT-SDS-K-KKRLPPTLKAWPLVG
H. sapiens CYP51A1        (1)  MAAAGMLLLGLLQAGGSVL-GQA------MEKVTGGNL-LSMLLIACAF-TLSLVLIRLAAGHLVQLPA--G-V-KSPPYIFSPIPFLG
R. oryzae CYP51F1         (1)  MAVISTLL----------------PTLESIPLYA-VLALGVFVIINILSQWFG-P-K-N-P-KEPPVVFSWIPFMG
R. oryzae CYP51F1-p       (1)  --------------------------------------------------------------------------
R. oryzae CYP51F5         (1)  MAIISTLL-----------SE------TSLSSLLYKA-IVFLATYVIHLLSQFIQ-P-K-D-P-KAVPLVPSWIPFFG
C. neoformans CYP51F1     (1)  MSAIIPQ----VQ-QLL--GQVAQFIP----PW-FA----ALPTSVKKVIAV-IGIPALVICLNVFQQICLP-R-R-K-DLPPVVFHYIPWFG
P. triticina CYP51F1      (1)  MSSVIGS-----LL-EPI------GS-----F-STFNQVLIYL-VLAVVSIISINFDQLAI-P-K-D-P-TAPPVVFHLFPFIG
U. maydis CYP51F1         (1)  MVASSSSA----TA-SLL--DQLFALTP----LAD----S-SAWIKTITVL-VLLPLLAVLNVASQLLI-A-T-P-K-NHPPVVFHFVPVIG
C. cinerea CYP51F1        (1)  M---------------------------LDLVKLGFFL-AVPVATILLNVLRQLLF-P-T-R-A-PNPPEVFHWIPIVG
C. cinerea CCIG_08364     (1)  MSTLAAQ----LN-GTL--PLSADAWSGFLAQAKQHVS----A-LDSRTFVLLL-INAPVIAIVLNVIRQLVI-P-K-A-TDPPEVFHWIPIVG
S. cerevisiae CYP51F1     (1)  MSATKSIV----ID------GLSHFLA----L-PLA----QRI-SLIIIPFIYNIVWQLLY-S--L-RKDRAPLVFYWIHPWFG
C. albicans CYP51F1       (1)  MAIVETV------TW------GINYFLA----L-SVT----QQI-SILLGVPFVYNLVWQYLY-S--L-RKDRVPLVFYWIPWFG
C. guilliermondii CYP51F1 (1)  MAIADIA------AW------VYSEFRG----L-PVS----AQI-SIVLFPFYNLLCQFWY-S--L-RKDRVPMVFHWIPWFG
C. lusitaniae CYP51F1     (1)  MALAEYV------LH------GYAQFCL----L-GLL----SKI-ALLVFTPIVFNVWQLLF-S--L-QKDRAPLVFHWHWPWLG
C. parapsilosis CYP51F1   (1)  MALVDLA------ID------GYNYFMT----L-STL----QQF-GLLVFAPFIYNIIWQLLY-S--L-RKDRVPMVFHWIPWFG
C. tropicalis CYP51F1     (1)  MAIVDTA------LH------GINYFLS----L-SLT----QQI-TILVVFPFIYNIAWQLLY-S--L-RKDRVPLVFYWIPWFG
D. hansenii CYP51F1       (1)  MALADYS------IA------LYNYFTS----L-AIV----QQA-SIILLFPFIYNLLWQFFY-S--F-RKDRVPLVFHWMPWVG
L. elongisporus CYP51F1   (1)  MALVDLA------IQ------GYTFFMS----L-TTF----QQV-FLIVVPFVYNIAWQLLY-S--L-RKDRVPLVFYWIPWFG
S. pombe CYP51F1          (1)  M---------------------------A-F--SLVSIL-LSIALAWYVGYINQLTS-R--N-S-KRPPIVFHWIPFVG
A. clavatus CYP51F1       (1)  MGLITDIL-----D--GV--CKY-------CST-----Q-SIWMLGGVGL-LSLLAVAVVNVLRQLLF-K--N-NEPPLVFHWFPFIG
A. flavus CYP51F1         (1)  MGILAVIL-----D--SV--CER-------CSG-----S-SLWMLSTVAL-LSILVVSVVINVLRQLLF-K--N-Y-KEPPLVFHWFPFIG
A. fumigatus CYP51F1      (1)  MGLIAFIL-----D--GI--CKH-------CST-----Q-STWLVIGIGL-LSILAVSVIINVLQLLF-K--N-P-HEPPVVFHWFPFIG
A. nidulans CYP51F1       (1)  MGLVSLVL-----D--NV--CER-------CSA-----L-SWALSGLGL-LSVIIAVLNVLRQLLF-K--N-P-NEPPVVFHWFPFIG
A. niger CYP51F1          (1)  MGLLAVVL-----D--SL--CER-------CSN-----T-SVLVVLGFGL-LSLLAVSVIFNILRQLFF-K--N-P-NEPPLVFHWFPFIG
A. oryzae CYP51F1         (1)  MGLIAVIL-----D--SV--CER-------CSG-----S-SLWMLSTVAL-LSILVVSVVINVLRQLLF-K--N-Y-KEPPLVFHWFPFIG
A. terreus CYP51F1        (1)  MGVFADII-----G--TL--CEH-------CST-----Q-SIWALGGAVL-LAVFVLSVVINVLRQLLF-K--N-P-NEPPVVFHWVPFIG
A. terreus CYP51F1-p      (1)  MGLFAAAL-----D--KI--CKQ-------CST-----Q-AIYMLSGVGV-ITTLVLSVVINVLIQLSF-K--N-P-NEPPVVFHWFPLIG
A. benhamiae CYP51F1      (1)  MGLLADIV-----S--RF--CEN-------CST-----L-STAALVASAV-SAFIVLSIVINVLQLLF-K--D-P-TKPPVVFHWVPIIG
B. dermatitidis CYP51F1   (1)  MGLLADAI-----V--RI--CAH-------CSA-----A-SLWVILLSGF-ATFIALSVVINVIRQLLF-K--N-P-NEPPVVFHWLPVIG
B. cinerea CYP51F1        (1)  MGILEAVT-----G--PL--AQE-------ISQ-----R-STGVVVAAGV-AAFIVLSVVLNVQVLF-A--N-P-NEPPMVFHWLPVIG
C. globosum CYP51F1       (1)  MGLIQELG-----G--PL--AEG-------FAR-----L-GLASQIGVAI-GGFLFLSVFLNVLKOVLI-K--N-P-NEPPLVFHWFPFVG
C. graminicola CYP51F1    (1)  MGLRQEVA-----G--PL--SQQ-------FQT-----L-GTGLQIFVIV-TAVISLSVVLNVAYQILF-K--N-S-NEPPMVFHWFPFIG
C. higginsianum CYP51F1   (1)  MGLLQEVA-----G--PL--SQQ-------FQT-----L-GTGLQIVTIV-GAVIFLSVVLNVASQILF-K--N-S-NEPPVVFHWFPEIG
C. immitis CYP51F1        (1)  MGLLANVL-----T--GV--SKA-------CSG-----Q-SPWTLVLLGI-CAFLTISVFVNVLKQVFF-K--N-P-NEPPVVFHWFPIIG
F. graminearum CYP51F1    (1)  MGLLQELA-----G--HPL--AQQ-------FQE-----L-PLGQOVGIGF-AVFLVLSVVLNVINLQLLF-K--N-R-NEPPLVFHWFPFVG
F. oxysporum CYP51F1      (1)  MGLLQELA-----S--HPL--AQQ-------YQE-----L-PLGQOIGF-GAFILSVVLNVINLQLLF-K--N-R-NEPPLVFHWFPFVG
F. verticillioides CYP51F1(1)  MGLLQELA-----S--HPL--AQQ-------YQE-----L-PLAQQIGIGF-GAFVVLSVVLNVINLQLLF-K--N-R-NEPPLVFHWFPFVG
H. capsulatum CYP51F1     (1)  MGLLADVV-----A--RV--CAH-------CST-----L-SPWAFLLTGS-ATLIVLSVVIINVLHQLLW-K--N-P-HEPPVVFHWSWFPVVG
M. oryzae CYP51F1         (1)  MGLLQDTT-----G--PL--VDA-------FYQ-----L-GTGAQVGVAF-VSFIFLSVFFH-VAQIFF-K--N-P-HEPPVVFHWSWFPVVG
M. canis CYP51F1          (1)  MGLLADIV-----S--RF--CEN-------CST-----L-STTALISSGI-SAFIVLSIIINVLQLLF-K--D-P-TKPPVVFHWVPIIG
M. gypseum CYP51F1        (1)  MGFLADII-----S--RF--CEN-------CST-----L-STTALVASAI-SAFIVLSIVINVIQLLF-K--D-P-TKPPVVFHWVPIIG
M. graminicola CYP51F1    (1)  MGLLQEVL-----A--QF--DAQ-------FGQ-----T-SLWKLVGLGF-LAFSTLAILLNVLSQLLF-R--GK-L-SDPPLVFHWVPFIG
N. fischeri CYP51F1       (1)  MGLIAFIL-----D--GI--CKH-------CST-----Q-STWLVGVGL-LSILAVSVIANVLQLLF-K--N-P-HEPPVVFHWFPFIG
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                                     (1)                                                                          (100)
N. crassa        CYP51F1  (1)  MGILQVVA------G-PL-SQQ--------FSQ------L-GTVSQIGVAI-ASFLFVAVLNVLQQFLF-K---K-P-NEPPLVFHWFPLIG
P. brasiliensis  CYP51F1  (1)  MGLLADVV------A-CI-CAH--------CSN------L-SLWVLLVGF-VSFIALSVLINVIRQLLF-K---P-NEPPVFHWFPIIG
P. tritici-repentis CYP51F1 (1) MGLLADVT------G-PL-ANF--------TSQ------S-STPVVASAAF-ASFILLSVLNVIKQLLF-K---A-NEPPMVFHWLPIIG
R. secalis       CYP51F1  (1)  MGIFEAVT------V-PL-AQQ--------VSQ------R-GLGVIAAGF-AAFLVVSVILNVISQILF-K---N-P-NEPPIVFHFFPIIG
S. sclerotiorum  CYP51F1  (1)  MGILETIA------G-PL-AQE--------ISQ------R-STFAVVAAGV-AAFVLSVILNVLKQVLLW-A---N-P-NEPPIVFHLFPIIG
S.nodorum        CYP51F1  (1)  MGILADVA------G-PV-GNF--------TSQ------S-STATLIAAGF-ASFVLSVVLNVLKQLLW----D-P-TAPPVVFHLFPIIG
T. equinum       CYP51F1  (1)  MGLLADIV------S-RF-CEN--------CST------L-STAALVASAV-SAFIVLSIVINVIQQLLF-K---D-P-TKPPVVFHWFPIIG
T. rubrum        CYP51F1  (1)  MGLLADIV------S-RF-CEN--------CST------L-STAALVASAI-SAFIVLSIVINVIQQLLF-K---D-P-TKPPVVFHWFPVIG
T. tonsurans     CYP51F1  (1)  MGLLADIV------S-HF-CEN--------CST------L-STAALVASAV-SAFIVLSIVINVIQQLLF-K---D-P-TKPPVVFHWVPVIG
T. verrucosum    CYP51F1  (1)  MGLLADIV------S-RF-CEN--------CST------L-STAALVASAV-SAFIVLSIVINVIQQLLF-K---D-P-TKPPVVFHWVPIIG
U. reesii        CYP51F1  (1)  MGVLADLF------A-GA-RQA--------CST------Q-PLWTIALLGL-GAFITLSVVVNVLNQILF-K---N-P-NEPPVFHWFPVIG
V. albo-atrum    CYP51F1  (1)  MGLLQEIT------G-PA-AQQ--------FQT------L-GTASQVGVVV-FGVVLLSVIHVANQLLF-K---N-P-NEPPMVFSWFPFIG
V. dahliae       CYP51F1  (1)  MGLLQEIT------G-PA-AQQ--------FQT------L-GTASQVGVVV-FGVVLLSVILHVANQLLF-R-LRN-R-TEPPMVFHWFPFIG
A. clavatus      CYP51F2  (1)  M---------------------------------------LSITLFGLYL-VSATAVILNVNVYQHLF-R-LRN-K-TRPPLVFHWIPFLG
A. flavus        CYP51F4  (1)  M---------------------------------------SWPR-IGAYA-LLAFVAIMALNVTYQFLF-R-MLN-K-TRPPLVFHWIPFLG
A. flavus        CYP51F2  (1)  M---------------------------------------ASFTLVSAYA-AAGLLAIIVLNLLRQLLF-R---K-TDPPLVFHWIPFLG
A. fumigatus     CYP51F2  (1)  M---------------------------------------VPMLWLTAYM-AVAVLTAILLNVVYLFF-R-LWN-R-TEPPMVFHWVPYLG
A. nidulans      CYP51F2  (1)  ML--------------------------------------SP-TLFSAYV-LVGAIVALVNVIRQIFF-R---K-NEPPMVFHWVPFVG
A. niger         CYP51F2  (1)  M---------------------------------------ALLAVAGVYA-FAALLVAIVLNVTYQLLF-R-MLN-K-TDPPLVFHWIPFLG
A. oryzae        CYP51F2  (1)  M---------------------------------------SWPR-IGAYA-LLAFVAIMALNVTYQFLF-R-MLN-K-TRPPLVFHWIPFLG
A. oryzae        CYP51F4  (1)  M---------------------------------------ASFTLVSAYA-AAGLLAIIVLLRQLLF-R---K-TDPPLVFHWIPFLG
A. terreus       CYP51F2  (1)  M---------------------------------------SLITI-TSYA-AVGILALIAWNVIRQLLF-Q---K-SEPPVFHWIPFLG
A. benhamiae     CYP51F2  (1)  M---------------------------------------AVLT-VGTYA-LLGFIAAIFFNVVYQVLF-R-LLN-K-SRPPLVFHWIPFLG
B. dermatitidis  CYP51F2  (1)  M---------------------------------------SWST-IGTYA-VLAFTAAVALNLVYQLAF-R-LLN-T-TRPPLVFHWIPFLG
C. immitis       CYP51F7  (1)  MESL------PF-PS-----------------------PTVQLLPTVV-IIGVLSIFIGNLLRQFAP---H-R-G-AKPPVFHWVPFIG
C. higginsianum  CYP51F2  (1)  MSTLLE------TI-QP---------------------VSTASIAVYG-FIGLVALILFNACQQVVF---R-S-K-TEPPVVFHWIPYIG
F. graminearum   CYP51F2  (1)  M---------------------------------------FHLIIYPLWV-LVALFAVIIANLLYQQLP-R-R-P-DEPPLVFHWFPFFG
F. oxysporum     CYP51F2  (1)  M---------------------------------------FSLLYPLWA-FASCLVIITLNVLYQKLP-R-N-A-NEPPIVFHWLPFVG
F. verticillioides CYP51F2 (1)  M---------------------------------------FSLLYPLWA-FASCLAIITLNVLYQKLP-R-N-A-NEPPLVFHWLPFFG
H. capsulatum    CYP51F2  (1)  MAFF------FP-SA-----------------------SWFT-IGTFG-VLAFVAAVTLNVLVYQLF-R-FLN-K-TRPPLVFHWIPFLG
M. oryzae        CYP51F2  (1)  M---------------------------------------PVWVYSAGAA-LLFIIGSIILNFIWQQLP-R-P-K-SEPPLVFHWLPFLG
M. canis         CYP51F2  (1)  M---------------------------------------AWYS-LTAYL-LLGFVAAIALNLIYQLLF-R-LLN-R-TRPPLVFHWIPFLG
M. gypseum       CYP51F2  (1)  M---------------------------------------AVYA-VGAYV-LLSSIAVISLNVVYQLLF-R-LLN-R-SRPPLVFHWIPFLG
N. fischeri      CYP51F2  (1)  M---------------------------------------VPMLLTAYM-AVAMLTAILLNVVYQLFF-R-LWN-R-TEPPMVFHWVPFLG
P. tritici-repentis CYP51F2 (1) MP------SP-----------L-LLA---SGF-LALCIAYIFANTIRQLLL-P---NTKEPPVFHWLPWLG
R. secalis       CYP51F2  (1)  MLGI------FS--VL----------------------VSSIRYGFYT-LTLLIVTVLLNALHQLLP---K-K-K-SEPPLWIWIPFIG
T. equinum       CYP51F2  (1)  M---------------------------------------AVFT-VGAYA-LLSFIAAIFLNVIYQLLF-R-LLN-K-SRPPLVFHWIPFLG
T. rubrum        CYP51F2  (1)  M---------------------------------------AVLT-VGAYA-LLSFIAAIFLNVIYQLLF-R-LLN-K-SRPPLVFHWIPFLG
T. tonsurans     CYP51F2  (1)  M---------------------------------------AVFT-VGAYA-LISFIAAIFLNVIYQLLF-R-LLN-K-SRPPLVFHWIPFLG
T. verrucosum    CYP51F2  (1)  M---------------------------------------AVFT-LGAYA-FLGFIAAIFLNVVYQLLF-R-LLN-K-SRPPLVFHWIPFLG
U. reesii        CYP51F7  (1)  MESF------LV--FS----------------------PTTQLLPAVL-IVGVLTVFIGNLLQFSS---R-G-G-AKPPVFHWVPFVG
F. graminearum   CYP51F3  (1)  ----M------E-SL-YET-------------LRT------L-PLSVSIPLTT-SIIILSIVTNVVKQIWF-P---N-P-HRPPVVFHFPFIG
F. oxysporum     CYP51F3  (1)  ----M------E-AL--YSN-------------WRS------F-PLSLSVPITA-FLIIAIATITNVIKQIWF-P---N-P-HRPPVVFHIFPLIG
F. verticillioides CYP51F3 (1)  ----M------D-AL--YTN-------------WRS------L-PLSLSIPITA-FLIVIATITNVIKQIWF-P---N-P-HRPPVVFHIFPLIG

Consensus        (1)           M                                         NVL QLLF          N          PP VFHW PF G
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260

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                                      101                                                                                           200
      Homo sapiens CYP7      (44)  CALQFGANPLEFLRANQRKH-GHVFTCKLMGKYVHFITNPLSYHKVLCHGKYFW---KKFHFATSAKAFGHRSIDPMDGNTENINDTFIKTLQGHALN
 M. tuberculosis CYP51B1     (18)  HLEEFRTDPIGLMQRVRDEC-GDVGTFQLAGKQVVLLSGSHAN-EFFFRAGDDL-DQAKAY-FFMTPIFGEGVFDASPERRE--MLHNAALRGEQMK
          T. cruzi CYP51E1    (44)  HIVQFGKNPLEFMQRCKRELKSGVFTVNLVHKKITFLIGGQRVTIVGDPHEH-SRFFSPRNEIL-SPREV-TIMTPVFGEGVAYSAFYPRMREQLNFLAEELTIAKFQ
      A. thaliana CYP51G1     (52)  SLIKFLKGPIIMLREEYPKL-GSVFTVNLVHKKITFLIGPEVS-AHFFKASESDL-SQQEVY-QFNVPTFGPGVFVDDYSVRQEFRFFTEALRVNKLK
       H. sapiens CYP51A1     (79)  HALAFSKSPIEFLENAYEKY-GPVFSFTMVGKTFTYILGSDAA-ALLFNSKNEDL-NAEDVYSRLTTPVFGKGVAYDVPNPVFLEQKMKSGLNIAHFK
        R. oryzae CYP51F1      (55)  NAIEFGINPIAFLQKCQKKY-GDVFTFYMVGKRYTVFFNADGN-QFVFYMVGKRVTVFNADGN-QFVFNAKQNLT-SAADAYNHMTKHVFGPEVVYDAPHSVFMEQKRFIKAGLNSESFR
        R. oryzae CYP51F1-p    (1)   --
        R. oryzae CYP51F5      (57)  NAIEFGKNPIEFLQTCQKKY-GDVFTFRLLNKRVTACLGPDGN-QFVFNAKQEVA-SAAAAYNHMTKYVFGNDIVFDTAHSVFMEQKRMIKSGLNIEAFR
    C. neoformans CYP51F1      (73)  SAAYGEDPYKFLFECRDKY-GDLFFILMGRRVTVALGPKGN-NLSLGKISQ-SAEEAYHLTTPVFGKGVVYDCPNEMLNQQKKFIKSGLITESLQ
      P. triticina CYP51F1     (62)  SAVSYGIDPYAFLESCRKKY-GNVFTFVLLNKKVTVALGLEGN-ALVLNGKLSQ-NAEEAYTALTTPVFGTDVVYDVPNAILQQKKFVKSGLTNENFR
         U. maydis CYP51F1     (72)  SAIYYGIDPYKFFFECREKY-GDVFTFVLLGRKITVALGPKGS-NLVFNAKHQQV-TAEDAYTHLITPVFGKEVVYDPNAVFMEQKFKVKGLSIENFR
        C. cinerea CYP51F1     (49)  SALQYGNDPINFFKCREKY-GDVFTFILLGRRITVALGPKGN-NFILGKSTVF-NAEDAYTHLITPVFGKDVVYDVPNEKFMQKFIKAGISTENLR
   C. cinerea CC1G_08364       (79)  SALQYGMDPLNFFKCREKY-GDVFTFVLLGRRVTVALGPKGH-NFILGKSIVF-NAEDAYTHLTTPVFGKDVVYDVPNEKFMQKRFVVKGISAENLR
     S. cerevisiae CYP51F1     (68)  SAVVYGMKPYEFFEECQKKY-GDIFSFVLLGRVMTVYLGPKGH-EFVFNAKLADV-SAEEAYAHLTTPVFGKGVIYDCPNSRLMEQKKFAKFALTTDSFK
       C. albicans CYP51F1     (60)  SAASYGQQPYEFFESCRQKY-GDVFSFMLLGKIMTVYLGPKGH-EFVFNAKLSDV-SAEDAYKHLTTPVFGKGVIYDCPNSRLMEQKKFAKFALTKDSFI
  C. guilliermondii CYP51F1    (60)  SSVVYSMQPYEFFESCRQKY-GDVFSFMVGRVMTVVLGPKGH-EFVFNSKATDV-SAEDAYTHLTTPVFGKGVIYDCPNHRLMEQKKFAKFALTKDSFQ
      C. lusitaniae CYP51F1    (60)  SAIPYGMQPYEFFESCREKY-GDLFSFVMLGRVMTVYLGPKGH-EFVFNAKLSDV-SAEEAYSHLTTPVFGKGVIYDCPNARLMEQKKFAKTALTTDSFR
    C. parapsilosis CYP51F1    (60)  SAASYGMQPYEFFENCRTKY-GDVFAFVLLGKVMTVYLGPKGH-EFIYNAKISDV-SAEEAYTHLTTPVFGKGVIYDCPNSRLMEQKKFAKYALTKDSFK
      C. tropicalis CYP51F1    (60)  SAVVYGMQPYEFFENCRQKY-GDVFAFVLLGKVMTVYLGPKGH-EFVLNSKLQDV-SAEEAYKHLTTPVFGKGVIYDCSNSRLMEQKKFAKFALTKDSFR
        D. hansenii CYP51F1    (60)  SAVGYGQQPYEFFENCRQKY-GDVFTFVCMGRKMTAFLGVQGN-DFLFNGKLADL-NAEEAYSHLTTPVFGKDVVYDIPNHVFMEHKHKFIKSGLGFSQFR
    L. elongisporus CYP51F1    (45)  SAVAYGMDPYKFFFDCRAKY-GDIFTFILLGKKTTVYLGTKGN-DFILNGKLKGN-CAEVYSLTTPVFGRHVVYDCPNFKFMKQKKFVKFGLTSDALR
          S. pombe CYP51F1     (64)  STISYGIDPYKFFFECRAKY-GDIFTFILLGKKTTVFLGTKGN-DFVLNAKIHDL-CAE--VYSSLTTPVFGNHVVYDCPNFKFMKQKKFVKFGLASDALR
        A. clavatus CYP51F1    (64)  STISYGMDPYRFFNCREKY-GDIFTFILLGKKTTVYLGTKGN-DFILNGKLRDV-CAEEVYSPLTTPVFGRHVVYDCPNAKLMEQKKFVKFGLTSDALR
          A. flavus CYP51F1    (64)  STISYGIDPYKFFFDCKEKY-GDIFTFVLLGKKTTVYLGTKGN-DFILNGKLRDV-CAEEVYSPLTTPVFGRHVVYDCPNAKLMEQKKFVKFGLTSDALR
       A. fumigatus CYP51F1    (64)  STISYGIDPYKFFFDCRAKY-GDIFTFVLLGKKTTVYLGRKGN-DFILNGKLRDV-CAEEVYSPLTTPVFGRHVVYDCPNAKLMEQKKFVKFGLTSDALR
        A. nidulans CYP51F1    (64)  STITYGMDPYKFFFDCRAKY-GDIFTFVLLGKKTTVYLGRKGN-DFILNGKIRDV-CAEEVYSPLTTPVFGRHVVYDCPNAKLMEQKKFVKFGLTSDALR
          A. niger CYP51F1     (64)  STTTYGMDPYKFFFDCRAKY-GDIFTFVLLGKKTTVYLGRKGN-DFILNGKLRDV-CAEEVYSPLTTPVFGRNVVYDCPNAKLMEQKKFVKFGLTSDALR
         A. oryzae CYP51F1     (64)  STVTYGMDPFPAFFQANREKY-GDIFTFVLLGKKTTVYLGPQGN-DFILNGKIKDV-CAEEIYTVLTTPVFGRDVVYDCPNSKLMEQKKFMKIALTTEAFR
        A. terreus CYP51F1     (64)  STVIYGMDPFPAFFKANEKY-GDIFTFILLGKKTTVYLGPAGN-DFILNGKLKDV-CAEEIYTVLTTPVFGRHVVYDCPNSKLMEQKKFVKFGLTSEALR
      A. terreus CYP51F1-p     (64)  STIRYGIDPYKFFFECRRKY-GDIFTFILLGKKTTVFLGTKGN-DFILNGKLKDV-CAEEIYTVLTTPVFGKDVVYDCPNSKLMEQKKFVKFGLTSEALR
      A. benhamiae CYP51F1     (64)  STISYTDPYKFFFECRAKY-GDIFTFILLGKKTTVFLGTKGN-DFVLNAKIHDL-CAE--VYSSLTTPVFGKDVVYDCPNFKFMKQKKFVKFGLTSEALR
   B. dermatitidis CYP51F1     (64)  STISYGIDPYKFFFLSCKEKY-GDIFTFVLLGKKTTVYLGRKGN-DFILNGKLKDV-CAEEVYSPLTTPVFGRHVVYDCPNAKLMEQKKFVKYGLTSESLR
         B. cinerea CYP51F1    (64)  STITYGMDPYKFFFDCRAKY-GDIFTFVLLGKKTTVYLGRKGN-DFILNGKLKDL-NAEEIYTVLTTPVFGKDVVYDCPNSKLMEQKKFMKIGLSTRAFR
        C. globosum CYP51F1    (64)  STTTYGMDPPRFFKENKEKY-GECFTFILLGKKTTVYLGPQGN-EFILNGKIRDV-CAEEIYTVLTTPVFGKDVVYDCPNSKLMEQKKFMKIALTTEAFR
     C. graminicola CYP51F1    (64)  STVTYGMDPPAFFQANREKY-GDIFTFVLLGKKTTVYLGPQGN-DFILNGKIKDV-CAEEIYTVLTTPVFGRDVVYDCPNSKLMEQKKFMKIALTTEAFR
    C. higginsianum CYP51F1    (64)  STVIYGMDPPAFFKANREKY-GDIFTFVLLGKKTTVYLGPAGN-DFILNGKIKDV-CAEEIYTVLTTPVFGRHVVYDCPNSKLMEQKKFMKIALTTEAFR
          C. immitis CYP51F1   (64)  STITYGMDPPTFFFRENRAKH-GDIFTFILLGKKTTVYLGRKGN-DFILNGKLKDV-CAEEIYTVLTTPVFGKDVVYDCPNSKLMEQKKFMKIALTTEAFR
     F. graminearum CYP51F1    (65)  STITYGMDPPTFFFRENRAKH-GVFTFILLGKKTTVFLGPKGN-DFILNGKLKDV-SAEEIYTVLTTPVFGKDVVYDCPNSKLMEQKKFMKIALTTEAFR
        F. oxysporum CYP51F1   (65)  STITYGMDPPKFFKENRAKH-GVFTFVLLGKKTTVFLGTKGN-DFILNGKLKDV-SAEEIYTVLTTPVFGKDVVYDCPNSKLMEQKKFMKIALTTEAFR
  F. verticillioides CYP51F1   (65)  STISYGIDPYKFFLNCREKY-GDIFTFVLLGKKTTVYLGTEGN-EFILNGKLRDV-NAEEIYGPMTTPVFGKDVVYDCPNSKLMEQKKFMKIALTTEAFR
      H. capsulatum CYP51F1    (65)  STVTYGKDPPQFFRDMAKKY-GNIFTFILLGKKTTVYLGTKGN-DFILNGKLKDV-CAEEIYSPLTTPVFGSHVVYDCPNSKLMEQKKFVKFGLTSESLR
          M. oryzae CYP51F1    (64)  STISYGIDPYKFFFDDCKAKY-GDIFTFILLGKKTTVYLGTKGN-DFILNGKLKDV-NAEEIYSPLTTPVFGKDVVYDCPNSKLMEQKKFVKFGLTSEALR
           M. canis CYP51F1    (64)  STISYGIDPYKFFFDDCKEKY-GDIFTFILLGKKTTVYLGTKGN-DFILNGKLKDV-CAEDVYSPLTTPVFGKDVVYDCPNSKLMEQKKFMKIALTTEAFR
         M. gypseum CYP51F1    (64)  STTTYGIDPYKFFFSCREKY-GDIFTFILLGKKTTVYCLGTKGN-DFILNGKLKDV-NAEEIYSPLTTPVFGKDVVYDCPNSKLMEQKKFVKFGLTTSALQ
      M. graminicola CYP51F1   (65)  STITYGIDPYKFFFDDCKEKY-GDIFTFILLGKKTTVYLGTKGN-DFILNGKLKDV-CAEEVYSPLTTPVFGRHVVYDCPNAKLMEQKKFVKFGLTSDALR
        N. fischeri CYP51F1    (64)  STISYGIDPYKFFFDCRAKY-GDIFTFILLGKKTTVYLGTKGN-DFILNGKLRDV-CAEEVYSPLTTPVFGRHVVYDCPNAKLMEQKKFVKFGLTSDALR
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N. crassa          CYP51F1 (64)  STITYGMDPERFKENREKY-GDCFTFILLGKKTTVVGPKGN-DFILNGKIRDV-NAEEIYTVLTTPVFGKDVVYDCPNSKLMEQKKFMKIALTTEAFR
P. brasiliensis    CYP51F1 (64)  STISYGIDPYKFFLSCREKY-GDIFTFILLGKKTTVFLGVKGN-DFILNGKLKDV-CAEEVYSPLTTPVFGRHVVYDCPNSKLMEQKKFVKFGLTSESLR
P.tritici-repentis CYP51F1 (64)  STVTYGMDPYAFFFANHKKY-GNVFTFILLGRKMTVCLDTAGN-NFILNGKIKDV-NAGEVYSPLTTPVFGKDVVYDCPNSKLMEQKKFVKYGLTQEALR
R. secalis         CYP51F1 (64)  STVTYGIDPYKFFFDNKAKY-GEVFTFILLGKKTNVYLGTHGN-EFILNGKIKDV-NAEEIYTVLTTPVFGKDVVYDCPNSKLMEQKKFMKIGLTTEAFK
S. sclerotiorum    CYP51F1 (64)  STVTYGMDPYKFFECRAKY-GDVFTFILLGRMTVCLDTKGN-NLILNGKLKEV-NAEEIYSPLTTPVFGKDVVYDCPNAKLMEQKKFVKFGLTSEALR
S.nodorum          CYP51F1 (64)  STVTYGIDPYKFFFAQQKKY-GDIFTFILLGKKTTVFLGTKGN-DFILNGKLKDV-CAEDVYSPLTTPVFGRHVVYDCPNSKLMEQKKFVKFGLTSEALR
T. equinum         CYP51F1 (64)  STISYGIDPYKFFDDCKEKY-GDIFTFILLGKKTTVFLGTKGN-DFILNGKLKDV-CAEDVYSPLTTPVFGRHVVYDCPNSKLMEQKKFVKFGLTSEALR
T. rubrum          CYP51F1 (64)  STISYGIDPYKFFDDCKEKY-GDIFTFILLGKKTTVFIRYKGE-RHFERQAQGCLPRRDVYSPLTTPVFGRHVVYDCPNSKLMEQKKFVKFGLTSEALR
T. tonsurans       CYP51F1 (64)  STISYGIDPYKFFDDCKEKY-GDIFTFILLGKKTTVFLGTKGN-DFILNGKLKDV-CAEDVYSPLTTPVFGRHVVYDCPNSKLMEQKKFVKFGLTSEALR
T. verrucosum      CYP51F1 (64)  STISYGIDPYNFFFSCREKY-GDIFTFILLGKKTTVFLGTKGN-DFILNGKLKDV-CAEEVYSPLTTPVFGRHVVYDCPNAKLMEQKKFMKIALTTEAFR
U. reesii          CYP51F1 (64)  NTVTYGMDPPAFFEANKKKY-GDVFTFILLGSKTTVAVGPQGN-DFILNGKQRDV-NAEEIYSVLTTPVFGRDVVYDCPNAKLMEQKKFMKIALTTEAFR
V. albo-atrum      CYP51F1 (64)  NTITYGMDPPAFFEANKKY-GDVFTFILLGSKTTVAVGPQGN-DVILNGKQRDV-NAEEIYSVLTTPVFGRDVVYDCPNAKLMEQKKFMKIALTTEAFR
V. dahliae         CYP51F1 (49)  STITYGIDPSKFFFACREKY-GNIFTFILLGQKVTVYLGVEGN-EFILNGKLKDV-NAEEIYSPLTTPVFGSDVVYDCPNSKFMEQKKFIKFGLTSQAALE
A. clavatus        CYP51F2 (48)  STIHYGMDPYGFFFSCREKY-GDIFTFILLGRPTTVYLGTQGN-EFILNGKLKDV-NAEEIYSPLTTPVFGSDVVYDCPNSKLIEQKKFIKFGLSQAALE
A. flavus          CYP51F2 (47)  STVTYGMDPYAFFSCRQKY-GDIFTFILLGRKITVYLGIQGN-EFILNGKLKDV-NAEEIYSPLTTPVFGSDIVYDCPNSKLMEQKKFIKYGLTQAALE
A. flavus          CYP51F4 (49)  STISYGIDPYKFFFACREKY-GDIFTFILLGQKTTVYLGVQGN-EFILNGKLKDV-NAEEVYSPLTTPVFGSDVVYDCPNSKLMEQKKFIKFGLTQAALE
A. fumigatus       CYP51F2 (47)  STISYGMNPYKFFFSCREKY-GDIYTFVMLGKKMTVYLGVKGN-DFILNGKLKDL-SAEEVYSPLTTPVFGSDVVYDCPNSKLMEQKKFIKFGLTQAALE
A. nidulans        CYP51F2 (47)  STIHYGTDPYGFFFSCREKY-GDIFTFILLGRPTTVYLGTQGN-EFILNGKLKDV-NAEEVYSPLTTPVFGSDVVYDCPNSKLIEQKKFIKFGLTQAALE
A. niger           CYP51F2 (48)  STISYGMDPYTFFFSCRKKY-GDIFTFILLGQKTTVYLGTQGN-EFILNGKLKDV-NAEEVYSPLTTPVFGSDVVYDCPNSKLIEQKKFIKYGLTQAALE
A. oryzae          CYP51F2 (47)  STIHYGTDPYGFFFSCREKY-GDIFTFILLGRPTTVYLGTQGN-EFILNGKLKDV-NAEEVYSPLTTPVFGSDIVYDCPNSKLIEQKKFIKFGLTQAALE
A. oryzae          CYP51F4 (46)  STVTYGMDPYAFFSCRQKY-GDIFTFILLGRKITVYLGIQGN-EFILNGKLKDV-NAEEIYSPLTTPVFGSDIVYDCPNSKLMEQKKFIKYGLTQAALE
A. terreus         CYP51F2 (48)  STISYGIDPYAFFASCRQKY-GDIFTFILLGRKITVYLGVQGN-EFILNGKLKDV-NAEEVYSPLTTPVFGSDVVYDCPNAKLMEQKKFIKYGLSSAALE
A. benhamiae       CYP51F2 (54)  NTISYGMDPYKFFFSCQKKH-GDIFTFVLLGRHTTVYLGIKGN-EFILNGKLKDV-NAEEVYSPLTTPVFGSDVVYDCPNSKLIEQKKFIKYGLTQAALE
B. dermatitidis    CYP51F7 (56)  STICYGIDPYKFFFSCRQKH-GDIFTFVLLGRHTTVYLGVKGN-EFILNGKLKDV-NAEEVYSPLTTPVFGPDVVYDCPNSKLIEQKKFIKYGLSSEALA
C. immitis         CYP51F2 (47)  SAISYGLDPYRFFTSCREKY-GDIFTFVLLGRKTVYLGVKGN-EFILNGKLQDV-NAEEVYSPLTTPVFGSDVIYDCPNQKLMEQKKFIKFGLMQRALE
C. higginsianum    CYP51F2 (47)  NAVSYGMDPYAFFIKHRKKH-GDVFTFLFGCGFFEKCREKH-DFILNGRLQDV-NAEEIYGPLTIPVFGQDVIYDCPNSKLMEQKKFVKFGLTQKALE
F. graminearum     CYP51F2 (47)  NAVAYGLDPCGFFEKCREKH-GDVFTFILFGRKIVACLGVDGN-DFVLNSRLQDA-NAEEVYGPLTIPVFGSDVVYDCPNSKLMEQKKFVKFGLTQKALE
F. oxysporum       CYP51F2 (48)  NAVAYGLDPYGFFVKCREKH-GDVFTFILFGRKIVACLGVDGN-DFVLNSRIQDA-NAEEIYGPLTTPVFGSDVVYDCPNSKLMEQKKFVKFGLTQKALE
F. verticillioides CYP51F2 (54)  STIRYRTDPYKFFFSCRQKH-GDIFTFVLLGRPTTVYLGIKGN-EFILNGKLQDL-NAEEVYGPLTTPVFGSDIIYDCPNSKLIEQKKFIKYGLTQKALD
H. capsulatum      CYP51F2 (48)  NAVSYGMDPYRFYSQCREKH-GDVFTFVLFGRRMTVFLGVQGN-DFILNGKLQDL-NAEEYAPLTTPVFGSDVVYDCPNSKLMEQKKFIKYGLTQAALE
M. oryzae          CYP51F2 (48)  STISYGIDPYRFFFSCREKH-GDIFTFVLLGRHTTVYLGIKGN-EFILNGKIKDV-NAEEVYSPLTTPVFGSDVVYDCPNSKLMEQKKFIKYGLSQSALE
M. canis           CYP51F2 (49)  STISYGIDPYKFFFSCREKH-GDIFTFVLLGQKTTVYLGVQGN-EFILNGKLKDV-NAEEVYSPLTTPVFGSDVVYDCPNSKLMEQKKFIKYGLTQRALE
M. gypseum         CYP51F2 (47)  SAITYGKDPYKFLFAAREKH-GPIFTYILFGKKMTVYLGLEGN-DFVFNGKETHV-NAEEIYGPLCNPVFGEGVYDCPNSKLMEQKKFVKFGLTQRALE
N. fischeri        CYP51F2 (54)  NAIEYGTDPLAFYTKCQRQH-GPIFTYILFGKKMTVYLGLEGN-EFILNGRLQDV-NAEDIYGPLATPVFGPNIYDCPNSKLMEQKKFIKYGLTDALK
P. tritici-repentis CYP51F2 (48) NTISYGMDPYKFFSCRKKH-GDIFTFVLLGRHTTVYLGIKGN-EFILNGKIKDV-NAEEVYSPLTTPVFGSNVVYDCPNSKLMEQKKFIKYGLSQAALE
R. secalis         CYP51F2 (48)  NTISYGMDPYKFFFSCRKKH-GDIFTFVLLGRHTTVYLGIKGN-EFILNGKIKDV-NAEEVYSPLTTPVFGSDVVYDCPNSKLMEQKKFIKYGLSQAALE
T. equinum         CYP51F2 (48)  NTISYGMDPYKFFFSCQKKH-GDIFTFVLLGRHTTVYLGIKGN-EFILNGKIKDV-NAEEVYSPLTTPVFGSDVVYDCPNSKLMEQKKFIKYGLSSAALE
T. rubrum          CYP51F2 (54)  NTISYGMDPYKFFFSCRKKH-GDIFTFVLLGRHTTVYLGIKGN-DFILNGKIKDV-NAEEVYSPLTTPVFGSDVVYDCPNSKLMEQKKFIKYGLSSAALE
U. reesii          CYP51F7 (57)  SAISYGLDPYRFASCREKY-GDIFTFVLLGKSTTVFLGPKGN-DFILNGKHADL-NAEDVYGKLTTPVFGEEVYDCSNARFMDQKRLLKLGLTTDSLR
F. graminearum     CYP51F3 (57)  STVQYGIDPYAFFDCRDKY-GDCFTFILLGKSTTVFLGPKGN-DFILNGKHADL-NAEDVYGKLTTPVFGKEVYDCSNARFMDQKRLLKLGLTTDSLR
F. oxysporum       CYP51F3 (57)  STVQYGIDPYKFFFDCQAKY-GDCFTFILLGKSTTVYLGPKGN-DFILNGKHADL-NAEDVYGKLTTPVFGEVYDCSNARFMDQKRLLKLGLTTESLR
F. verticillioides CYP51F3 (57)  STVQYGIDPYKFFFDCQAKY-GDCFTFILLGKSTTVYLGPKGN-DFILNGKHADL-NAEDVYGKLTTPVFGEVYDCSNARFMDQKRLLKLGLTTESLR

Consensus                (101)  STI YG DPY FF CR KY GDIFTF LLGKKTTVYLG KGN  FILNGKL DV  NAEEVYSPLTTPVFG DVVYDCPNSKLMEQKKF K GLT  ALR
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             Homo sapiens CYP7  (140)  SLTESMENLQRIMRPP----VSSNS----K-TA---AWTEGMYSFCYRVMFEAGYLTIFGRDLTRR-DT-QKAHILN
     M. tuberculosis CYP51B1  (112)  GHAATIEDQVRRMIAD------------W-GE---AGEIDLLDFFAELTIYSS-ACLIGKKFRDQLDG-RFAKLYH
           T. cruzi CYP51E1  (141)  NFVPAIQHEVRKFMAE----N---W----K-KD---EGVINLLEDCGAMINFAC-QCLFGEDLRKRLNARHFAQLLS
        A. thaliana CYP51G1  (148)  GYDMMVTEAEDVFSK-------------W-GE---SGEKNIKVELERLILLTAS-HCLLGREVDQLFD-DVSALFH
         H. sapiens CYP51A1  (176)  QHVSIIEKETKEYFES----W--------GE---SGEKNVFEALSELILLTAS-HCLHGKEIRSQLNE-KVAQLYA
          R. oryzae CYP51F1  (152)  QHVPMIVEEVEGFKNY----K--------P---TGAFDAYHTLGELIICTASR-CLMGKEIRASLDD-SVAGLYY
        R. oryzae CYP51F1-p    (1)  ---MIVEEVSFFKDF-----K--------P---TSASDAYHILEKLIICTASR-CITDKETRVYLDD-SAASLYY
          R. oryzae CYP51F5  (154)  KDVPLIIEECDAFFDG----L--------P---QGEMDLYKMFGTLIIYTASR-TLLGPEIRQALDS-GVSELYY
      C. neoformans CYP51F1  (170)  SYPPMITSECEDFFTKEV---G--I---E--P---SATLDLLKSMSELILTAS-RTLQGKEVRESLNG-QFAKYYE
       P. triticina CYP51F1  (159)  KYVSLIAEETIS-LED-H---V--FE---SPQ-KP---QTVKDTFKVASEITICIAS-ATLQGPEVREALNK-SFAQLYH
         U. maydis CYP51F1  (169)  VIVPQIVDEVRYIKSDA---R--FS-ALKT-RK---TITVDIFQAMSELILTAS-RTLQGEVRQGLDK-EFAQTYN
         C. cinerea CYP51F1  (146)  AYVGMIEEVEDFLEKDP---S-FATYQQRNPA-NE---WGRFNAVKTMQEITILTAS-RTLQGREIRNGLTK-EFAQTYN
  C. cinerea CC1G_08364 CYP51F1 (176)  AYVGMIEEVEGFLNNDP---A-FSVFQQ-NDV-NE---WGTFNAVKTMQEITILTAS-RTLQGREIRNGLTK-EFAQTYN
      S. cerevisiae CYP51F1  (165)  SYVPLIAEEVYKYFRDS----KN---FR---LNE-RT---TGTIDVMVTQPEMTIFTAS-RSLLGKEMRAKLT-DFAYLYS
        C. albicans CYP51F1  (157)  RYVPKIREEILNFVTD----ES---FK---LKE-KT---HGVANVMKTQPEITIFTAS-RSLFGDEMRRIFDR-SFAQLYS
   C. guilliermondii CYP51F1  (157)  KYVPKIQDEVLSYFVE----HD---FK---MKE-KD---SGVVNVMKTQPEITIFTAS-RSLMGDEMRKRFDT-SFAQLYS
       C. lusitaniae CYP51F1  (157)  KYVPRIQEILDYFKSC----LD---FS---MTE-RN---SGVANVMKTQPELTILTAS-KSLMGDDMRARFPT-SFAQLYS
     C. parapsilosis CYP51F1  (157)  RYVPLIRGEILDYFTKS---KV---FN---MKK-QK---SGVDDVLQSQPEITIFTAS-RSLLGEAMRKRFDA-SFAQLYA
       C. tropicalis CYP51F1  (157)  TYVPKIREEVLNYFVND----VS---FK---TKE-RD---HGVASVMKTQPEITIFTAS-RCLFGDEMRRIFDR-SFAQLYA
         D. hansenii CYP51F1  (157)  KYVPKIKEEVLSYFTDS----EN---FN---MKG-KS---SGVADVMKTQPEITIFTAS-RSLMGDEMRKKFDA-SFAQLYA
     L. elongisporus CYP51F1  (157)  KYVPLIREEVLNYFTSN----SN---FN---MKN-QK---SGVADVMKTQPEITIFTAS-RSLMGKEMRSRFDE-SFAELYS
            S. pombe CYP51F1  (142)  SYVPLILNEMDAFLSTSP---D--EG----P-GK---EGVALLKTMPVMTIYTAS-RTLQGAEVRKGFDA-GFADLYH
         A. clavatus CYP51F1  (161)  SYVHLITDELESFVKSS----SA---F----Q-GP---KGVFDVCKTIAEITIYTAS-RSLQGKEVRNKFDS-TFAELYH
          A. flavus CYP51F1  (161)  SYVRLITEEVEDFVQKS---SA---L----Q-GP---NCVFDVCKTIAEITIYTAS-RSLQGKEVRSRFDS-TFAELYH
        A. fumigatus CYP51F1  (161)  SYVPLITDEVESFVKNS---PA---F----Q-GH---KGVFDVCKTIAEITIYTAS-RSLQGKEVRSRFDS-TFAELYH
        A. nidulans CYP51F1  (161)  SYVQLITAEVEDFAQKS---SV---F----Q-NA---KGVFDVSRTIAEITIYTAS-RSLQGKEVRDKFDS-TFAELYH
          A. niger CYP51F1  (161)  SYVRLITGEVENFVEHS---AA---F----K-GS---GCVFDVCKTIAEITIYTAS-RSLQGKEVRSRFDS-TFAELYH
         A. oryzae CYP51F1  (161)  SYVRLITEEVEDFVQKS---SA---L----Q-GP---NGVFDVCKTIAEITIYTAS-RSLQGKEVNRFDS-TFAELYH
        A. terreus CYP51F1  (161)  SYARLITAEVDDFVKNS---PT---L----Q-QP---KGTFDVCKTIAEITIYTAS-RSLQGKEVNRFDS-TFAEMYH
       A. terreus CYP51F1-p  (160)  SYVELITKEVDEYITNA---NA---F----K-QP---RGTIDVGKVIADITIYTAS-RLLQGKEVEKFDS-TFAELYH
       A. benhamiae CYP51F1  (161)  SYVTLITKEVEQFFESS----PI---F----K-GD---SGLFNVSKVMAEITIYTAS-RSLQGKEVRGKFDS-SFAELYS
     B. dermatitidis CYP51F1  (161)  SYVTLITDEFNHYLETS---SA---L----K-GE---KGVLDVCKSIAEITIFTAS-RSLQGKEVRSKFDS-SFAQLYH
          B. cinerea CYP51F1  (161)  SYVPIIQMEVENFMKRS---SA---F----K-GP---KGTADIGPAMAEITIYTAS-HTLQGKEVRDFDT-SFASLYH
         C. globosum CYP51F1  (161)  SYVPIISDEVTNYFKRT----AD---F----K-GQ---SGVVNICPKMAQITIFTAS-HALQGKEIRDKFDV-SLADLYH
      C. graminicola CYP51F1  (161)  SYTPIVADEVTNYFKSS----PD---F----K-GK---SGVVNIPTKMAEITIFTAS-HALQGAFIPNKFDE-SLASLYH
      C. higginsianum CYP51F1  (161)  SYTPIVADEVTNYFKRS----LD---F----K-GK---SGVVNIPTKMAEITIFTAS-HALQGAFINKFDE-SLASLYH
          C. immitis CYP51F1  (161)  SYVTLITRESYLENS----PA---F----Q-GD---SGVFENVAKSVAEITIYTAS-RSLQGKEVRDKFDS-SFAELYA
     F. graminearum CYP51F1  (162)  SYVPIISSEVRDYFKKS----PD---F----K-GK---SGIADIPKKMAEITIFTAS-HALQGSAIRSKFDE-SLAALYH
        F. oxysporum CYP51F1  (162)  SYVPIISAEVRDYFKKS----PD---F----K-GK---SGIVDIPKKMAEITIFTAS-HALQGSVIRNKFDE-SLAALYH
   F. verticillioides CYP51F1  (162)  SYVPIISSEVRDYFKKS----PD---F----K-GK---SGIVDIPKKMAEITIFTAS-HALQGSVIRNKFDE-SLAALYH
       H. capsulatum CYP51F1  (161)  SYVTLITDENQYIKTS----PA---F----Q-GD---KGVLDVCKSIAEITIYTAS-RSLQGKEVRSKFDS-SFAQLYH
          M. oryzae CYP51F1  (161)  SYVPIIADEVSSYLKRT----PA---F----K-GP---SGVVNIPPKMAEITIFTAS-HALQGKEIRDQFDE-TLADLYH
           M. canis CYP51F1  (161)  SYVTLITKEVEQFFETS----PI---F----K-GD---SGAFNVSKVMAEITIYTAS-RSLQGKEVREKFDS-SFAELYS
          M. gypseum CYP51F1  (161)  SYVTLITKEVEQFFETS----AV---F----K-GD---SGAFNVSKVMAEITIYTAS-RSLQGKEVRGKFDS-SFAELYC
       M. graminicola CYP51F1  (162)  SYVTLIAAETRQFFDRNNPHKK-------F----A-ST---SGTIDLPPALAEITIYTAS-RSLQGKEVREGFDS-SFADLYH
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Multiple sequence alignment (residues 161–300):

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                                        (201)                                              (300)
N. fischeri        CYP51F1 (161) SYVPLITDEVESFVKNS---PA--F---Q-GH  KGVFDVCKTIAELTIYTAS-RSLQGKEVRSKFDS-TFAELYH
N. crassa          CYP51F1 (161) QYVPIISDEVTSYLKRT---AD--F---K-GK  SGIVDIPPKMAQTIFTAS-HALQGKEIRDKFDE-TLADLYH
P. brasiliensis    CYP51F1 (161) IYVTLITDEFNQYMETS---SA--F---Q-GN  KGILDVCKSMAEITIYTAS-RSLQGKEVRSKFDS-SFAQLYH
P.tritici-repentis CYP51F1 (161) SYVTLITQECEDFMKRH---KA--F---K-GE  KGTFDVTKVMAELTIYTAS-RSLQGEIRKSFDS-KFAELYH
R. secalis         CYP51F1 (161) TVVPIIQDEVETFIKGS---AA--F---K-GH  KGTVNIPAQMAEITIYTAS-HTLQGKEVRDFDT-TFASLYH
S. sclerotiorum    CYP51F1 (161) SYVPIIQMEVENFMKRS---SV--F---K-GQ  KGTADIGPAMAEITIYTAS-HSLQGKEIRKSFDS-KFADLYH
S.nodorum          CYP51F1 (161) SYVGIITQECEDFFKRH---KA--F---K-GQ  KGTFDVTKVMAELTIYTAS-RSLQGKEVRGKFDS-SFAELYS
T. equinum         CYP51F1 (161) SYVTLITKEVEQFFESS---PV--F---K-GD  SGVFNVSKVMAEITIYTAS-RSLQGKEVRGKFDS-SFAELYS
T. rubrum          CYP51F1 (161) SYVTLITKEVEQFFESS---PI--F---K-GD  SGVFNVSKVMAEITIYTAS-RSLQGKEVRGKFDS-SFAELYS
T. tonsurans       CYP51F1 (162) SYVTLITKEVEQFFESS---PV--F---K-GD  SGVFNVSKVMAEITIYTAS-RSLQGKEVRGKFDS-SFAELYS
T. verrucosum      CYP51F1 (161) SYVTLITKEVEQFFESS---PI--F---K-GD  SGLFNVSKVMAEITIYTAS-RSLQGKEVRGKFDS-SFAELYS
U. reesii          CYP51F1 (161) SYVTLITREVEQYLENS---PA--F---K-GD  SGILSVAKVVAEITIYTAS-RSLQGKEVRDKFDS-SFAELYG
V. albo-atrum      CYP51F1 (161) SYVPIISHEVQNYFKTS---KY--F---K-EQ  SGVVNIPPRMAEITIFTAS-HALQGSEIRNKFDT-SLAELYH
V. dahliae         CYP51F1 (161) SYVPIISHEVQNFFKTS---KY--F---K-EQ  SGVVNIPPRMAEITIFTAS-HALQGSEINKFDT-SLAELYH
A. clavatus        CYP51F2 (146) AHVPLIEKEVEDYLETS---PR--F---Q-GT  SGLVDIAAAMAEITIFTAA-RALQGEEVRSKLTA-EFADLYH
A. flavus          CYP51F2 (145) AHVPLIEKEVEDYLAMS---PN--F---H-GT  SGEVDIPAAMAEITIFTAG-RALQGEVRSKLTA-EFAVLYH
A. flavus          CYP51F4 (144) SHVPLIEKEVLDYLKTS---PN--F---K-GT  SGRVEITDAMAEITIFTAG-RALQGEEVRKLTA-EFADLYH
A. fumigatus       CYP51F2 (146) SHVPLIEKEVLDYLRDS---PN--F---Q-GS  SGRVDISAAMAEITIFTAA-RALQGEVRSKLTA-EFADLFH
A. nidulans        CYP51F2 (144) SHVPLIEKEVLDYIKTS---PR--F---K-GD  SGVLDAPAAMAELTIYTAG-SALQGKEVRKKLTA-EFAELYH
A. niger           CYP51F2 (144) SHVQLIEKETLDYLRDS---PR--F---N-GA  SGVIDIPAAMAEITIYTAG-RALQGEEVRKKLTA-EFADLYH
A. oryzae          CYP51F2 (145) AHVPLIEKEVEDYLAMS---PN--F---H-GT  SGEVDIPAAMAEITIFTAG-SALQGEEVRSKLTT-EFAVLYH
A. oryzae          CYP51F4 (144) SHVPLIEKEVLDYLKTS---PN--F---K-GT  SGRVEITDAMAEITIFTAG-RALQGEEVRKLTA-EFADLYH
A. terreus         CYP51F4 (143) SHVQLIEREVLDYLRTS---PR--F---H-GA  SGVVDISAAMAELTIFTAG-RALQGEEVRRKLTA-EFAVLYH
A. benhamiae       CYP51F2 (145) AHVPLIEKEVRDYLDTS---PN--F---H-GK  SGDVDISSAMAEITIFTAG-ITLQGEVRSKLTT-EFAALYH
B. dermatitidis    CYP51F2 (145) SHVPLIEKEVRDYLDAS---AN--F---L-GA  SGEVDIAAAMAEITIFTAG-SALQGEVRSKLTT-EFAALYH
C. immitis         CYP51F7 (151) SYVPLIQHEVEQYIASS---PH--F---K-GE  SGTMNVLQVMAEITILTAA-RTLQGEVRSKLDS-SFANYYH
C. higginsianum    CYP51F2 (153) SHVPLIEKEVDYIETS----PA--W---K-GA  SGVVDVSAAMSEVLFTAA-RSLQGEVRKKLTA-EFAELYH
F. graminearum     CYP51F2 (144) SHVQLIEREVLEYIQAV---PS--F---S-GR  TSTIDVPKAMAEITIFTAS-RSLQGEVRRKLTA-EFAALYH
F. oxysporum       CYP51F2 (144) SHVQLIEREVLEYIQAV---PS--F---S-GK  SGTVDVSKAMAEITIFTAA-RSLQGEVRRKLTA-EFAALYH
F. verticillioides CYP51F2 (144) SHVPLIEKEVMDYLDSS---PV--F---L-GA  SGEVDISAVMAEITIFTAG-SALQGEVRSKLTT-EFAVLYH
H. capsulatum      CYP51F2 (151) SYVPLIEREVLDYIESS---PN--F---QAGN  HGIVDIPSMMAEITIFTAS-RTLQGEVRKKLTG-EFARLYH
M. oryzae          CYP51F2 (145) SHVPLIEKEVLDYLDTS---PN--F---H-GP  SGEVDISDAMAEITIFTAG-SALQGEVRSKLTT-EFAILYH
M. canis           CYP51F2 (145) SHVPLIEKEVLDYLNTS---PN--F---H-GQ  SGEVDISSVMAEITIFTAG-ITLQGEVRSKLTT-EFASLYH
M. gypseum         CYP51F2 (146) SHVPLIEKEVLDYLRNS---PN--F---Q-GS  SGQVDISAAMAEITIFTAG-RALQGQEVRSKLTT-EFADLYH
N. fischeri        CYP51F2 (144) AHVQLIEQEVVDYIKAS---RE--F---K-GE  SGTINVPPVMAQTIFTAA-IALQGEVRSKLTN-EFASLYH
P. tritici-repentis CYP51F2 (151) QHVPLIENEVLSYMASS--PS--F---I-PQSPTSPSSSDQSTTKMEQKLQTAIIDLPHTMAQTIFTAG-RALQGPEVRTKLTD-EFAGLYD
R. secalis         CYP51F2 (151) AHVPLIEKEVLDYLNTS---PN--F---H-GK  CGEVDISSAMAEITIFTAG-ITLQGEVRSKLTT-EFAALYH
T. equinum         CYP51F2 (145) AHVPLIEKEVLDYLDTS---PN--F---H-GK  SGDVDISGAMAEITIFTAG-ITLQGEVRSKLTT-EFAALYH
T. rubrum          CYP51F2 (145) AHVPLIEKEVLDYLNTS---PN--F---H-GK  CGEVDISSAMAEITIFTAG-ITLQGEVRSKLTT-EFAALYH
T. tonsurans       CYP51F2 (145) AHVPLIEKEVLDYLNTS---PN--F---H-GK  SGDVDISSAMAEITIFTAG-ITLQGEVRSKLTT-EFAALYH
T. verrucosum      CYP51F2 (145) AHVPLIEKEVLDYLNTS---PH--F---R-GE  SGTVNIAQIMAEITILTAA-RTLQGEVRSKLDS-SFANYYH
U. reesii          CYP51F7 (151) SYVPLIQHEVEQIATS----Y---F---K-GD  TGIVNITEVMAEITIYTAS-GSLGNEVRSMFDS-TFATLYR
F. graminearum     CYP51F3 (154) CYIPKFVKEVEDYVKNSP--Y---F---K-GS  TGIVNITEVMAEITIYTAA-GSLLGNEVRSMFDS-TFATLYR
F. oxysporum       CYP51F3 (154) CYIPKFVKEVEEYIATSP--Y---F---K-GS  TGIVNITEVMAEITIYTAA-GSLLGNEVRSMFDS-TFATLYR
F. verticillioides CYP51F3 (154) CYIPKFVKEVEYIATSP---Y---F---K-GN  TGIVNITEVMAEITIYTAA-GSLLGNEVRSMFDS-TFATLYR

Consensus         (201) SYVPLI EV Y S        F       G    SG D  MAEITI TAS  R LQG EVR KFD  FA LYH
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                                         301                                                                                      400
        Homo sapiens CYP7 (205)   NLD-NFKQFDKYFPALVAG---------------REKLAESLRHENLQKRE----------------------------------SISELISLRMFLNDTLST-FDDLKAKTHL
  M. tuberculosis CYP51B1 (171)   ELERGTDPLAYV---DPY----LPIESFRRDEARNGLVALVADIMNGRIA-NP-P--TD---KSD-RDMLDVLIA-VKA-ETGT-PRFSADEITGMFI
        T. cruzi CYP51E1 (203)   KMESSLIPAAVF----MPWLLRLPLPQSARCREARAELQKILGEIVAREK-EEAS-KD---TE-SDLLGGLLK-AVF-RDGT-R-MSLHEVCGMIV
     A. thaliana CYP51G1 (207)   DLDNGMLPISVL---EPY----LPIPAHRRDRAREKLSEIFAKIIGSRKR-SG-K----TE-NDMLQCFIE-SKY-KDGR-Q-TTESEVTGLLI
     H. sapiens CYP51A1 (235)   DLDGFSHAAWL---LPG----WLPLPSFRRDRAHREIKDIFYKAIQKRQ-SQEK-ID---DILQTLLD-ATY-KDGR-P-LTDDVAGMLI
       R. oryzae CYP51F1 (212)   DLDQGFKPINFIF---PN----LPSHSYGKHDVVRQKMADFYSNIIACKKVEND----N-ADLLQALMD-ANY-KDGS-N-VPDHHIAGMMI
     R. oryzae CYP51F1-p (57)   VLGQRFKSISFIF---PN----LPSHSYGKHDVVRQKMADFYSNIIACKKVEND
       R. oryzae CYP51F5 (213)   DLDQGFRPINFMF---PN----LPLPSYRRDREAREKLAKIYAGIQKRRQ-SE-H---G---E-SDLLQTLIE-ARY-KDGS-A-VPDAQICGILT
   C. neoformans CYP51F1 (235)   DLDGGFTPLNFM---FPN----LPLPSYKRRDEAQKAMSDFYLKIMENRRK-GESD-H---E-HDMIENLQS-CKY-RNGV-P-LSDRDIAHMMI
     P. triticina CYP51F1 (224)   DLDGGFTPLHFA---FPN----LPLPSYRRDRAQLAMRNFYMNIIKKRRE-DDRE-GQ---L-GDMIDSIQG-QT-KDGR-P-LNDKEIAHIMI
       U. maydis CYP51F1 (236)   DLDSGFTPINFV---IPN----LPLPSNFKRDRAQKKMSQFYQDIVAKRRA-AGAS-TSADDASGE-NDMIAALIE-QKY-KNGR-A-LSGVEIAHMMI
       C. cinerea CYP51F1 (217)   DLDGLTPLNFL---FPN----LPLESYRRDRAHKKMSDFYIDIKKRKL-AQVE----E-HDMIASLSE-QRY-RSGA-P-LPDHEIAHIMI
  C. cinerea CC1G_08364 (246)   DLDGGFTPLNFL---FPN----LPLESYRRDRAHKKMSDFYIDIARRKA-GHSD-ET---E-HDMISALLE-QRY-RNGT-A-LKDHEIAHIMI
    S. cerevisiae CYP51F1 (231)   DLDKGFTPINFV---FFN----LPLEHYRKRDHAQKAISGTYMSLIKERRK-NNDI-Q---D-RDLIDSLMKNSTY-KDGV-K-MTDQEIANLLI
      C. albicans CYP51F1 (223)   DLDGFTPINFV---FPN----LPIPHYWRDAQKKISATYMKEIKSRRD-RGDI-DP---N-RDLIDSLIHSTY-KDGV-K-MTDQEIANLLI
 C. guilliermondii CYP51F1 (222)   DLDKGFHPIHFA---FPH----LPLPMSRNDRAQQKISATYKGLIDTRRS-SGNI-DP---T-RDLIDSIMATSTY-KDGV-K-MTDQEIANLLI
    C. lusitaniae CYP51F1 (223)   DLDKGFTPINFV---FPH----LPIPAYRRDEAQRKISATYMSLINDRRK-SGDI-VP---D-RDLIDSLMLNSTY-KDGV-K-MTDQEIANLLI
  C. parapsilosis CYP51F1 (223)   DLDKGFTPINFV---FPH----LPLPHYWKRDAAQQKISETYMTEIARRRE-TGDI-DE---N-RDLIDSLLVNSTY-KDGV-K-MTDQEIANLLI
    C. tropicalis CYP51F1 (223)   DLDKGFTPINFV---FPN----LPLPHYWRDAAQQKISSTYMSLSKRRD-TGDI-DP---K-RDLIDSLMTHSTY-KDGV-K-MTDQEIANLLI
       D. hansenii CYP51F1 (223)   DLDKGFTPNFV---FPN----LPLPHYWRDAAQRKISAHYMKEIKRRE-SGDI-DG---D-EDLISSLMVHSTY-KDGV-K-MTDQEIANLLI
  L. elongisporus CYP51F1 (223)   DLDKGFTPNFV---FPW----LPLPRNRRRDRAHKIMQKTYLKIIKDRRS-ST-E-NP---G-TDMIWTLMS-CKY-RDGR-P-LKEHEIAGMMI
         S. pombe CYP51F1 (206)   DLDQGFSPVNFV---FPW----LPLPRNRRRDRAHKIMQKTYLKIIKARRQ-AGNE-KD---S-EDMVWNLMS-CVY-KNGV-P-VPDEIAHMMI
       A. clavatus CYP51F1 (224)   DLDMGFAPINFM---LPW----APLPHNRKRDAAQKRLTETYMEIIKERRK-AGSK-KD---S-EDMVWNLMS-CMY-KDGT-P-VPDEIAHMMI
        A. flavus CYP51F1 (224)   DLDMGFAPINFM---LPW----APLPHNRKRDAAQKRMTETYMEIIKERRK-AGSK-KD---S-EDMVWNLMS-CMY-KDGT-P-VPDEIAHMMI
     A. fumigatus CYP51F1 (224)   NLDMGFAPINFM---LPW----APLPHNRKRDAAQKRLTETYMEIIKARRQ-AGSK-KD---S-EDMVWNLMS-CVY-KNGT-P-VPDEIAHMMI
      A. nidulans CYP51F1 (224)   DLDMGFAPINFM---LPY----APLPHNRKRDAAQKRKMAETYMEIIKERRK-SGEK-KD---S-EDMVWNLMS-CVY-KNGT-P-LSDEIAHMMI
        A. niger CYP51F1 (224)   DLDMGFAPINFM---LPW----APLPHNRKRDAAQKRKMAETYMEIIKERRN-GGNK-KD---S-EDMVWNLMS-CIY-KDGT-P-VPDEIAHMMI
       A. oryzae CYP51F1 (224)   DLDMGFAPINFM---LPW----APLPHNRKRDAAQKRMTETYMEIIKERRK-AGSK-KD---S-EDMVWNLMS-CMY-KDGT-P-VPDEIAHMMI
      A. terreus CYP51F1 (224)   DLDMGFAPINFV---LPW----APLPWNKRKRDAAQKRKMAETYMEIIKERRQ-GGEK-KD---S-EDMVWNLMS-CVY-KDGT-P-LPDEIAHMMI
    A. terreus CYP51F1-p (223)   DLDMGFAPINFI---LPW----APLPHNCKRDAAQKKMAQTYMEIIQKRRS-GGEK-N---S-EDMVWNLMS-CVY-KDGT-P-VPDIEVAHIMI
     A. benhamiae CYP51F1 (224)   DLDMGFAAINFM---FPW----FPFPHNRKRDAAQKRMAQVTDIIRQRRE-AGGE-KD---S-EDMVWNLMS-SVY-KNGT-P-IPDIEVAHMMI
  B. dermatitidis CYP51F1 (224)   DLDMGFTAINFM---LPW----APLPHNRKRDAAQKMAKVYTDIIRQRRE-AGCQ-KD---S-EDMVWNLMS-CVY-KDGT-P-LPDIEIAHMMI
        B. cinerea CYP51F1 (224)   DLDMGFSPINFM---LHW----APLPHNRARDHAQRTVAKTYMDIIQNRRA-QATE-AE---FKSDIMWQLMR-SSY-KDGT-P-VPDKEIANMMI
      C. globosum CYP51F1 (224)   DLDMGFSPINFM---LHW----APLPWNNRRDHAQRTVAKIYMTIKSRRE-RGET-GA---QDIMYHLMN-STY-KNGV-N-VPDHEIAHMMI
  C. graminicola CYP51F1 (224)   DLDMGFTSINFV---LHW----APLPWNKKRDLAHDTIAQIYMTIKERRE-QGRT-NG---LDIMSHLMN-STY-KNGI-K-VPDHEIANMMI
  C. higginsianum CYP51F1 (224)   DLDMGFTSINFV---LHW----APLPWNKKRDLAHDTIAQIYMTIKERRE-QGRT-NG---LDIMSHLMN-STY-KNGT-K-VPDHEIANMMI
         C. immitis CYP51F1 (224)   DLDMGFAAINFM---FPW----APLPWNKRKRDHAQRTVAKIYMDTIKERRA-AGGK-ND---E-HDMMKHLMN-STY-KDGT-P-VPDEVAHMMI
   F. graminearum CYP51F1 (225)   DLDMGFTPINFM---LHW----APLPWNRKRDHAQRTVAKIYMDTIKERRA-KGNN-ES---E-HDMMKHLMN-STY-KNGI-R-VPDHEVAHMMI
     F. oxysporum CYP51F1 (225)   DLDMGFTPINFM---LHW----APLPWNRKRDHAQRTVAKIYMDTIKERRA-KDND-DT---E-HDMMKHLMN-STY-KNGT-P-VPDHEVAHMMI
 F. verticillioides CYP51F1 (225)   DLDMGFTPINFM---LHW----APLPWNRKRDHAQRTVAKIYMDTIKERRA-KDND-DT---E-HDMMKHLMN-STY-KNGT-P-LPDIEAHMMI
    H. capsulatum CYP51F1 (224)   DLDMGFTPINFM---LHW----APLPHNRKRDAAQKTKIYTDIIRQRRE-AGGK-KD---S-EDMVWNLMS-CVY-KDGT-P-LPDIEAHMMI
        M. oryzae CYP51F1 (224)   DLDMGFHPVNFK---LHW----LPLPRIIRRDKAQKTIAKIYMTIQRRRA-GKGKD-SE---A-KDMMYHLMN-STY-KNGT-P-VPDIEVAHMMI
         M. canis CYP51F1 (224)   DLDMGFAAINFM---FPW----APLPHNRKRDAAQKMTKIYTDIIRQRRE-SGGK-KD---S-EDMVWNLMS-SVY-KNGT-P-VPDIEVAHMMI
       M. gypseum CYP51F1 (224)   DLDMGFAAINFM---FPW----FPFPHNRKRDAQRKMAQVYTDIIRQRRE-SGGE-KD---S-EDMVWNLMS-SVY-KNGT-P-IPDIEVAHMMI
   M. graminicola CYP51F1 (228)   YLDMGFTPINFM---LPW----APLPQNRRDYAQKKMSETYMSLIQKRRE-SKTG-EH---E-EDMHNLMQ-CKY-KDGN-A-IPDKEIAHMMI
      N. fischeri CYP51F1 (224)   NLDMGFAPINFM---LPW----APLPHNRKRDAAQKRLTETYMEIIKARRQ-TGSK-KD---S-EDMVWNLMS-CVY-KNGT-P-VPDEEIAHMMI
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Multiple sequence alignment (CYP51 family, residues ~301–400):

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                                      (301)                                                                                    (400)
N. crassa           CYP51F1 (224)  DLDMGFSPINFM----LHW----APLPWNNRRDYAQRTVAKIYMDTIKERRA-RGET-GA------QDIMWHLMN-STY-KGDV-P-VPDHEIAHHMI
P. brasiliensis     CYP51F1 (224)  DLDMGFTAVNFM----FPW----APLPHNRKRDAAQKKAAIYIDIIKRRE-AGI--KD----T-EDMVWNLMS-CVY-KDGT-P-LPDIEIAHHMI
P.tritici-repentis  CYP51F1 (224)  DLDMGFSPINFM----LSW----APLPHRARDNARETMIKLYSDLVRKRRS-GAVK-KD----S-HDMIWHLMD-CKY-KDGT-Q-VPEHEIAGIMI
R. secalis          CYP51F1 (224)  ALDMGFSPINFM----LHW----APLPHNRARDHAQRTVAKAYMEMETRRK-DRKS-LD----N-MDIMSQLMR-STY-KDGV-P-VPDMEIAHHMI
S. sclerotiorum     CYP51F1 (224)  DLDMGFSPVNFM----LSW----APLPHNRARDVARETMIKLYSEIVRKRA-QATE-AD----S-HDMIWHLMD-CKY-KDGT-Q-VPEHEIAGIMI
S.nodorum           CYP51F1 (224)  DLDMGFAAINFM----FPW----FPFPHNRKRDRAQKKWAQVTDIIRQRRE-AGGE-KD----S-EDMVWNLMS-SVY-KNGT-P-IPDIEVAHHMI
T. equinum          CYP51F1 (224)  DLDMGFAAINFM----FPW----FPFPHNRKRDRAQRKWAQVTDIIRQRRA-AGGE-KD----S-EDMVWNLMS-SVY-KNGT-P-IPDIEVAHHMI
T. rubrum           CYP51F1 (224)  DLDMGFAAINFM----FPW----FPFPHNRKRDRAQRKWAQVTDIIRQRRA-AGGE-KD----S-EDMVWNLMS-SVY-KNGT-P-IPDIEVAHHMI
T. tonsurans        CYP51F1 (225)  DLDMGFAAINFM----FPW----APLPHNRKRDRAQRKWAQVTDIIRQRRA-AGGE-KD----S-EDMVWNLMS-SVY-KNGT-P-IPDIEVAHHMI
T. verrucosum       CYP51F1 (224)  DLDMGFAAINFM----FPW----FPFPHNRKRDRAQRKWAQVTDIIRQRRA-AGGE-KD----S-EDMVWNLMS-SVY-KNGT-P-IPDIEVAHHMI
U. reesii           CYP51F1 (224)  DLDMGFAAINFM----FPW----APLPHNRKRDRAQQKWAQIYTDIIRERRA-AGGE-KD----S-EDMVWNLMS-SVY-KDGT-P-VPDIEVAHHMI
V. albo-atrum       CYP51F1 (224)  DLDMGFSAINFA----LHW----APLPWNRKRDAAQKAVAQIFMDTIKDRRE-SGRT-DG----LDMISHLMR-STY-KNGI-N-VPDHEIAHHMI
V. dahliae          CYP51F1 (224)  DLDMGFSAINFA----LHW----APLPWNRKRDAAQKAVAQIFMDTIKDRRE-SGRT-DG----LDMISHLMR-STY-KNGI-N-VPDHEIAHHMI
A. clavatus         CYP51F2 (209)  DLDRCFTPVNFM----LPW----APLPRNKKRDAAHVRRAIYDIINKRRN-DGE------DTQK-SDMIWNLMN-CSY-KNGQ-Q-VPDKEIAHHMI
A. flavus           CYP51F2 (208)  DLDKGFTPINFM----LPW----APLPRNKKRDAAHARWRSIYIDIINKRRN-AG------DNVPEK-LDMIGNLMQ-CTY-KNGQ-P-LPDKEIAHHMI
A. flavus           CYP51F4 (207)  DLDRGFTPINFM----LPW----APLPRNRRDAAHARWREIYMDIINERRK-NPDR-----ET-SDMIWNLMH-CTY-KNGQ-P-LPDKEIAHHMI
A. fumigatus        CYP51F2 (209)  DLDKGFTPINFM----LPW----APLPRNKKRDAAHARWRSIYIDIITQRRL-DGEK----DSQK-SDMIWNLMN-CTY-KNGQ-Q-VPDKEIAHHMI
A. nidulans         CYP51F2 (207)  DLEMGFTPINFI----LPW----APLPQNRKRDIAHARWRETYMEINQRRK-NPD------AQD-HDMIWNLMH-STY-KNGN-P-VPDKEIAHHMI
A. niger            CYP51F2 (208)  DLDKGFSPINFM----LPW----APLPHNKKRDAAHARWREIYTDIINERRK-NDE------EK-SDMIWNLMH-CTY-KSGQ-P-VPDKEIAHHMI
A. oryzae           CYP51F2 (207)  DLDRGFTPINFM----LPW----APLPRNKKRDAAHARWRSIYIDIINKRRN-AG------DNVPEK-LDMIGNLMQ-CTY-KNGQ-P-LPDKEIAHHMI
A. oryzae           CYP51F4 (207)  DLDRGFTPINFM----LPW----APLPRNRRDAAHARWREIYMDIINERRK-NPDR-----ET-SDMIWNLMH-CTY-KNGQ-P-LPDKEIAHHMI
A. terreus          CYP51F2 (206)  DLDKGFTPINFM----LPW----APLPHNKKRDAAHARWRAIYTDIIEKRRR-EGAK-----SNNTQ-TDMIHNLMQ-CTY-KDGQ-P-VPDKEIAHHMI
A. benhamiae        CYP51F2 (208)  DLDKGFTPINFM----LPW----APLPHNKKRDAAHSRWRAIYIDIIDKRRL-AGTI-----SDAPQV-LDMIGNLMQ-CTY-KNGQ-K-LPDKEIANIMI
B. dermatitidis     CYP51F2 (208)  DLDKGFTPINFL----LPW----APLPHNKKRDAAHARWHAIYIDIIDKRRL-SQE------RG-HDMLWNLMDNCVY-KDGT-A-LPDKEIAHLMI
C. immitis          CYP51F7 (214)  DLDSGFTPINFL----FPR----APLPRNRRDAAHAKKAVYEGIIKQRRE-TGL------GAEAE-HDMLWNLMS-SSY-KNGT-P-VPDSEIACMMI
C. higginsianum     CYP51F2 (216)  DLDLGFRPVNFL----FPW----LPLPHNRRDAAHIKWREVYMDIINDRRK-GGI------RTEDG-TDMIANLMG-CTY-KNGQ-P-LPDKEIAHHMI
F. graminearum      CYP51F2 (207)  DLDLGFTPVNFL----FPW----LPLPHNRRDAAHAKWKREIYMGIINGRR-GVG------DLEKG-TDMIANLMN-CEY-KNRQ-P-IPDKEIAHHMI
F. oxysporum        CYP51F2 (207)  DLDLGFTPVNFL----FPW----LPLPHNRRDAAHSKWREIYMGIINERR-VG-------DLEXR-TDMIANLMS-CAY-KNRQ-P-IPDKEIAHHMI
F. verticillioides  CYP51F2 (207)  DLDLGFTPVNFL----FPW----LPLPHNRRDAAHSKWREIYMGIINERR-AARN-----ANGSQT-QDMIENLMQ-CTY-KNGQ-K-LPDKEIANIMI
H. capsulatum       CYP51F2 (208)  DLDKGFSPINFM----LPW----APLPHNKKRDAAHTLMHAIYLDIIEKRRR-QKDD-----QEEEEEAE-PDMIRHLMGSCVY-KNGQ-A-LPDKEIAHHMI
M. oryzae           CYP51F2 (215)  DLDLGFRPINFL----APW----APLPQNRRRDVAHARWRDVYMDLINKRRR-AGTN-----INASQR-SDMIQNLMQ-CTY-KNGQ-P-VPDKEIAHIMI
M. canis            CYP51F2 (208)  DLDKGFSPINFM----LPW----APLPHNKKRDSAHSRWRAIYTDIIDKRRR-AGAA-----STNSQ-SDMIHNLMQ-CTY-KNGK-P-VPDKEIAHIMI
M. gypseum          CYP51F2 (208)  DLDKGFSPINFM----LPW----APLPHNKKRDAAHARWRSIYIDIINQRRL-DGEK-----DSQK-SDMIWNLMN-ST--KNGQ-Q-VPDSEIANMMI
N. fischeri         CYP51F2 (209)  DLDKGFTPINFM----LPR----APFPHNIKRDRAQLKRRKIYETIIAERR-AGKI-PP---T-TDMISHLMQ-CSY-KDGR-P-VPDSEIANMMI
P. tritici-repentis CYP51F2 (207)  DLDGGFSPINFV----APW----LPLPQNWRDKAHAKMSVYMDIISRRRE-KSGGGVVGRGMEREE-IDMIDNLMT-CV--KSGE-T-IPDSEIACMMI
R. secalis          CYP51F2 (234)  DLDHGFRPINFL----LPW----LPLPYNKKRDAAHARWRAIYTDIIEKRRR-EGAK-----RNHTQ-TDMIHNLMQ-CAY-KNGQ-L-LPDKEIAHHMI
T. equinum          CYP51F2 (208)  DLDKGFTPMNFM----LPW----VPLPYNKKRDAAHARWRAIYTEIIEKRRR-EGAK-----SNNTQ-TDMIHNLMQ-CTY-KNGQ-P-LPDKEIAHHMI
T. rubrum           CYP51F2 (208)  DLDKGFTPINFM----LPW----APLPHNKKRDAAHARWRAIYTDIIEKRRR-EGAK-----RNHTQ-TDMIHNLMQ-CAY-KNGQ-P-LPDKEIAHHMI
T. tonsurans        CYP51F2 (208)  DLDKGFTPINFM----LPW----APLPHNKKRDAAHSRWRAIYTDIIEKRRR-EGAK-----SNNTQ-TDMIHNLMQ-CTY-KNGQ-P-LPDKEIAHHMI
T. verrucosum       CYP51F2 (208)  DLDKGFTPINFL----LPW----APLPHNKKRDAAHKKMRDVYMGIIKQRRA-SQT------RG-HDMLWNLMDNCVY-KDGS-A-LPDKEIAHIMI
U. reesii           CYP51F7 (214)  HLDDGFQPINFV----MPG----LPLPQNFRRNHARKVMEKLFSDIISKRRE-TGNQ-GD---E-TDMIWMLMN-AQ--KDGE-D-LPHHAARMLI
F. graminearum      CYP51F3 (217)  HLDDGFQPINFV----MPG----LPLPQNFRRDHARKVMEELFSDIIRKRRE-IGNQ-GG---E-TDMVWTLMN-AKY-KDGE-D-LPNHHAARMLI
F. oxysporum        CYP51F3 (217)  HLDDGFQPINFV----MPG----LPLPQNFRRDHARKVMEELFSDIIRKRRE-MGNQ-GD---E-TDMWTLMN-AKY-KDGE-D-LPNHHAARMLI
F. verticillioides  CYP51F3 (217)  HLDDGFQPINFV----MPG----LPLPQNFRRDHARKVMEELFSDIIRKRRE-MGNQ-GD---E-TDMWTLMN-AKY-KDGE-D-LPNHHAARMLI
Consensus           (301)          DLD GF PINFM    LPW    APLPHNRKRD    AQ  M  Y  DII  RR     G      DMI NLM  Y  KNG  P  PD  EIAHMI
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Homo sapiens CYP7          (281)  VVLWASQANTIPATFWSLFQMIR--NPEAMKAATEVKRTLENAGQKVS-LEGNPICLSQAELN-DLFVLDSIIKESLRISSASLNI-RTAKEDFTL-H
M. tuberculosis CYP51B1    (252)  SMMFAGHHTSSGTASWTLIE MR--HRDAYAAVIDELDELYGDG------R-SVSFHALR-QIFQLENVLKETLRLHPPILIMRVAKGEFEV--
T. cruzi CYP51E1           (287)  AAMFAGQHTSTITTSWSMLH MHPKNKKWLDKLHKEIDE--F------D-KIDHDILS-EMDVLYRCIKEALRLHPPLIMLMRASHSDFSV--T
A. thaliana CYP51G1        (284)  AAIFAGQHTSSITSTWTGAYMR--YKEYFSAALDEQKNLIAKHG------NL-P-LTYDQLK-DKTLQKKCYLEKTVCG---ELYEAVKNPMPV
H. sapiens CYP51A1         (313)  GLLAGQHTSSAWGFFLAR--DKTLQKKCYLEKTVCG---E------NL-P-LTYDLK-DENLLDRCIKETLRLRPPVMIMMARTPQTV-A
R. oryzae CYP51F1          (290)  AVLFGQHTSATTSAWTLLEAA--RPDLIRDLREQITKLG--S------L-KADLTFDNLK-ELTLLDSCVRETLRLHPIFQMMRRVTANKVVFE
R. oryzae CYP51F1-p        (104)                                                                                    F--
R. oryzae CYP51F5          (290)  AAIFGQHTSSTTAAWTIELAQ--RPDIQALRQEMITQCG--S------L-EVDFTYDHLE-RITLLEHVKETLRLHPIFNMMRRVVAPKCVFS
C. neoformans CYP51F1      (313)  ALLMAGQHTSSATSSWTLLHLAD--RPDVVEALYQEKQKLGNP------DGTF-R-DYKYEDLK-ELFIMDSIIRETLRMHAPIHSIYRKVLSDIPV-P
P. triticina CYP51F1       (303)  ALLMAGQHTSAATGSWLLHLAS--RPDIVAELRQEQIDLFGKPGQ-TDDQEL-D-PLDLERVQ-SPIMACIKEVLRLHPPIHSIMRKVKSPITV-P
U. maydis CYP51F1          (321)  ALLMAGQHTSSATSSWAFLRLAS--RPEIEELYEQLNVYSDG------HGGL-R-ELDYETQKTSVPLLDAVVKETLRLHPPELHSIMRYVKSDLAV-P
C. cinerea CYP51F1         (296)  ALLMAGQHTSSWALLHLAH--NVEVAEALYNEQVEHFSNP------DGSF-R-PLTYDELR-ALPILDSVIRETLRMHPPIHSIMRYVRDDVVV-P
C. cinerea CC1G_08364      (325)  ALLMAGQHTSSATGAWTLLHLAN--RPDLIRDLREQLEHFTNP------DGSF-R-EMTYDELR-ALPILDSVIRETLRMHPPIHSIMRYVRDDVAV-P
S. cerevisiae CYP51F1      (310)  GVLMGGQHTSAATSAWILLHLAE--RPDVQQELYEQEMRVD--GG--K--E-KLTYDLLQ-EMPLNQTIKETLRMHPPLHSLFRKVMKDMHV-P
C. albicans CYP51F1        (303)  GILMGGQHTSASTSAWFLLHLGE--KPHLQDVIYQEVVELKEKG--D--I--N-DITYEDLQ-KLFSVNNTIKETLRMHPPLHSIFRKVTNPLRI-P
C. guilliermondii CYP51F1  (302)  GILMGGQHTSAATSAWFLLHLGE--RPDLQEEMYKEICGVFEKKNK-S--F-K-DLSYDDLQ-EMPIVNNVIKETLRMHPPLHSIFRKVKKPLLV-P
C. lusitaniae CYP51F1      (303)  GVLMGGQHTSAATSAWFLLHLAE--KPELQEELYNEVLSVLTKKG--K--I-T-DLSYEDLQ-EMHIVNQTIKETLRLHPPLHSIFRKVVSPLVV-P
C. parapsilosis CYP51F1    (303)  GVLMGGQHTSATTSAWFLLHLAE--KPQLQDELYQEVINALSGKG--N--I--D-DLSYEDLQ-QMPIVNNTIKETLRLHPPLHSIFRKVMNPLRV-P
C. tropicalis CYP51F1      (303)  GVLMGGQHTSASTSAWFLLHLAE--QFQLQDDLYEELTNLIKEKG--D--L-N-DITYEDLQ-KLPIVNNTIKETLRLHPPLHSIFRKVMNPLRV-P
D. hansenii CYP51F1        (303)  GVLMGGQHTSASTSAWFLLHLAE--KPELQDELYEEILSVLKQNG--S--F-N-DLDYDDLQ-NMPLLNNIIKETLRLHPPLHSIFRKVMSPMVV-P
L. elongisporus CYP51F1    (303)  GILMGGQHTSASTSAWFLLHLGD--KPELQDYEILQQALQSKG--N--I-S-DLSYEDLQ-KMTIVNNTIKETLRMHPPLHSIFRKVMSPMVV-P
S. pombe CYP51F1           (284)  ALLMAGQHTSAATIVWVLALLGS--KPEIIEMLWEEKRVVG--------EN--L-ELKFDQYK-DMPLNYVIQETLRLHPPIHSHMRKVKRDLPV-E
A. clavatus CYP51F1        (303)  ALLMAGQHSSSSTAAWIVLRLAT--RPDIMEELYQEQLRVL-----GS---D--L-P-PLTYDSLQ-KLDLHAKVIKETLRLHAPIHSIIRAVKNPMPV-E
A. flavus CYP51F1          (303)  ALLMAGQHSSSSTAAWIVHLAA--SPEITEELYQEQLRIL-----GH---D--M-P-PLTYENLQ-KLDLHAKVIKETLRIHAPIHSIIRAVKNPMPV-E
A. fumigatus CYP51F1       (303)  ALLMAGQHSSSSTASWIVLRLAT--RPDIMEELYQEQIRVL-----GS---D--L-P-PLTYDLQ-KIDLHAKVIKETLRLHAPIHSIIRAVKNPMAV-D
A. nidulans CYP51F1        (303)  ALLMAGQHSSSSTLSWILLHLAR--HPEIVEELYQELKVL-----GS---D--M-H--MTYDDLQ-KIELHSKIIKETLRIHAPIHSIIRAVKSPMPV-P
A. niger CYP51F1           (303)  ALLMAGQHSSSSTASWIVLHLAR--NPQIMEELYAEQIRVL-----GS---D--L-P-PLTYDNLQ-KIDLHAKVIKETLRLHAPIHSIIRAVKNPMPV-D
A. oryzae CYP51F1          (303)  ALLMAGQHSSSSTAAWIVHLAA--SPEITEELYQEQLRIL-----GH---D--M-P-PLTWDSLQ-KLDLHAKVIKETLRLHAPIHSIIRAVKNPMPV-E
A. terreus CYP51F1         (303)  ALLMAGQHSSSSTASWIVLRLAE--NPEITEELYQEQLRVL-----GS---D--L-P-PLTWDSLQ-KLDLHAKVIKETLRLHAPIHSIIRAVKNPMPV-E
A. terreus CYP51F1-p       (301)  ALLMAGQHSSSSTSIIWIILRLAS--QPEITEQLYQEQIRVL-----GA---D--L-P-PLTYENLQ-KELQTNVIKETLRLHAPIHSMLRTVKYPLQV-E
A. benhamiae CYP51F1       (303)  ALLMAGQHSSSSTGSWIVLRLAS--RPDILEELYEQKRVL-----GE---D--L-P-PLTYESLQ-KIDLHNNVIKETLRIHAPIHSILRAVKSPMPV-E
B. dermatitidis CYP51F1    (304)  ALLMAGQHSSASTLSWIVLRLAS--CPKIVEELYEQKEVL-----GE---D--L-P-PVTYETLQ-KITLNSHVIRETLRLHAPIHSIIRAVKSPMPV-D
B. cinerea CYP51F1         (304)  ALLMAGQHSSSSISWIMLRLAS--RPDIMEELYQEIQVL-----GA---D--L-P-ALKYEDLS-KLPIHQNVLKETLRLTPIHSIMRKVTTPMPI-S
C. globosum CYP51F1        (302)  ALLMAGQHSSSSTSSWIMYLAA--RPDIMEDLYQEIKNL-----GA---D--L-P-PLKYEDLA-KLPINQAVIKETLRINAPIHSIIRAVKSPMPV-P
C. graminicola CYP51F1     (302)  ALLMAGQHSSSSTSSWIMLRLAQ--NPHIMEELYEEQVRVL-----GA---D--L-P-PLAYEDLA-KLPINQAIIKETLRLHAPIHSIIRAVKSPMPV-P
C. higginsianum CYP51F1    (302)  ALLMAGQHSSASTGSWIVLRLAS--RPDIMEELYQEQLRVL-----GP---D--L-P-PLTYESLQ-RLELHSNVIKETLRLHAPIHSILRAVKSPMPV-D
C. immitis CYP51F1         (304)  ALLMAGQHSSSSTSSWMLRLAQ--YPHIMEELYQEQVKNL-----GA---D--L-P-PLINQAIVKETLRLHAPIHSIIRAVKNPMPV-E
F. graminearum CYP51F1     (304)  ALLMAGQHSSSSTSSWIMLRLAQ--YPHIMEELYQEQVREL-----GA---D--L-P-PLTYDNIA-KLPINQAIIKETLRLHAPIHSILRAVKSPMPV-P
F. oxysporum CYP51F1       (304)  ALLMAGQHSSSSTSSWIMLRLAQ--YPHIMEELYQEQVREL-----GA---D--L-P-PLTYDDIA-KLPINQAIIKETLRLHAPIHSILRAVKSPMPV-P
F. verticillioides CYP51F1 (303)  ALLMAGQHSSSSTFSWIILRLAS--CPHIIEELYEQKQVL-----GE---D--L-P-PLTYETIQ-KININSHVIRETLRLHAPINSILRAVKSPMPV-P
H. capsulatum CYP51F1      (303)  ALLMAGQHSSSSTSSWIMLRLAS--RPDIMEELYQEVRAL-----GA---D--L-P-PLRYEDLA-NLPILHLAVIKETLRLHAPINSILRAVKQDLPV-P
M. oryzae CYP51F1          (303)  ALLMAGQHSSSSTGSWIVLRLAS--RPDIEELYQCLRVL-----GK---D--L-P-PLTYESLQ-KIDLHNNVIKETLRLHAPIHSILRAVKSPMPV-E
M. canis CYP51F1           (303)  ALLMAGQHSSSSATESWITLRLAS--RPDIEELYQEQKRVL-----GS---D--L-P-PLTYESIQ-KIDLHNNVIKETLRLHAPIHSILRKVKSPMPI-E
M. gypseum CYP51F1         (307)  ALLMAGQHSSSSATESWITLRLAS--RPDIQDEILQEKDML----GVNAD--GSI-K-ELTYANLS-KLTLLNQVVKETLRLHAPIHSILRKVKSPMPI-E
M. graminicola CYP51F1     (303)  ALLMAGQHSSSSATESWITLRLAS--RPDIMEELYQEQIRVL-----GS---D--L-P-PLTFDNLQ-KIDLHAKVIKETLRLHAPIHSIIRAVKNPMAV-D
N. fischeri CYP51F1        (303)
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                                          (401)                                                                                                              (500)
        N. crassa CYP51F1 (302)  ALLMAGQHSSSSTSSWIMRLAS--RPDIMEDLYNEQVKNL---GA--D----L-P-PLTYEDIA-KIPLHAAIVKETLRLHAPIHSIMRAVKTPMPV-P
      P. brasiliensis CYP51F1 (303)  ALLMAGQHSSSSTLSWIVLRLAT--CPHIVEELYEEQKRVL---GD--S----L-P-PVTYETLQ-QLTINSHVIRETLRLHAPIHSILRAVKSPMPV-E
  P.tritici-repentis CYP51F1 (303)  ALLMAGQHSSSSTIAWLLRLAQ--NPHVIEELLAEQKSIL---GK--N----L-P-ALTYDLQ-KLPHAQVVKETLRIHAPIHSIMRKVKQPLVV-D
          R. secalis CYP51F1 (304)  ALLMAGQHSSSSSTWILRLAA--RPDILEELYOEQLEVI---GA--D----V-P-ALQYEDIA-KLTKHQNVLKEVLRHTPIHSIMRKVTTPMEI-S
     S. sclerotiorum CYP51F1 (303)  ALLMAGQHSSSSSWILLRLAS--RPDIMEELYOEQIQVL---GA--D----L-P-ALKYEDIA-KLPLHQNILKETLRIHTPIHSIMRKVKSPMPI-A
          S.nodorum CYP51F1 (303)  ALLMAGQHSSSSTIAWIIRLAQ--YPHLLEELLAEQKAVM---GE--D----L-P-AVTYEDLN-KLPLHAQVVKETLRIHAPIHSIMRTVKSPIVV-E
          T. equinum CYP51F1 (303)  ALLMAGQHSSSSTGSWIVLRLAS--RPDILEELYEEQKRVL---GE--D----L-P-PLTYESLQ-KLDLHNNVIKETLRLHAPIHSILRAVKSPMPV-E
           T. rubrum CYP51F1 (304)  ALLMAGQHSSSSTGSWIVLRLAS--RPDILEELYEEQKRVL---GE--D----L-P-PLTYEALQ-KLDLHNNVIKETLRLHAPIHSILRAVKSPMPV-E
        T. tonsurans CYP51F1 (303)  ALLMAGQHSSSSTGSWIVLRLAS--RPDILEELYEEQKRVL---GE--D----L-P-PLTYESLQ-KLDLHNNVIKETLRLHAPIHSILRAVKSPMPV-E
       T. verrucosum CYP51F1 (303)  ALLMAGQHSSSSTGSWIVLRLAS--RPDILEELYEEQKRVL---GE--D----L-P-PLTYEALQ-KLDLHNNVIKETLRLHAPIHSILRAVKSPMPV-E
          U. reesii CYP51F1 (302)  ALLMAGQHSSSSTGSWIVLRLAS--RPDILEELYEEQLRVL---GP--D----L-P-PLTYEDIS-RLEIHSNVIKETLRIHAPIHSILRKVKSPMPV-D
       V. albo-atrum CYP51F1 (302)  ALLMAGQHSSSSTSSWIMARLAQ--NPAVIDDLKEQVEAF---GA--D----L-P-PLTYEDLS-KLPLNQAIIKETLRLHAPIHSILRKVKSPMPV-P
          V. dahliae CYP51F1 (289)  ALLMAGQHSSSSTSSWIMARLAQ--NPSVIDDLKEQVEAL---GA--D----L-P-PLTYEDLS-KLPLNQAIIKETLRLHAPIHSILRKVKSPMPV-P
         A. clavatus CYP51F2 (288)  TLLMAGQHSSSSIGSWIMRLAS--QPEVLEKLYQEQIDKLAQGGP--G--SNI--R-PLQYKDLE-LLPYHQHVIRETLRLHSSIHSILRKVKNTLSV-
           A. flavus CYP51F2 (285)  TLLMAGQHSSSSISSWIMRLAS--QPAVVEELYQEQLANLERTGP--N--GSL--A-PLQYKFD-NLPLHQNVIRETLRLHSSIHSLLRKVKNPLPV-P
           A. flavus CYP51F4 (289)  TLLMAGQHSSSSISSWIMRLAS--EPAVMEELYQEQITKLSPD---G--RTL--P-PLQYRDLD-LLPLHQNLIKETLRLIHSSIHSIMRKVKSPLPV-P
        A. fumigatus CYP51F2 (285)  TLLMAGQHSSSSISAWIMRLAS--QPKVLEELYQEQLANLGPAGP--D--GSL--P-PLQYKDLD-KLPFHQHVIRETLRLIHSSIHSIMRKVKNPIPV-P
         A. nidulans CYP51F2 (285)  TLLMAGQHSSSSISAWILRLAS--EPQILEELYQEQLANLKRDPR--T--GAF--E-PLQYODLD-KLPFLQSVIKETLRVLSIHSILRKVKNFLPV-P
           A. niger CYP51F2 (288)  TLLMAGQHSSSSISSWILRLAS--EPQVLEELYQEQLASLSNR---N--GVF--E-PLQYODLD-NLPLHQNVIRETLRLHSSIHSLLRKVKNPLPV-P
          A. oryzae CYP51F2 (285)  TLLMAGQHSSSSISSWIMRLAS--QPAVVEELYQEQLANLERTGP--N--GSL--A-PLQYKFD-NLPLHQNVIRETLRLHSSIHSLLRKVKNFLLV-P
          A. oryzae CYP51F4 (290)  TLLMAGQHSSSSISSWIMRLAS--EPAVMEELYQEQITKLSPD---G--RTL--P-PLQYRDLD-LLPLHQNLIKETLRLIHSSIHSIMRKVKNPMPV-P
          A. terreus CYP51F2 (289)  TLLMAGQHSSSSISAWIFLRLAS--EPRVLEELYQEQVASLGKPDA--D--GVF--P-PLQFRDLD-RLPLHQNVVKETLRLHSSIHSIMRKVKNFLPV-P
        A. benhamiae CYP51F2 (290)  TLLMAGQHSSSSISSWIMRLAS--QPDIVEELYREQLANLPHTGP--N--GCL--A-PLQYKDLD-NLPLHQNVIRETLRLNSSIHSLMRKVKNPMPV-P
     B. dermatitidis CYP51F2 (293)  TLLMAGQHSSASISTWIMRLAS--EPTVVEKLYQEQLSNLPQTGP--N--GSL--A-PLQYKDLD-RLPLHQNVIRETLRLHTSIHSLLRKVKNPLPV-P
          C. immitis CYP51F7 (296)  TLLMAGQHTSSAISAVAILQLAA--HPEVMEGLYQEQVQVFGP-----S--K--SITLGDID-RLSFSQMVIKETLRLHGSIHSILRKVKRRPMAV-
      F. graminearum CYP51F2 (287)  TLLMGGQHSSSSSASAWIMRLAA--HPDIAEEYEEQARNLGEGDR--G--LRI--P-PLQYADLD-KLPLLRNVIKETLRLVHSSIHSILRKVKRRPMOV-P
        F. oxysporum CYP51F2 (287)  TLLMAGQHSSSSASSWIVIHLAS--SPDITEELYQEQIVNLSV----N--GAL--P-PLQYDLD-KLPLLQNVIKETLRVSSIHSILRKVKRRPQA-P
  F. verticillioides CYP51F2 (290)  TLLMAGQHSSASSWIVILHLAS--STDIAEELYQEQLINLSA----S--GAL--P-PLQYDLD-KLPLLQNVIKETLRVHSSIHSILRKVKRRPQA-P
       H. capsulatum CYP51F2 (300)  SLLMAGQHSSSTTSSWIMHLAS--EPAVVEQLYQEQLDNLPRTGP--N--GSL-G-PLQYGDIN-RLPLHRNVIRETLRLHTSIHSLLRKVMNPMPV-A
           M. oryzae CYP51F2 (290)  TLLMAGQHSSSSSAWIMRLAS--RPDIAEEVIQEVQRL-G------H-A-SLQHSDID-KLPLLANVIRETLRLVKETLRLVHSSIHSTMRKVKRPMRI-P
            M. canis CYP51F2 (289)  TLLMAGQHSSSSISSWIMRLAS--QPAVVEELFQEQLANLPRIGP--D--GSL--A-PLQYKDLD-LLPLHQNVIRETLRLNSSIHSLMRKVKNQMPV-P
          M. gypseum CYP51F2 (289)  TLLMAGQHSSSSISSWIMRLAS--QPEVVEELYQEQLANLSHTGP--D--RSL--A-SLQYKDID-NLPLHQNVIRETLRLNSSIHSLMRKVKSPLPV-P
          N. fischeri CYP51F2 (321)  TLLMAGQHSSSSISAWIMRLAS--QPKVLEELYQEQLDQLADEHG--N---I--P-KIDLQTE-KLKIHSNVKETLRLRMHNSIHSIMRKVKSPIPV-P
P. tritici-repentis CYP51F2 (289)  TLLMAGQHNSSNIASWIMHLAN--KPQLCEELYQEQDANLYL----AGN-R-GLQYQDLE-KLKLLSDVKETLRHSSIHSVMRAVNDLPI-P
          R. secalis CYP51F2 (289)  TLLMAGQHSSSSSCWIMHLAS--RPDLQEELYREQDANLYL----N--GRL-A-PLQYKDLD-SLPLHQNVIRETLRLNSSIHSLMRKVKNPMPV-P
          T. equinum CYP51F2 (289)  TLLMAGQHSSSSISSWIMRLAS--QPDILEELYREQLANLPHTGP--N--GRL-A-PLQYKDID-SLPLHQNVIRETLRLNSSIHSLMRKVKNPMPV-P
           T. rubrum CYP51F2 (289)  TLLMAGQHSSSSISSWIMRLAS--QPDIVEELYREQLANLPHTGP--N--GHL-A-PLQYKDID-SLPLHQNVIRETLRLNSSIHSLMRKVKNPMPV-P
        T. tonsurans CYP51F2 (293)  TLLMAGQHSSSSISSWIMRLAS--QPDIVEELYREQLANLPHTGP--N--GCL-A-PLQYKDID-NLPLHQNVIRETLRLNSSIHSLMRKVKNPMPV-P
       T. verrucosum CYP51F7 (296)  TLLMAGQHSSSSISSWIMRLAS--QPDIVEELYREQLANLPHTGP--N--GCL-A-PLQYKDID-NLPLHQNVIRETLRLNSSIHSLMRKVKNPMPV-P
          U. reesii CYP51F3 (296)  TLLMAGQHTSSAVSSWAMLQLAA--HPEIGEDLYQEIRVLES-----S--Q-ALTLRDID-RLTLNQMVLKETLRLHSPIHSIMRKANTMSI-P
      F. graminearum CYP51F3 (296)  AILMGGQHNTAVSGAWLLNIAH--KPHLVQELYEEQTQVLG-----S--P----Q-E-PLTWENLQ-KITLNGQVIKETLRLHSPIHSILRQVKSPMRV-P
        F. oxysporum CYP51F3 (401)  AILMGGQHNTAASGAMLLNIAH--KPHLVQELYDELEVLG-----S--P----Q-E-PLTWENLQ-KITLNGQVIKETLRLHSPIHSILRQVKSPMRV-P
  F. verticillioides CYP51F3      AILMGGQHNTAASGAMLLNIAH--KPHLVKELYDEQVELG-----S--P----Q-E-PLTWENLQ-KITLNGQVIKETLRLHSPIHSILRQVKSPMRV-P

           Consensus (401)  ALLMAGQHSSSSTSSWI LRLA  P I EELYQEQ  L  G  L P PL Y DL  KLPL  VIKETLRLH PIHSI R VK PMPV P
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This page presents a multiple sequence alignment (residues 501–600) of CYP51 family proteins.

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                                              501                                                                                         600
Homo sapiens CYP7        (374)  -----LEDGSYNIRKDDIIALYPQLMHLDPEIYPDLTFKYDRYLDENGK----------TKTTFYCNGLKLK----YYYMPFGSGAT
M. tuberculosis CYP51B1  (335)  --------QGH---RIHEGDLVAASPAISNRIPEDFFDPHDFVPARYEQPRQ-----------EDLLN--R----WTWIPFGAGRH
T. cruzi CYP51E1         (370)  --------GS---YVVKGDIIACSPLLSHHDELAFENPRLWDPERD---------------EKVD----GAFIGFGAGVH
A. thaliana CYP51G1      (369)  AR------DGK--TYDIPKGHIVATSPAFANRLPHIFKDPDTYDPERFSPGRE-----------GA----FSYIAFGGRH
H. sapiens CYP51A1       (398)  --------YTIPPGHQVCVSPTVNQRLKDSWVERLDFNPDRYLQDNPAS------------EDKAA--GA--GEKFAVPFGAGRH
R. oryzae CYP51F1-p      (105)  -------VIPL-------------------------------------------------------------------
R. oryzae CYP51F5        (376)  -----KT--GHEIPKGNFLCAVPGVTQVDSQYENEPLKYDPLRWINLTDPVHS-ME----AGDDSNIDYGFGAVGISSKNPFLPFGAGRH
C. neoformans CYP51F1    (401)  PSLSAPSE----GREIPQGDYLLAAPGVTQLDPHYFHQPTVWDPYRWSQLKDPVHQ-LE----QGEDANADYGFGVGISSKSPFLPFGAGRH
P. triticina CYP51F1     (394)  RTLASRNE----NG--QYIIPKGHYIMAAPGVSQMDPRIWQDAKVNEPARWHDEKGFAAA-AMAQY----SKAEQVDYGFGVSVKGTESPYQPFGAGRH
U. maydis CYP51F1        (410)  PTLSSPTSTKSEPDA-DT--PYIIPSSNFVLAAPTAQLDGSIWSSPHEFDPSRWLKLQSPFKA-GET----QEEMVDYGFGMISSGANSPFLPFGAGRH
C. cinerea CYP51F1       (384)  GSLAAASE----HYVIPKGHYIMAAPGVSQVDPQIWKSSDQEFDPHRWLDATTAAAM-QDS----GEDKQFDFGMISTGANSPYLPFGAGRH
C. cinerea CC1G_08364    (413)  GSLSAPSK----DK--TYIIPKGNYVLASPVVSQMDPRIWRNPEKHEPARWSDPNGVAAQ-AYSTYV---DGNGEKIDYGFGAVSKGTESPYQPFGAGKH
S. cerevisiae CYP51F1    (395)  --------NT--SYIIPAGYHYLVSPGYTHLRDEYFPNAHQEHIHRWNKDSASSY-----SV-GEEVDYGFGAISKGVSSPYLPFGGGRH
C. albicans CYP51F1      (391)  --------ET--NYIVPKGHYLVSPGYAHTSERYFDNEDEFDTFRWDTAAAKANS-VS--F---NS-SDEVDYGFGKVSKGVSSPYLPFGGGRH
C. guilliermondii CYP51F1(390)  --------GT--KYTVPKGHYVMVSPGYAMVNDRWFDAASFNPRRWDEQPLPTD-----GQ-DETVDYGFGNISKGVSSPYLPFGGGRH
C. lusitaniae CYP51F1     (391)  --------NT--SYVVPKGHHVIVSPGYAQTSERWFEKADTFDPHRWDNDWASSV-----SE-GDAVDYGFGKITKGVSSPYLPFGGGRH
C. parapsilosis CYP51F1  (391)  --------NT--KYIVPRGHHVIVSPGYAHTNERFYKDASDFNPHRWESDDTKASA-VS--F---NS--EDTVDYGFGKISKGVSSYLPFGGGRH
C. tropicalis CYP51F1    (391)  --------NT--KYVIPKGHHVIVSPGYAHTSDRWFEHDEHFNPRRWESDETAKATG-----N--EDTVDYGFGAITKVASPYLPFGGGRH
D. hansenii CYP51F1      (391)  --------NT--QYVVPKGHHVIVSPGYAMTNEKWFPEAAKFKPHRWDETAKATG-----D-SDEVDYGFGKVSKGVASYLPFGGGRH
L. elongisporus CYP51F1  (391)  --------GT--SYVIPAGYHVLVSPGYAHTNERYKNANQFFTHATDEPDKRWNDRVNEDE-----NAEQIDYGYGLVTKGAASPYLPFGAGRH
S. pombe CYP51F1         (368)  GS-------KIVIPANNYLLAAPGLTATEEEYFTHATDEPLEWNDEHIVEND------ED--EEKIDYGYGLVNKGTNSPYLPFGAGRH
A. clavatus CYP51F1      (388)  --------GT--PYVIPTSHNVLSSPGVTARSEEHFADPLEWNPHRWDEAIAVSS------ED--EEKVDYGYGLVITKGTNSPYLPFGAGRH
A. flavus CYP51F1        (388)  --------GT--SYVIPTSHNVLSSPGVTARSEEHFFPDLEWKPHRWDENIAASA------ED--EEKVDYGYGLVSKGTNSPYLPFGAGRH
A. fumigatus CYP51F1     (388)  --------GT--SYVIPTSHNVLSSPGVTARSEEHFPNPLEWNPHRWDSNPIANST-----ED--EEKIDYGYGLVSKGTNSPYLPFGAGRH
A. nidulans CYP51F1      (387)  --------GT--PYVIPTNHVLLAAPGVSADAQYFQPLWEPHRWEKESPLAPS-IVRNEEAEDADEEKIDYGYGLVSKGAASYLPFGAGRH
A. niger CYP51F1         (388)  --------GT--PYVIPTSHNVLSSPGVTARSEEYFFPNPLKWDPHRWDETIAASA------EE--EDQIDYGYGLVTKGTNSPYLPFGAGRH
A. oryzae CYP51F1        (388)  --------GT--PYVIPTSHNVLSSPGVTARSEEYFFPNPLKWDPHRWDETIAASA------ED--EEKVDYGYGLVTKGTNSPYLPFGAGRH
A. terreus CYP51F1       (388)  --------GT--PYVIPTSHNVLSSPGVTARSEEHFFPDPLKWDPHRWDETAAPST-----EE--EEEIDYGYGLVTKGTNSPYLPFGAGRH
A. terreus CYP51F1-p     (386)  --------GT--SYVIPNSHNVLSSPGFTA-SPEYFSKPLDWPHRWNGVN--AT------GD-DEQVDYGYGLVSKGAGSPYLPSGAGRH
A. benhamiae CYP51F1     (388)  --------GT--NYVPISHNLLAAPGVPSRDPQYFFDPLVWNPHRWENVVVV------EASEEKTDYGYGLVSKGANSPYLPFGSGRH
B. dermatitidis CYP51F1  (388)  --------GT--RYTIPTTHNLLAAPGVTSRLPEYFFDPMTWDPHRWETSTMEKEE-----DQNEEKLDYGYGLISKGANSPYLPFGSGRH
B. cinerea CYP51F1       (389)  --------GT--KYVIPTSHTLMASPGCTSRDDEFFEALEWDPHRWDLGSGRV--VG--N--DQDEEFQDYGYGMISKGAASYLPFGAGRH
C. globosum CYP51F1      (387)  --------GT--KYVVPTNHVLLAAPGVSAADAQYFQPDLWEPHRWEKESPLAPS-IVRNEEAEDADEEKIDYGYGLVSKGAASYLPFGAGRH
C. graminicola CYP51F1   (387)  --------GT--KYVVPTTHTLLAAPGVSGSGDPAYFFEPDQWNPHRWDADSPLAPR-LGAND---AAEEEKIDYGYGLVSKGAASYLPFGAGRH
C. higginsianum CYP51F1  (387)  --------GT--KYVVPTTHTLLAAPGVSGSGSDPTYFFEPEQWNPHRWDADSPLAPR-LGGND---ATEEETIDYGYGLVSKGAASYLPFGAGRH
C. immitis CYP51F1       (388)  --------GT--KYVVPTSHNLLAAPGVPSRMADYFFEPMLWDPHRWEKGGGVNATGVQD-----EAAEEKVDYGYGLVSKGASYLPFGAGRH
F. graminearum CYP51F1   (389)  --------GT--KYTIPTSHTLLAAPGVSATDSAYFFNPDEWDPHRWEADSPNFPR-MASK----GDDEEKIDYGYGLVSKGASPYLPSGAGRH
F. oxysporum CYP51F1     (389)  --------GT--KYTIPTSHTLLAAPGVSATDSAYFFNPDEWDPHRWEADSPNFPR-MATR----GDDEEKIDYGYGLVSKGASYLPFGAGRH
F. verticillioides CYP51F1(389) --------GT--RYTIPTTHNLLAAPGVTSRLPEHFFNPMTWDPHRWENPAMAPEG-----DQSDEKLDYGYGLISKGANSPYLPFGSGRH
H. capsulatum CYP51F1    (388)  --------GT--NYVIAKDTVLAAPGYSAGDPNHFFEPLWEPHRWEADSRLAPR-ISMSND----NDEEEKIDYGYGLVSKGANSPYLPFGAGRH
M. oryzae CYP51F1        (388)  --------GT--SYIVPTSHNLLAAPGVSRDPQYFFDPLVWNPRRWENNAGVTVV------ESSEEKTDYGYGLVSKGANSPYLPFGSGRH
M. canis CYP51F1         (388)  --------GT--NYVVPTSHNLLAAPGVPSRDPQYFFDPMLWNPHRWENNAGVTIV------ESSEEKTDYGYGLVSKGANSPYLPFGSGRH
M. gypseum CYP51F1       (388)  --------GT--AYVIPTHTLLAAPGTSRMDEHFFPDCLHWEPHRWDESPSEKYKHLSPTTALGSIAEEKDYGYGLVSKGAASPYLPFGAGRH
M. graminicola CYP51F1   (396)  --------GT--SYVIPTSHNVLSPGVTARSEEHFFPNLEWDPHRWDEDIAASA------ED--DEKVDYGYGLVNKGTNSPYLPFGAGRH
N. fischeri CYP51F1      (388)
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                                        (501)                                                                                           (600)
N. crassa CYP51F1         (387) --------GT--KYVIPTDHVLLAAPGVSATDESYFPQPDLWEPHRWEKDSPLAPT-IVRNVPSEE--DDEKIDYGYGLVSKGANSPYLPFGAGRH
P. brasiliensis CYP51F1   (388) --------GT--RYTIPVSHNLLAAPGVTSRLPEHFPDWDPHRWERMATDEAE-----DKSEEKFDYGYGLISKGGNSPYLPFGSGRH
P. tritici-repentis CYP51F1 (388) ------GT--NYVVPTSHTLMSSPGFSAQLDTHFVNPAVWDPHRWDPDQNNYDE-ERD----DADQEKIDYGWGVVSKGTNSPYLPFGAGRH
R. secalis CYP51F1        (388) --------GT--KFVIPTSHVLLASPGFSSREATYFPDPLKEPHRWDPESGGV--LG-T---EVEEESYDYGYGLISKGAKSPYLPFGAGRH
S. sclerotiorum CYP51F1   (389) --------GT--KYVIPTSHTLMASPCTSRDADYFPEPLEWDPHRWDIGSGRV--IG-N---DQDEFQDIYGWGMISKGASSPYLPFGAGRH
S. nodorum CYP51F1        (388) --------GT--NYVIPTSHNLMSSPGYSALLDSYFVNAATWDPHRWDAGQHNYDE-AEN----EASEEKTDYGYGLVSKGANSPYLPFGSGRH
T. equinum CYP51F1        (388) --------GT--NVVVPTSHNLLAAPGVPSRDPQYFPDPLVVNPHRWENNVGVTVV-----EASEEKTDYGYGLVSKGANSPYLPFGSGRH
T. rubrum CYP51F1         (388) --------GT--NVVVPTSHNLLAAPGVPSRDPQYFPDPLVVNPHRWENNVGVTVV-----EASEEKTDYGYGLVSKGANSPYLPFGSGRH
T. tonsurans CYP51F1      (389) --------GT--NVVVPTSHNLLAAPGVPSRDPQYFPDPLVVNPHRWENNAGVTVV-----EASEEKTDYGYGLVSKGANSPYLPFGSGRH
T. verrucosum CYP51F1     (388) --------GT--NVVVPTSHNLLAAPGVPSRDPQYFPDPLVVNPHRWENNENNAGVTVV----EASEEKTDYGYGLVSKGANSPYLPFGSGRH
U. reesii CYP51F1         (388) --------GI--KYVIPTHNLLAAPGVASRLPDYFPDPMLVNPHRWEKGGAVNTTGVLD---EGAEEKIDYGYGLVSKGANSPYLPFGSGRH
V. albo-atrum CYP51F1     (387) --------GT--KFVIPTSHNLLSAPGTSATDPTYFPNPAIWDFYRWLPDSPNAPT-FARAE--SADEEKIDYGYGLVSKGANSPYLPFXAGRX
V. dahliae CYP51F1        (387) --------GT--KYVVPTSHNLLSAPGTSATDPTYFPNPAIWDFTAGSPTRP--------MNRPFSSPYLPFGAGRH
A. clavatus CYP51F2       (379) --------GT--SYVIPPGRVLLASPGVTALSDEHFPNASRWDPQRWENQTTKE---DS--GEMVDYGYGAVSKGTASPYLPFGAGRH
A. flavus CYP51F4         (378) --------GT--YVIPTSHVLLAAPGVTALSDEYFPNAMAWDPHRWETQAPQEN---NK--DDIVDYGYGAMSKGTSSPYLPFGAGRH
A. flavus CYP51F2         (373) --------GT--PYVVPADHVLLASPGVTALSDEYFPNASRWDPHRWENRVEKED---E---EDIVDYGYGTVSKGTSSPYLPFGAGRH
A. fumigatus CYP51F2      (379) --------GT--PYMIPPGRVLLASPGVTALSDEHFPNAGCWDPHRWENQATKEQ---EN--DKVVDYGYGAVSKGTSSPYLPFGGGRH
A. nidulans CYP51F2       (375) --------DT--PYIIPTSHTLLASPGATALSDEYFPNANMWDPHRWENQRPDKE-----DEEGELIDYGYGAVSKRMSSPYLPFGGGRH
A. niger CYP51F2          (373) --------GT--SYIIPEDHVLLASPGVTALSDEYFPNATRWDPHRWENQPDKEE---D--GEMVDYGYGSVSKGTASPYLPFGAGRH
A. oryzae CYP51F2         (378) --------GT--PYVIPTSHVLLAAPGVTALSDEYFPNAMAWDPHRWETQAPQEN---NK--DDIVDYGYGAMSKGTSSPYLPFGAGRH
A. oryzae CYP51F4         (373) --------GT--PYVVPADHVLLASPGVTALSDEYFPNASRWDPHRWENRVEKED---E---EDIVDYGYGTVSKGTSSPYLPFGAGRH
A. terreus CYP51F2        (380) --------GT--PYVVPTSHVLLASPGVTATSDEYFPNASRWDPHRWENQAEPDD----D--GEMVDYGYGVMAKGSNSPYLPFGAGRH
A. benhamiae CYP51F2      (379) --------GT--PYVVPTSHVLLASPGVTSAQSDEHFPNAMKWDPYFNAMVWDPHRWETQEPKLE---EKEEDMVDYGYGTISKGTSSPYLPFGAGRH
B. dermatitidis CYP51F2   (380) --------GT--PYVIPRSDIVLSAPGVTALSDEYFNAMVWDPHRWETQEPKLE------EK--EDMVDYGYGTISKGTSSPYLPFGAGRH
C. immitis CYP51F7        (377) --------GT--NLVIPESHILLSAPGFTARSEDHFKDAAKWNPHRWLSVIERSE-------ADEKIDYGYGVTIKGTKSPYIPFGAGRH
C. higginsianum CYP51F2   (386) --------GS--PYVVGTDKVLLASPMVTAMSDEFFPDAGSWDPHRWDEPLAGEGA-------VDVDTTVDYGYGAVSKGTKSPYIPFGAGRH
F. graminearum CYP51F2    (374) --------NS--PYTITTDKVIMASPTVTAMSEEYFENAKTWNPHRWDNRAKEE------VDTEDVIDYGYGAVSKGTKSPYLPFGAGRH
F. oxysporum CYP51F2      (374) --------GS--PYTITTDKVLLASPTVTALSEEHFTDAQRNPHRWDNKPQEE------AVTDDVIDYGYGAVSKGTKSPYLPFGAGRH
F. verticillioides CYP51F2 (374) -------GP--PYTITTDKVILASPTVTALSEEHFDAQRNPHRWDNKPQEE-------AVTDDVIDYGYGAVSKGTKSPYLPFGAGRH
H. capsulatum CYP51F2     (380) --------GS--DYVVTPGKVIVSAPIMTHLDEEHFRDARAWEPHRWETQEPKVD-----EK--EDMVDYGYGATSKGTKSPYLPFGAGRH
M. oryzae CYP51F2         (382) --------GS--DYVVTPGKVIVSAPIMTHLDEEHFRDARAWEPHRWETQEPKED-------DDEIVDYGYGATSKGTKSPYLPFGAGRH
M. canis CYP51F2          (380) --------GT--PYVIPTSHVLIASPGITALSDEHFSNAKAWDPHRWETQEPKED-----EK--EDMVDYGYGAMSKGSSSPYLPFGAGRH
M. gypseum CYP51F2        (379) --------ST--PYVIPTSHVLLASPGVTALSDEHFPNAMEWDPYRWETQKPEGS------EKEEDMVDYGYGVTSKGSSSPYLPFGAGRH
N. fischeri CYP51F2       (379) --------GT--PYMIPPGRVLLASPGVTALSDEHFPNAGCWDPHRWENQAAKEQ------EN--DEVVDYGYGAVSKGTSSPYLPFGAGRH
P. tritici-repentis CYP51F2 (373) -----NT--SWTIPPGHALLASPGISANSEEYFYNPDKWNPHRWDDRVIEED------DE--SEMVDYGYGRMSRGTKSAYLPFGAGRH
R. secalis CYP51F2        (408) --------GT--PYVVTTDKVLIASPLVTHLSPEHFSDPEEWDPYRWATPGDIE-----K--DEEVDYGYGVISKGTRSPYLPFGAGRH
T. equinum CYP51F2        (379) --------GT--SYVVPTSHVLLASPGVTAQSDEHFPNAMKWDPYRWNAQKPNPN-----EQEEDMVDYGYGVMSKGSNSPYLPFGAGRH
T. rubrum CYP51F2         (379) --------GT--PYVVPTSHVLLASPGVTAQSDEHFPNAMKWDPYRWDAQKPNTD-----EKEEDMVDYGYGVMSKGSNSPYLPFGAGRH
T. tonsurans CYP51F2      (379) --------GT--SYVVPTSHVLLASPGVTAQSDEHFPNAMKWDPYRWDAQKPNPN-----EKENDMVDYGYGVMSKGSNSPYLPFGAGRH
T. verrucosum CYP51F2     (379) --------GT--SYVVPTSHVLLASPGVTAQSDEHFPNAMKWDPYRWDAQKPKTD----D--DDEKIDYGYGVTMKGTKGFYLSFGGGRH
U. reesii CYP51F7         (377) --------GT--DLVVPESHILLSAPGFTARSEDHFRDAVEWNPRRWLSAPEPSE------AEDGQTVDYGFGVMSKSVSSPYLPFGAGRH
F. graminearum CYP51F3    (381) --------GT--EWVVPPSHTLLSSPGTMARSEEFFPRSEWDPHRWDKIEPLVKT-----KGNGETVDYGFVMNKSVSSPYLPFGAGRH
F. oxysporum CYP51F3      (381) --------GT--DWVVPPSHTLLASPTQARSEEFFPRDMEWDPHRWDKIESLEDS-----K--NGETVDYGFGMMSKSVSSPYLPFGAGRH
F. verticillioides CYP51F3 (381) -------GT--DWVVPPSHTLLASPTQARSEEFFEREWDPHRWDKIESLDDA------K--NGETVDYGFGMMSKSVSSPYLPFGAGRH

Consensus               (501)    GT    YVIPTSH LLiASPGVTA   E FP P  WDPHRW     EE    DYGYG VSKG   SPYLPFGAGRH
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                                      601                                                              677
        Homo sapiens CYP7    (443)   ICPGRLFAIHEIKQFLIIMLSYFELEIEGQ-AKC---P--P-LDQSRAGLGILPPL-NDIEFKYKFKHL----------
  M. tuberculosis CYP51B1    (393)   RCVGAAFAIMQIKAIFSVLLREYEFEMAQP---------PESYRNDHSKMVVQ-LA-QPACVRYRRTG-----V
           T. cruzi CYP51E1   (421)   KCIQKFALLQVKTILATAFREYDFQLLRDE---------V-PDPDYHTMVVG-PTLNQCLVKYTRKKLP---S
       A. thaliana CYP51G1   (432)   GCLGEPFAYLQIKAIWSHLLRNFELEIVSP---------F-PEIDWNAMVVG-VK-GNVMVRYKRRQL---S
         H. sapiens CYP51A1   (454)   RCIGENFAYVQIKTIWSTMLRLYEFDLIDY---------F-PTVNYTTMIHT-P--ENPVIRYKRRS---K
          R. oryzae CYP51F1   (454)   RCIGEQFGLQIKTIIATIIRLFDIELEDGK-----G-V-PKSDYTSMVVVP-E-RPSNIKYTWR---E
        R. oryzae CYP51F1-p   (109)   ---------FA------------------------------------------------------------F
          R. oryzae CYP51F5   (452)   RCIGEKFGYLQIKTIIGTFVKRFDVEPLSHT---------V-FKPDYTSMVVVP-E--NSHIRYRARK---Q
      C. neoformans CYP51F1   (489)   RCVGEQFAYTQISTIFTYVRNFTLKLAVPK---------F-PETNYRTMIVQ-P-NNF-LVTFTLRNAEV---K
        P. triticina CYP51F1   (479)   RCIGEQFAYIQLSTFAATVIRNCDLELTAPE---------F-PKPDYTTMLVC-P-LKPRDIKFTRRNH----L
         U. maydis CYP51F1   (500)   RCIGEQFAYLQIGVLLATFVRIFWHLDSK---------V-PDPDYQSMVVL-PSKNGCAIVLTPRAESLH---LD
         C. cinerea CYP51F1   (474)   RCIGEQFAYLQLGTLIATIIRKLELR-VDK---------V-PEHNYHTMILM-P-KAPDVISYRRRN-----V
   C. cinerea CC1G_08364     (503)   RCIGEQFAYLQIGVLISTVIRKLELR-VDI---------I-PEHNYHTMIMM-P-KTPHTISYRRRKA----D
     S. cerevisiae CYP51F1   (469)   RCIGEHFAYCQLGVLMSIFIRTLKWHYPEGK-----T--V-PDPDFTSMVTL-PT-GPAKIWEKRNPEQK----I
         C. albicans CYP51F1   (469)   RCIGEQFAYVQLGTILASYVYNVTWELKDK-----L--I-PDPDYSSMVVL-PT-EPAEIIWEKRETCM----F
 C. guilliermondii CYP51F1   (464)   RCIGEQFAYVQLGTILATYVYNIKWHYAKGH-----S--L-PGVDYASMVTL-PL-EPADIVWEKRRTCV----F
      C. lusitaniae CYP51F1   (465)   RCIGEQFAYVQLGTILTFVYNLKWKLANGK---------V-PDVDYTSMVTL-PM-APAEIVWEKRETCV----I
   C. parapsilosis CYP51F1   (463)   RCIGEQFAYVQLGTILTTYINYFKWRLNGDK---------V-PDVDYQSMVTL-PQ-HPAEIVWEKRDTCV----I
      C. tropicalis CYP51F1   (469)   RCIGEQFAYVQLGTILSTYVYNMKWSLKDGK---------V-PDVDYQSMVTL-PL-EPAEIVWEKRDTCM----V
        D. hansenii CYP51F1   (464)   RCIGEQFAYVQLGTLLTFIYNIKWKLKGGH-----M--I-PEIDYASMVTL-PM-EPADICWEKRENCV----I
    L. elongisporus CYP51F1   (464)   RCIGEQFAYMHISTIISKFVHDYTWTLIGK-----V-PHVDYASMVTL-PE-LPAEIVWEKRDTCV----V
           S. pombe CYP51F1   (441)   RCIGEQFAMHISTIISKFVHDYTWTLIGK---------V-PNVDYSSMVAL-P-LGPVKIAWKRRN----
        A. clavatus CYP51F1   (462)   RCIGEQFAYVQLGTITAGLARLFKFRNLPDI---------E--G-I-GKSVVQFEKREPALK----A
          A. flavus CYP51F1   (462)   RCIGEQFAYVQLGATAGLVRLFKFSNLPGV---------Q--T-L-PDTDYSSLFSK-PL-GNSKIQFEKREPVTK----A
       A. fumigatus CYP51F1   (462)   RCIGEQFAYLQIGTITAVLVRLFRFRNLPGV---------D--G-I-PDTDYSSLFSK-PL-GRSFVEFEKRESATK----A
        A. nidulans CYP51F1   (462)   RCIGEQFAYVQIITVTAALVRLFKFDTVSES---------DKSS-V-PETDYSSLFSR-PA-GKCFVQYEKRNVTTK----A
           A. niger CYP51F1   (462)   RCIGEQFAYVQLGAITAALVRLFKFRNLPNV---------K--D-I-PETDYSSLFSK-PA-GKSIIQFEKRATTIK----S
          A. oryzae CYP51F1   (462)   RCIGEQFAYVQLGAITAALVRLFKFSNLPGV---------Q--T-L-PDTDYSSLFSK-PL-GNSKIQFEKREPVTK----A
         A. terreus CYP51F1   (463)   RCIGEQFAYVQLGAITAALVRLFKFSNPPGA---------N--K-L-PDTDYSSLFSK-PL-GKSYVQFEKRETNTK----A
       A. terreus CYP51F1-p   (457)   RCIGEQFAYVQLCAICVALVRLFKFKKLPNV---------E--D-V-PATNYSSLFSR-PW-ANSVQYERRDAAAK----D
       A. benhamiae CYP51F1   (464)   RCIGEQFAYVQLGTVTALARLMKWKQVEGT---------K--DV--PPTDYSSLFSK-PL-GNPMVSWEKRKQPSQ----K
     B. dermatitidis CYP51F1   (464)   RCIGEQFAYVQLGTLLVAIVRHLKLSKVENE---------T--G-V-PETDYSSLFSK-PL-GRPLVAWEKRNPTK----K
          B. cinerea CYP51F1   (466)   RCIGEQFATVQIVTIMATVRLFKFKNIDGS---------K--D-V-IGTDYASLFTR-PL-APAVVAWERR----
        C. globosum CYP51F1   (471)   RCIGENFANVQLQTIVAMTVRLFKFRNVDGS---------N--K-V-IGTDYASLFSR-EL-EPANIHWERRDK----E
     C. graminicola CYP51F1   (468)   RCIGEHFANLQLQTITAMIVRNFKFRNVDGS---------N--N-I-NGTDYASLFSR-PL-EPANIYWEKRNP----
    C. higginsianum CYP51F1   (468)   RCIGEHFANLQLQTITAMIVRNFKFRNVDGS---------N--R-I-NGTDYASLFSR-PL-EPANIYWEKRNP----
          C. immitis CYP51F1   (469)   RCIGEHFANAQLQTITAALVRALKLRKLDGD---------K--E-V-PDTDYSSLFSK-PL-GNPKVAWEKRKPSSK---Q
     F. graminearum CYP51F1   (469)   RCIGEHFANAQLQTIVAEVREFKFRNVDGG---------H--T-L-IDTDYASLFSR-PL-EPANIHWERRQQ----
       F. oxysporum CYP51F1   (469)   RCIGEHFANAQLQTIVAEVVREFKFRNVDGG---------N--T-L-IDTDYASLFSR-PL-EPANIHWERRQQ----
  F. verticillioides CYP51F1   (464)   RCIGEHFANAQLQTIVAEVVREFKFRNVDGG---------N--T-L-IDTDYASLFSR-PL-EPANIHWERRQQ----
     H. capsulatum CYP51F1   (470)   RCIGEHFANVQLQTIVAMIVREFKFRNVDGS---------G--K-V-PATDYSSLFSK-PL-GKPLVAWERRNPTQ---K
         M. oryzae CYP51F1   (464)   RCIGEHFANVQLGTVTASLVRLLKWQVEGT---------K--PEV--VGTNYASLFSR-PE-EPAKIYWERR----
          M. canis CYP51F1   (464)   RCIGEQFAYVQLGTVTATLVRLMWKQTEGT---------K--DV--PLTDYSSLFSK-PL-GSPMVAWEKRKQPSQ---K
        M. gypseum CYP51F1   (464)   RCIGEQFAYVQIQTITATMVRDFKFYNVDGS---------K--D-V-VGTDYSSLFSK-PL-GNPMVSWEKRKQPSQ---K
    M. graminicola CYP51F1   (481)   RCIGEQFAYLQIGTITAVLVRDFKFYNVDGS---------D--N-V-VGTDYSSLFSK-PL-SPAVVKWEREEKEEK---N
        N. fischeri CYP51F1   (462)   RCIGEQFAYLQIGTITAVLVRLFKFRNLPGV---------D--G-V-PDTDYSSLFSK-PL-GRSFVEFEKRESATK----A
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                                          (601)                                                                                    (677)
N. crassa CYP51F1          (470) RCIGEQFANVQLQTIAIIVRNFKFRNVDGS---D-K-V--IGTDYASLFSR-PL-EPAKIYWERREGCQ----L
P. brasiliensis CYP51F1    (464) RCIGEQFAYVQLGTLLVAIVRHLKLKELDGD---T-A-L--PETDYSSLFSK-PL-GKPLVAWEKRNPAK----N
P.tritici-repentis CYP51F1 (467) RCIGERFAYLQLQTILVAFVREFKFRNVGGS---K-D-I--VSTDYTSLFSR-PL-APGIVEWERRQN----L
R. secalis CYP51F1         (465) RCIGERFANVQLITITAVMRYFKFRNLDGS---S-K-V--VETDYTSLFSR-PL-APAVVEWEREKVK----V
S. sclerotiorum CYP51F1    (466) RCIGEQFAYLQLQTILVAFVREFKFRNVDGS---K-D-V--IGTDYTSLFSR-PL-APAVIAWERR------
S.nodorum CYP51F1          (466) RCIGEQFAYLQLQTILVAFVREFKFRNVNGS---K-D-I--VGTDYTSLFSR-PL-APGTVEWERES-----
T. equinum CYP51F1         (464) RCIGEQFAYVQLGTVTATLARLMKWKQVEGT---K--DVV--PPTDYSSLFSK-PL-GDPMVSWEKRKQPSQ----K
T. rubrum CYP51F1          (464) RCIGEQFAYVQLGTVTATLARLMRWKQVEGT---K--DVV--PPTDYSSLFSK-PF-GNPMVSWEKRKQASQ----K
T. tonsurans CYP51F1       (465) RCIGEQFAYVQLGTVTATLALMKWKQVEGT---K--DVV--PPTDYSSLFSK-PL-GDPMVSWEKRKQPSQ----K
T. verrucosum CYP51F1      (464) RCIGEQFAYVQLGTVTALLARLMKWKQVEGT---K--DVV--PPTDYSSLFSK-PL-GNPMVSWEKRKQPSQ----K
U. reesii CYP51F1          (468) RCIGEQFAYVQLGTITAALVRAILKIKKLDGD---K--E-I--PNTDYSV-------SRKGIC----H
V. albo-atrum CYP51F1      (468) RCIGEQFANVQLQTITVEIGS-QFSNADGA---S--N-I--NGTDYASLFSR-PL-EPVNIRYEREK----T
V. dahliae CYP51F2         (452) RCIGEKFAYVNIGVILATLVRHLRLSNMDGK---E--G-V--PATDYSSLFSG-PM-KPSIIQWGKRSND----LSK
A. clavatus CYP51F2        (452) RCIGEKFAYLNLAVIVATMVRHLRFSNLDGQ---T--G-V--PDTDYSSLFSG-PM-KPARIWERRAAK----SG
A. flavus CYP51F4          (446) RCIGEKFAYVNLGVIVATMARHMKLFNVDGK---K--G-V--PATDYSMFSG-PS-KPAIIGWERRFPE----KS
A. flavus CYP51F2          (446) RCIGEKFAYVNLGVILATIVRHLRLFNVDGK---K--G-I--PETDYSSLFSG-PM-KPSIIGWEKRSKN----TSK
A. fumigatus CYP51F2       (453) RCIGEKFAYVNLGVIATIVRNLKLYNVDGK---T--G-V--PATDYSMFMG-PM-KPAVVGWERRFPA----RS
A. nidulans CYP51F2        (450) RCIGEKFAYVNLGVIIATIVRHLKLFNVDGR---K--G-V--PGTDYSTLFSG-PM-KPAIVGWERRFPDNIKGSMN
A. niger CYP51F2           (446) RCIGEKFAYLNLAVIVATMVRHLRFSNLDGQ---T--G-V--PDTDYSSLFSG-PM-KPARIWERRAAK----SG
A. oryzae CYP51F2          (452) RCIGEKFAYVNLGVIVATMARHMKLFNVDGK---K--G-V--PATDYSMFSG-PS-KPAIIGWERRFPE----KS
A. oryzae CYP51F4          (446) RCIGEKFAYINLGVIVATVVRHLKLANVDGR---K--G-V--PQTDYSSLFSG-PV-KPAIIGWERRFPT----A
A. terreus CYP51F2         (446) RCIGEKFAYVNLTVIVATVLGG-------E--E-W---RTCIRLLITV-FW-TNE-TRADSLGTT----S
A. benhamiae CYP51F2       (453) RCIGEKFAYLNITVIVATMVRHMQFSNIEGK---T--G-V--PSTDYSSLFSG-PI-KPARIWRRRNAK----SD
B. dermatitidis CYP51F2    (454) RCIGEKFAYVNLGVLAVMVRHFKLRNPDNR---V--G-V--PATDYRSLLSR-PM-EPSSVHWEKRNFV----SE
C. immitis CYP51F7         (450) RCIGEKFAYLNLGVIIATIVRNFKLETMDGR---H--GVV--PPTHSSMFAR-PM-QPSKIRWTRRE-----I
C. higginsianum CYP51F2    (462) RCIGEKFAYVNLGVIVATLVRNFRLSTIDGR---P--G-V--PETDYTSLFSR-PA-QPAFIWERRKK----A
F. graminearum CYP51F2     (448) RCIGEKFAYVNLGVVATLVRNFRLSTLDGK---P--G-I--PATDYTSLFSR-PA-QPAYNWERRR------A
F. oxysporum CYP51F2       (448) RCIGEKYAYVNLGVVATLVRNFRLSTLDGK---P--G-V--PATFETSLFSR-PA-QPAYIWERRR------A
F. verticillioides CYP51F2 (454) RCIGEKFAYVNLTVVAIMVRHLQFSNIGGK---T--G-V--PRTDYSSMFSA-PM-KPARIWKRRTAK----SG
H. capsulatum CYP51F2      (454) RCIGEKFAYLNLAAIVSTLVRNFKFSTLDGK---A--T-V--PPTDYTSMFSR-PM-QEATVRWERRSPK----TA
M. oryzae CYP51F2          (454) RCIGEKFAYVNITVIVATMVRHLRVYNLDGK---R--G-V--PETDYSSLFSG-PM-KFTRIWERRAAI----SK
M. canis CYP51F2           (454) RCIGEKFAYVNLTVIVATVVRHLRLYCLEGK---K--G-V--PATDYSSLFSG-PM-KPARIWERRSNS----R
M. gypseum CYP51F2         (454) RCIGEKFAYVNLTVIVATVVRHLRLYNPEGK---D--G-I--PATDYSSLFSG-PM-KPARIHWERRPQS----H
N. fischeri CYP51F2        (453) RCIGEKFAYVNLGVLATIVRHLRLFNVDGK---K--G-V--PETDYSSLFSG-PL-KPSIIGWEKRSKD----TSK
P. tritici-repentis CYP51F2(480) RCIGEKFAFVNLLPIATLVRGYKWSLVPGGEGG---Q--I--PGTDYSSMVSR-PM-PGSRVRWEKRV----
R. secalis CYP51F2         (454) RCIGEKFAFVNLLFIATLVRGYKWSLVPGEGG---Q--L--PATDYSSLFSG-PM-KPARIWERRPQS----H
T. equinum CYP51F2         (454) RCIGEKFAYVNLTVATVVRHLRLYNDGK---K--G-V--PATDYSSLFSG-PM-KPARIWERRSNS----R
T. rubrum CYP51F2          (454) RCIGEKFAYVNLTVIVATVVRHLRLYNPEGK---D--G-I--PATDYSSLFSG-PM-KPARIHWERRPQS----H
T. tonsurans CYP51F2       (454) RCIGEKFAYVNLTVIVATVLGG-------E--E-W---CTCYRLLITV-FW-TNE-TRADSLGTT----S
T. verrucosum CYP51F2      (454) RCIGEKFAYVNLTVIVATVLGG-------E--E-W---LVF--LATDYTVSXX--------XX---XX
U. reesii CYP51F7          (451) RCIGEKFAYLNLGSDHGPSWFAISVGGTQTS---G--LVF--LATDYTVSXX--------XX---XX
F. graminearum CYP51F3     (457) RCVGENYAVAQLGAIVATFIRLVHIEQDPK-----AP-L--PAPDYSSMFSR-PM-NFAEIRWRRETV----E
F. oxysporum CYP51F3       (457) RCVGENYAVAQLGAIIATFVRLLHIEQDPN-----AP-L--PAPDYSSMFSR-PM-NFAVIRWTRRNTEA----N
F. verticillioides CYP51F3 (456) RCVGENYAVAQLGAIIASFVRLLHIEQDPK-----AP-L--PAPDYSSMFSR-PM-NFAVIRWTRRNAET----G

Consensus                  (601) RCIGE FAYVQLGTI AT VR  K   DG   V  P TDYSSLFS P P I WERR
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