INVESTIGATING THE MOLECULAR MECHANISMS OF INSECTICIDE RESISTANCE IN THE TOMATO LEAF MINER, *TUTA ABSOLUTA*

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Abstract

Tuta absoluta is an economically significant pest of tomatoes, which has undergone a rapid expansion in its range during the past six years. One of the main means of controlling this pest is through the use of chemical insecticides including pyrethroids and spinosad. However, their intensive use has led to the development of resistance. The aim of this PhD was to understand the mechanisms underlying resistance to pyrethroids and spinosad.

The target site of pyrethroids, the sodium channel, was cloned and three known knockdown resistance mutations, L1014F, M918T and T929I were found. High-throughput diagnostic assays were developed and the prevalence of the three mutations was then assessed. All three mutations were found at high frequencies in populations across the range of *T. absoluta*. Additionally, a fourth novel mutation L925M was found in 14% of samples. Therefore, pyrethroids are unlikely to be effective at controlling *T. absoluta*.

Bioassays were conducted to determine the sensitivity of five populations of *T. absoluta* to spinosad. One population, from an area where control failure using spinosad was reported in 2012, exhibited a high level of resistance after selection in the laboratory with spinosad. Synergist bioassays did not show enhanced activity/expression of P450s and esterases. The transcriptome of *T. absoluta* was sequenced and used, in combination with degenerate PCR, to identify the target site of spinosad, the nicotinic acetylcholine receptor (nAChR) α6 subunit. Analysis of Taα6 revealed that two mutually exclusive exons (3a and 3b) that encode loop D of the ligand binding domain are both absent in all transcripts from the selected strain. Additionally, QPCR showed that α6 is down regulated in both larvae and adults of the selected strain. Taken together this study has provided new data on the molecular basis of resistance of *T. absoluta* to pyrethroids and spinosad.

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List of abbreviations

Ace = acetylcholinesterase

CCE = Carboxyl/choline esterase

cDNA = complimentary deoxyribonucleic acid

DEseq2 = Differential gene expression sequencing analysis 2

DNA = deoxyribonucleic acid

dNTPs = deoxyribo-nucleoside triphosphates

EdgeR = Empirical analysis of digital gene expression data

EDTA= Ethylenediaminetetraacetic acid

Et-Br = Ethidium Bromide

FastQC = fast quality control

g = gram

GABA = gamma-Aminobutyric acid

gDNA = genomic DNA

Gem5 = Gemini 5

GSTs = Glutathione S tranferases

IRAC = Insecticide Resistance Action Committee

Kdr = knock-down resistance

L = litre

LB = Lysogeny broth

LC50 = Lethal concentration for 50% of insects in a population

min = minute

mg = milligram

ml = millilitre

ng = nanogram

nAChR = Nicotinic acetylcholine receptor

P450s = cytochrome P450s

PBO = Piperonyl butoxide

PCR = Polymerase chain reaction

QPCR = Quantitative polymerase chain reaction

RACE = Rapid amplification of cDNA ends

rbp1 = RNA-binding protein 1

RNA = ribonucleic acid

sec = second

sKdr = super knock down resistance

SNP = single-nucleotide polymorphism

SWAP = protein suppressor of white apricot

Taq polymerase = *Thermus aquaticus* polymerase

TGAC = The Genome Analysis Centre (Norwich)

Tm = melting temperature

TM = transmembrane domain

µl = microlitre

µM = micro molar

Introduction

1.1 Food security

Currently, 11% of the world's population are undernourished (FAOSTAT, 2015) and by 2050, it is expected that the world will need between 50 and 100% more food, due to a growing population (The Royal Society, 2009). In 2013 the world population was 7.2 billion and this is expected to rise to 9.6 billion in 2050 and 10.9 billion by the end of the century (Bongaarts, 2015). In addition to having more people to feed, the amount of land available to grow food is threatened by urbanisation, desertification, soil erosion and climate change (Godfray et al., 2010). Modelling the effects of changing temperature and precipitation on crop yields in 12 regions with high human malnourishment found that many crops are expected to be adversely affected by climate change. South Asian wheat and rice, Southern African maize and wheat, West African ground nut, Brazilian rice and wheat, and Central American rice and wheat all have a greater than 95% probability of decreased production by 2030 (Lobell et al., 2008).

One way to increase food production is to reduce the yield gap. The yield gap is defined as the difference between actual productivity and the highest productivity that could be achieved with the best possible management strategy (Godfray et al., 2010). One factor affecting the yield gap is the loss of crops due to abiotic factors such as light, water, temperature and nutrients, and biotic factors including weeds, fungi, bacteria, viruses, and animal pests. As well as directly reducing crop yield, pests can negatively affect the quality of harvested crops meaning that less of the harvest is fit for consumption (Oerke, 2006). During the period 2001-2003 an estimated 40% of maize, 37% of rice, 40% of potatoes and 28% of wheat crop was lost to weeds, animal pests and disease. If no pesticides had been used, the potential yield loss would have been 50% in wheat, 69% in maize and more than 70% in rice and potatoes (Oerke, 2006).

1.2 Insect pests

Many insect pests attack crops worldwide, for example there are currently 457 on the UK plant health risk register (Defra, 2015). Furthermore non-native pests can spread to new areas through natural migration or through human transport of goods between countries e.g. plants, fruit and vegetables and wood furniture. Insects are serious pests of both food and non-food crops and in certain cases are also important vectors of human and animal diseases. Damage to crops can be through direct feeding damage or through indirect routes such as the transmission of plant viruses (Navot et al., 1991, Hogenhout et al., 2008). Large numbers of insects are required to cause direct damage to plants when feeding. However, indirect damage can be caused by a single insect infecting an entire plant with a virus (Jones and Jones, 1984). An example of an insect causing direct damage is the diamondback moth, *Plutella xylostella,* a serious pest of cruciferous crops including turnips, cabbage, broccoli, Brussel sprouts and swedes where the larvae eat large amounts of the foliage (Jones and Jones, 1984). On the other hand the peach-potato aphid, *Myzus persicae* can cause indirect damage to crops through the transmission of viruses. It is able to transmit over 100 viruses and infests a wide range of crops making it one of the world's most destructive pests (Jones and Jones, 1984).

1.2.1.1 *Tuta absoluta*

T. absoluta (Meyrick) is a diploid pest in the Lepidoptera order, family Gelechiidae. As the larvae feed they 'mine' the leaves, stem and fruit of *Solanum lycopersicum* (Tomato), causing significant damage and leading to up to 100% yield loss if not controlled (Desneux et al., 2010). Whilst tomato is the preferred host, *T. absoluta* has been shown to be capable of developing, reproducing and increasing in population on *S. tuberosum* (potato), making this a potential alternative host (Pereyra and Sánchez, 2006). Larvae can also mine other cultivated Solanaceae including *S. melongena* (aubergine), *S. muricatum* (pepper), *Physalis peruviana* (Cape gooseberry) and *Nicotiana tabacum* (tobacco) (Urbaneja et al., 2013, Garzia et al., 2011). Wild Solanaceae such as *S. bonariense, S. nigrum*, *Datura ferox, D. stramonium* can also act as secondary hosts (Urbaneja et al., 2013). In addition, *T. absoluta* has been

recorded on *Phaseolus vulgaris* (bean) in the family Fabaceae (Garzia et al., 2011). The ability of *T. absoluta* to inhabit non-cultivated plants facilitates the its dispersal into new areas and could additionally provide population reservoirs in the absence of cultivated crops (Cifuentes et al., 2011). *T. absoluta* reproduces sexually and is multi-voltine, with the length of the life-cycle dependent on temperature (Urbaneja et al., 2013). The average development time ranges from 76 days at 14°C to 24 days at 27°C (Barrientos et al., 1998) in (Desneux et al., 2010). When reared on tomato at 25°C, the mean generation time is 28 days, with an average of approximately 130 eggs per female (Pereyra and Sánchez, 2006). The combination of a short life cycle and high reproductive potential can result in a rapid increase in population numbers in a short period of time (Garzia et al., 2011).

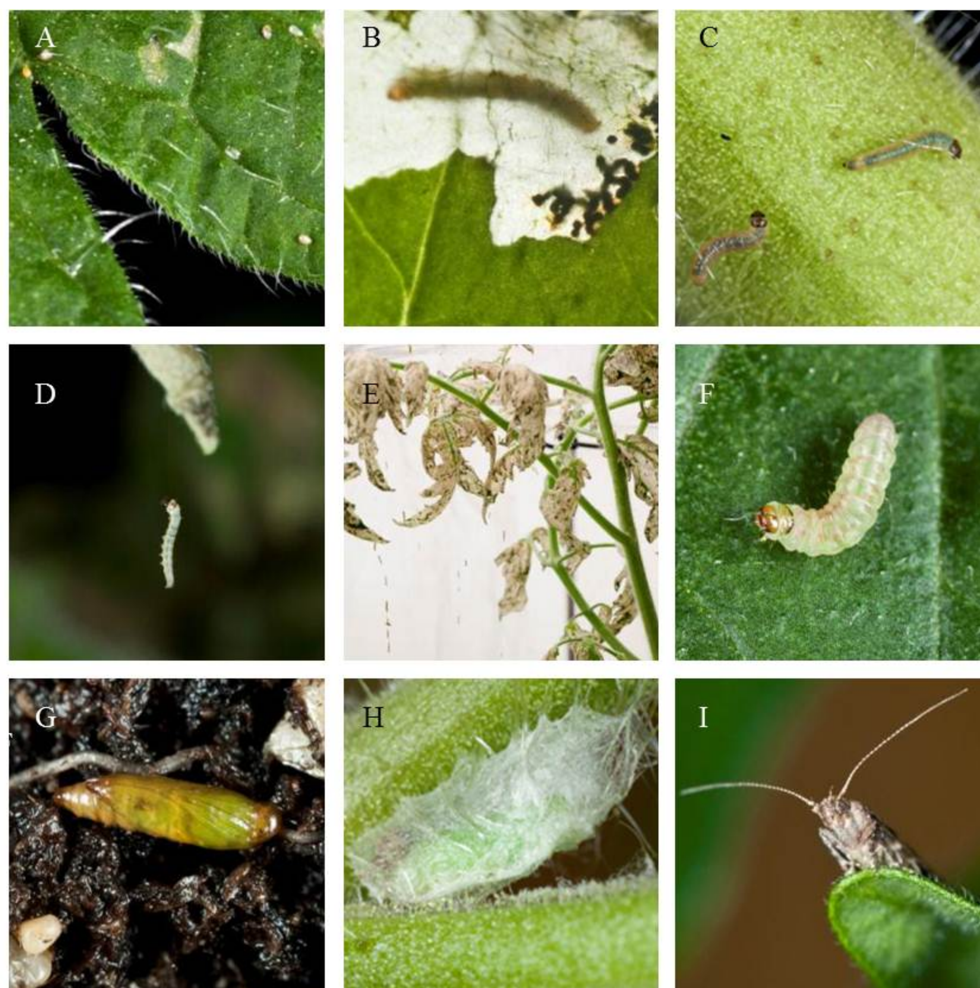

Figure 1.1. *T. absoluta*, the tomato leaf miner. A) Eggs B) Leaf-mine C) L2 Larvae D) Larva suspended from silk-thread E) Mined *Solanum lycopersicum* F) L4 Larva G) Pupa in soil H) Pupa on plant H) Adult. Photos Copyright Rothamsted Research.

T. absoluta has four life stages – egg, larvae, pupae and the adult moth (Figure 1.1). The eggs are laid on both sides of the leaf and measure 0.2 mm diameter and 0.4 mm length. They are creamy-white when first laid but turn yellow prior to hatching (Urbaneja et al., 2013). Within the larval stage there are four instars - L1, L2, L3 and L4. After hatching, the L1 larvae take approximately an hour to mine into the leaf (Cuthbertson et al., 2013). The larvae eat the leaf mesophyll but leave the epidermis intact. The larvae are c. 1.6 mm initially and cream-coloured, growing to c. 2.8 mm as second instar larvae, c. 4.7 mm in the third instar and c. 8 mm in the fourth instar (Urbaneja et al., 2013). The larvae can drop to the ground using a silk thread in order to pupate or to look for fresh leaf material. Most larvae pupate in the soil but some pupate on the leaves or stem of the plant, enclosed in a silk cocoon. The pupae are c. 4.3 mm in length and greenish becoming darker as they get close to adult emergence (Urbaneja et al., 2013). Adults are c. 7 mm with filliform antenna, silver-grey scales and black spots on the anterior wings. Females are normally larger than males (Urbaneja et al., 2013).

T. absoluta is native to South America, and was first described in Peru in 1917. It has been referred to previously as *Phthorimaea absoluta, Gnorimoschema absoluta, Scrobipalpua absoluta and Scrobipalpuloides absoluta* (Guedes and Picanço, 2012). Common names include the South American tomato pinworm, tomato borer, and tomato leafminer (Guedes and Picanço, 2012, Desneux et al., 2010, Urbaneja et al., 2013). Agricultural trade is thought to have aided the spread of *T. absoluta* between South American countries and it has been an agricultural pest in Ecuador, Chile, Columbia and Argentina since the 1960s, Bolivia, Paraguay and Uruguay since the 1970s and Brazil since the 1980s (Guedes and Picanço, 2012).

The first report of *T. absoluta* in Europe was in Spain in 2006. By 2008 it had also been detected in Italy and France as well as North Africa (Morocco and Algeria). In 2009 it spread to the Netherlands, Portugal, Tunisia, Libya, Germany, Switzerland, Greece, Romania, Bulgaria, Cyprus, Turkey, Albania, Bahrain, Kuwait, Malta, Denmark and the UK and by the end of 2011, *T. absoluta* had invaded 35 countries in Europe, North Africa and Asia (Desneux

et al., 2011). A recent microsatellite analysis found high genetic homogeneity between all European, Middle Eastern and North African samples but variability between different populations within South America (Guillemaud et al., 2015). This suggests a single point of origin for all *T. absoluta* in Europe, North Africa and the Middle East. Phylogenetic analysis clustered the non-native samples with those from central Chile, implicating that this is the likely source of *T. absoluta* in the Mediterranean basin (Guillemaud et al., 2015). From 2009-2012 there were 41 outbreaks of *T. absoluta* in the UK (Cuthbertson et al., 2013) where most tomato plants are grown in glasshouses. A small laboratory study found that adults of *T. absoluta* have the potential to survive for more than 30 days at a temperature of 10°C when provided with sucrose solution (Cuthbertson et al., 2013). The estimated minimum thermal requirement for *T. absoluta* is 7-10°C for eggs, 6-8°C for larvae and 9°C for pupae with 454-463 degree days needed for development from eggs to adult (Urbaneja et al., 2013). The ability to survive low temperatures may allow *T. absoluta* to disperse over large distances between glasshouses in temperate countries like the UK (Cuthbertson et al., 2013). A map showing the current distribution of *T. absoluta* and main tomato-producing regions is shown in Figure 1.2. In 2013 the world produced 163,963,770 tonnes of tomatoes and the three highest producing countries were China, India and the USA (FAOSTAT, 2013). *T. absoluta* has not been detected in these three countries to date, so prevention of spread to these areas is of upmost importance.

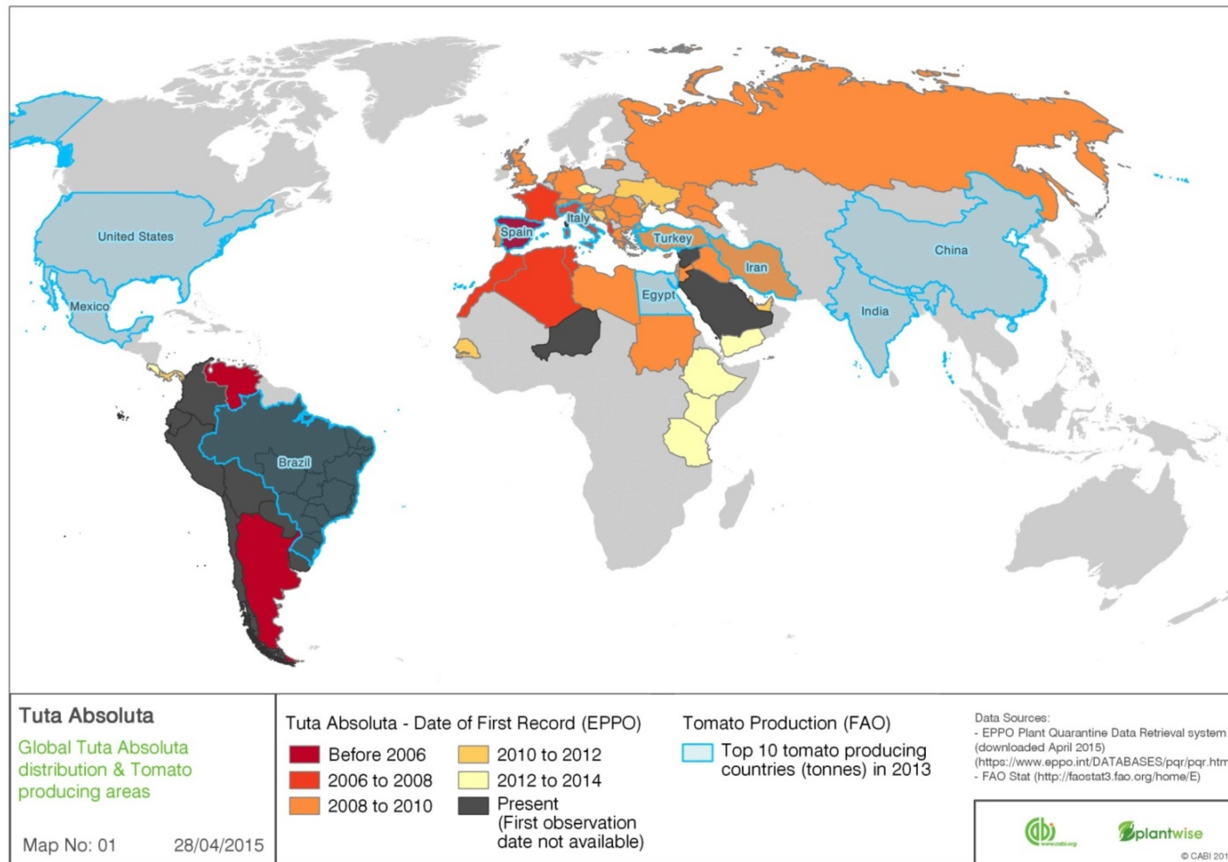

Figure 1.2. Map of *T. absoluta* distribution and tomato producing areas. Copyright CABI 2015. Reprinted with permission from CABI.

1.3 Control of insect pests

1.3.1 Biological control

Biological control of insects uses natural predators, parasitoids or pathogens to regulate the density of host pests (Bale et al., 2008). There are three main types of such control: classical, conservation and augmentative (Bale et al., 2008). Classical biological control is normally used to tackle invasive species which have spread to new regions. Natural enemies from the pests' place of origin are collected and released into the new environment and gradually increase in numbers until they can control the pest (Caltagirone, 1981). Conservation control is used to manage native pests by assisting natural enemies, for example through the provision of overwintering refuges, or additional food sources (Mensah, 1997, Corbett and Rosenheim, 1996). These types of biological control work best on perennial crops or in forests as they rely on the control species becoming permanently established. For short-term crops augmentative control is more effective (Bale et al., 2008). Large numbers of the biological control species are released so that they outnumber the pest species and can control the pest quickly. More biological control agents are released at regular intervals (Figueiredo et al., 2015). For example egg parasitoids in the genus *Trichogramma* are used in the augmentative control of a wide range of lepidopteron pests (Bale et al., 2008). Three releases of *T. pretosium* to control the fall armyworm *Spodoptera frugiperda* were found to significantly increase the yield of organic maize plots in Brazil (Figueiredo et al., 2015).

One of the advantages of biological control is that the control organisms are usually fairly specific to the pest species, and therefore do not harm beneficial non-target insects. Furthermore, some predators actively search for their prey whereas, in contrast to insecticides must be applied directly to the affected crops (Bale et al., 2008). However, biological control is normally slower acting than conventional insecticides and often doesn't completely eradicate the pest. Additionally, manufacturing and distribution of biological control can be difficult and the shelf life of most natural enemies is lower than that of insecticides (Bale et al., 2008). Moreover, some biological control species can

damage crop plants. For example *Nesidiocoris tenuis* is a predatory bug used to control whitefly and moths, but is phytophagous under certain conditions, causing necrotic rings on the crop plants (Mollá et al., 2011).

1.3.1.1 Biological control of *T. absoluta*

Natural enemies from 15 genera and 9 different families have been reported to attack *T. absoluta* eggs or larvae in the Mediterranean basin (Urbaneja et al., 2012). Parasitic wasps in the genus *Trichogramma* are natural parasites of *T. absoluta* eggs in both South America and Europe and *T. achaeae* is commercially available to control *T. absoluta* in some countries in Europe and North Africa. However, large numbers of *T. achaeae* must be released every week for successful parasitism (Chailleux et al., 2012). European mirid bugs including *Macrolophus pygmaeus* and *N. tenuis* prey on eggs and larva of *T. absoluta.* Laboratory experiments showed that female mirid bugs can consume over 50 eggs per day and males over 30 eggs per day, depending on the density of eggs. Whilst they can also eat larvae, the numbers predated are much lower, with an average of about two L1 larvae or one L1-L3 larva consumed in 24 hours (Urbaneja et al., 2009). Natural populations of *M. pygmaeus* and *N. tenuis* have been observed consuming *T. absoluta* in fields in Spain (González-Cabrera et al., 2011). These predators also prey on whiteflies, so can be used to manage both whiteflies and *T. absoluta* (Urbaneja et al., 2012). In South America, the mirid bugs *M. basicornis, Campyloneuropsis infumatus* and *Engytatus varians* all displayed high predation of *T. absoluta* eggs, consuming 50-100 eggs per day under laboratory conditions (Bueno et al., 2013). However these species were not found in tomato crops in the field, possibly due to high rates of insecticide application in the tomato crops (Bueno et al., 2013).

The bacteria *B. thuringiensis* can also be used to control *T. absoluta*. In laboratory experiments there was significantly less leaf damage in tomato plants treated with a *B. thuringiensis* formulation than in untreated controls. First instar larvae were the most sensitive with just 1% of the area of leaves damaged when the larvae were exposed to *B. thuringiensis,* whereas 77% of the area of control leaves was mined (González-Cabrera et al., 2011). In glasshouse experiments, no damaged fruit was obtained in areas sprayed with

B. thuringiensis compared to an average of three fruits per plant damaged in the control crops. In open-field conditions significantly fewer infested leaflets and infested fruits were recorded in *B. thuringiensis*-treated plots compared to non-treated (González-Cabrera et al., 2011). The use of *B. thuringiensis* early in the growing season can be combined with the release of the predatory bug *N. tenuis* to provide effective control of *T. absoluta*. Once *N. tenuis* has established *B. thuringiensis* sprays are no longer required to keep *T. absoluta* under control. Plants treated with *B. thuringiensis* once a week for two months in combination with a single release of *N. tenuis* had no fruit damage and higher yields than control plants and half of the fruit from untreated tomatoes were infested with *T. absoluta* (Mollá et al., 2011).

1.3.2 Chemical control

Synthetic insecticides are the main method used in the control of insect pests of crops, livestock, humans and pets. Most insecticides target proteins in the insect nervous system (Casida and Durkin, 2013) such as the voltage-gated sodium channel, the nicotinic acetylcholine receptor (nAChR), acetylcholinesterase (AChE) and the *gamma*-aminobutyric acid (GABA) receptor (Casida and Durkin, 2013).

1.3.2.1 Chemical control of *T. absoluta*

Despite attempts to control *T. absoluta* with biological agents, at present insecticides are the main way of managing populations and twelve different classes of chemical are registered for control (IRAC, 2011), Table 1.1. As a result the insecticides have been used intensively, which has led to the development of resistance to many classes of insecticides. In Brazil in 1997-1998 farmers were spraying tomato crops with insecticides 7- 22 times per cultivation cycle. A comparison of resistance between field populations collected in Brazil in this time period found resistance ratios (the concentration required to kill 50% (LC_{50}) of the most resistant population / LC_{50} most susceptible population), of 7 for permethrin, 9 for abamectin, 4 for methamidophos and 22 for cartap. A significant positive correlation between the number of sprays of a particular insecticide at a given location and the

resistance of *T. absoluta* in that location to that insecticide was found for abamectin, cartap and permethrin. However, this correlation was not observed for the organophosphate methamidophos (Siqueira et al., 2000b).

Two glasshouse populations of *T. absoluta* (Bella Vista and Rosario) collected in 2000 in Argentina showed resistance to deltamethrin and abamectin but there was no resistance to methamidophos. The resistance ratio to abamectin was 2.5 and 3.6 in Rosario and Bella Vista respectively. The resistance to deltamethrin in both populations was so high that most larvae were alive at the highest dose tested, close to the solubility limit of the insecticide, so the exact resistance ratio could not be determined. The Rosario population had received 16 sprays of which 12 were pyrethroids (deltamethrin or λ-cyhalothrin), so the resistance observed was unsurprising. The Bella Vista population had received seven sprays of abamectin but just one of pyrethroid in the past year. The authors suggested that the pyrethroid resistance in this strain could be due to migration of resistant insects from nearby glasshouses or cross-resistance between abamectin and pyrethroids (Lietti et al., 2005).

A more recent study of Brazilian populations of *T. absoluta* published in 2011, found significant resistance in at least one population to six classes of insecticide: avermectins, spinosyns, pyrethroids, oxadiozines, benzoylureas and *B. thuringiensis* (Silva et al., 2011). It is important to note that significant resistance means that there is variability between populations, but doesn’t necessarily equate with control failure in the field. Therefore, the authors predicted the likelihood of control failure by estimating the percentage mortality of insects treated with the recommended label rate of insecticide. Mortality was predicted to be significantly lower than 80% in at least one population for the insecticides bifenthrin, indoxacarb, permethrin, diflubenzuron, teflubenzuron, triflumoron and *B. thuringiensis*. In contrast, all populations had 100% estimated mortality at the label rate of abamectin and spinosad (Silva et al., 2011).

Table 1.1. Insecticide classes registered for use against *T. absoluta* (IRAC, 2011).

MOA Group	Chemical class	Target	Example
1	Organophosphates	Acetylcholinesterase	Methamidophos
3	Pyrethroids	Sodium channel	Deltamethrin
5	Spinosyns	NAChR	Spinosad
6	Avermectins	Chloride channel	Abamectin
11	Microbes	Insect midgut	*Bacillus thuringiensis*
13	Pyrroles	Oxidative phosphorylation	Chlorfenapyr
14	Nereistoxin analogues	NAChR	Cartap
15	Benozylureas	Chitin biosynthesis	Diflubenzuron
18	Diacylhydrazines	Ecdysone receptor	Tebufenozide
22	Oxadiozines	Sodium channel	Indoxacarb
28	Diamides	Ryanodine receptor	Chlorantraniliprole
Un	Tertranortiterepenoid	Unknown	Azadirachtin

1.3.2.2 Pyrethroids

The first synthetic insecticide was DDT which targets the voltage-gated sodium channel. Pyrethrins, natural flower extracts used in insecticide control, and synthetic pyrethroids (Figure 1.3) also target this protein. The insect voltage-gated sodium channel was originally cloned from *Drosophila melanogaster* and named '*para*' due to its position within the paralysis locus on the x chromosome (Loughney et al., 1989). Sodium channels are made up of four transmembrane domains (I-IV), each with six segments (S1-S6) connected by intracellular or extracellular loops (Catterall, 1988). The S1-S4 helices form voltage-sensing domains, whilst S5 and S6 create a central ion-conducting pore

(Payandeh et al., 2011). Sodium channels have three states: deactivated (closed), active (open), and inactivated (Bezanilla and Armstrong, 1977). At resting potential the activation-gate, consisting of S6 helices, blocks the channel, keeping it closed (Payandeh et al., 2011). The sodium channel activates in response to depolarisation of the membrane as a result of nerve stimulation (Hodgkin and Huxley, 1952). During activation the S4-S5 linker and voltage-sensing domain are thought to rotate together, pulling the S5-S6 helices outwards to open the channel pore (Payandeh et al., 2011). The pore contains a selectivity filter which allows Na+ ions to flow through (Payandeh et al., 2011). After a few milliseconds the inactivation-gate closes, so that the sodium channel becomes inactive (Bezanilla and Armstrong, 1977). Membrane repolarisation then reverses the conformational change of the activation-gate so that it again blocks the pore. Finally, after a short refractory period the inactivation-gate re-opens bringing the channel back to its resting (deactivated) state (Davies et al., 2007).

Pyrethroids are predicted to dock between the IIS5 and IIS6 helices of the sodium channel, surrounded by amino acids on the IIS4-S5 linker (Met^{918}), IIS5 helix (Leu^{925}, Thr^{929} and Leu^{932}) and the cytoplasmic end of IIIS6 (O'Reilly et al., 2006). When pyrethroids bind they are thought to stabilise the open-state of the channel, prolonging the period of time in which the channel is open and conducting sodium ions, creating a state of hyper-excitability (O'Reilly et al., 2006). This causes incapacitation of the insect, known as ‘knock-down’(Sawicki, 1962). Eventually this hyper-excitability rises too high for the cell to maintain the activity of the sodium pump. Insects exposed to lethal doses of pyrethroids display symptoms of uncoordinated movement, followed by paralysis and then death (Davies et al., 2007).

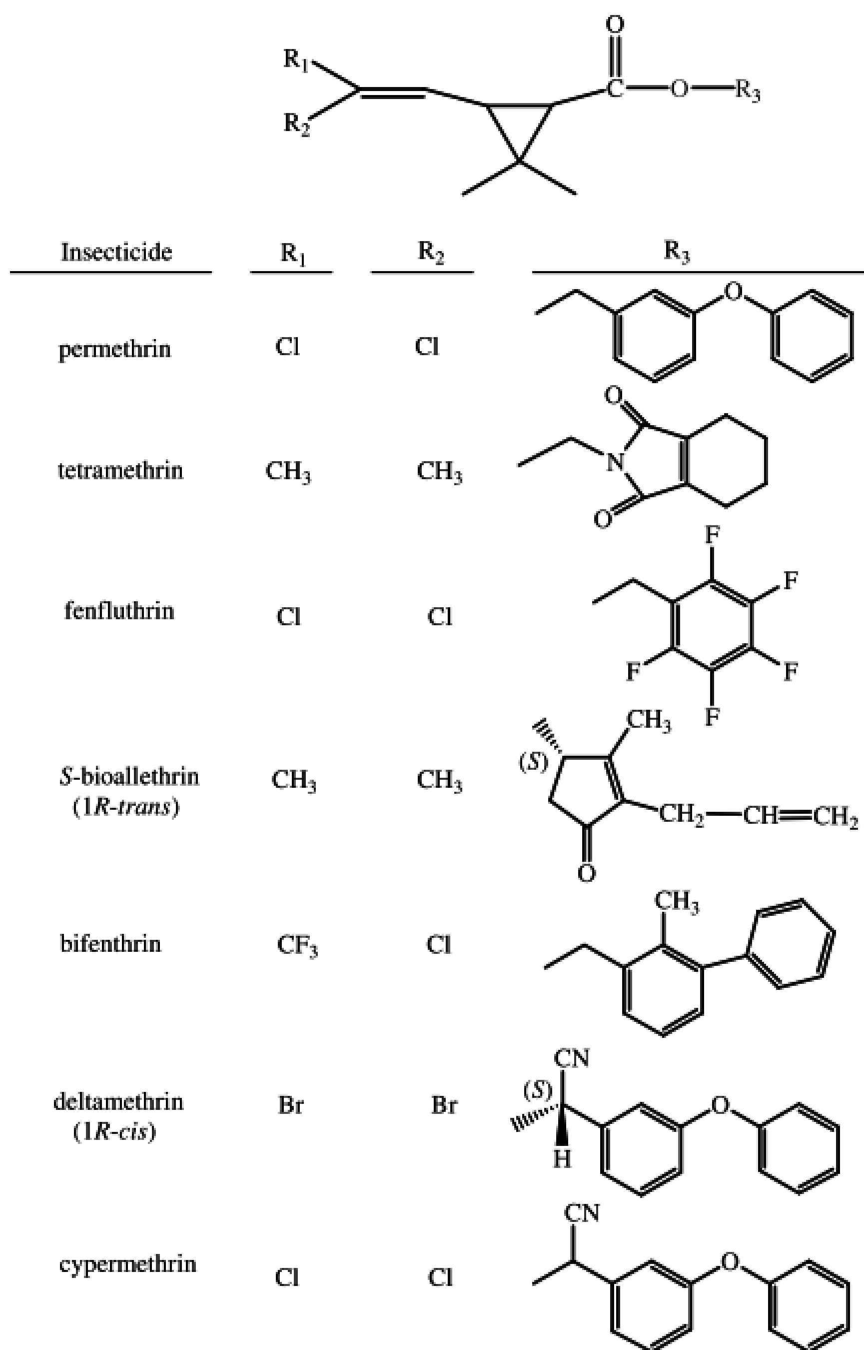

Figure 1.3. Chemical structures of pyrethroid insecticides which target the sodium channel of insect nervous systems. From (Hardstone et al., 2007) reprinted with permission from AAAS.

1.3.2.3 Spinosyns

Spinosyns are secondary metabolites derived from the soil bacteria *Sachharopolyspora spinosa*. The insecticide spinosad is a mixture of spinosyn A and spinosyn D (Figure 1.4). Contact or ingestion of spinosad causes

involuntary muscle contractions, tremors and paralysis in insects, leading to death. Many insect pests can be controlled with spinosad including *Ostrinia nubilalis*, *P. xylostella*, *Spodoptera frugiperda*, *Leptinotarsa decemlineata* and *Thrips palmi* (Thompson et al., 2000). A second spinosyn insecticide, Spineoraturm, was registered in 2007 (Dripps et al., 2008). Spinosyns target the nAChR a member of the transmitter-gated ion channel family that is also the target site of neonicotinoids and nereistoxin analogues (Nauen et al., 2012).

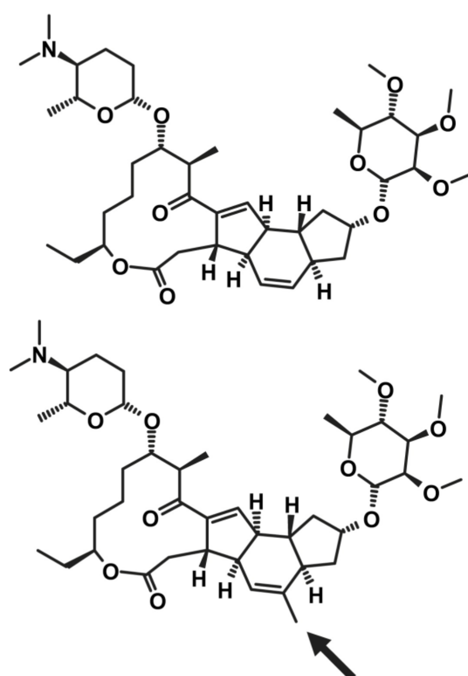

Figure 1.4. Structure of spinosyn A (top) and spinosyn D (bottom). The arrow indicates the area of difference between the two compounds. From (Watson et al., 2010) reprinted with permission from AAAS.

nAChRs bind acetylcholine, a neurotransmitter which causes conformational change in acetylcholine receptors to allow the influx of sodium ions and the efflux of potassium ions. The nicotinic prefix nAChR refers to their sensitivity to the plant toxin nicotine as opposed to muscarinic AChRs which instead show sensitivity to the mushroom toxin muscarine (Casida and Durkin, 2013). nAChRs are pentamers with a cation-selective pore (Figure 1.5). There are four transmembrane domains within each subunit, with the second transmembrane domain forming the gate of the closed channel (Miyazawa et al., 2003). In

vertebrates, nAChRs are formed from a combination of α, β, δ, γ and ε subunits. Different subunit combinations are found in muscular and neuronal nAChRs (Tomizawa and Casida, 2001). The diversity of possible subunit combinations is less well-understood in insects. Most insects have around ten genes encoding different subunit types and in the model lepidopteran, *Bombyx mori*, 9 α and 3 β subunit genes have been identified (Shao et al., 2007).

Spinosads target the α6 subunit (Perry et al., 2007, Watson et al., 2010) and functional expression studies in *Xenopus* oocytes found that the α6 subunit of *D. melanogaster* could not be expressed on its own, but co-expression of α5 and α6 with the chaperone protein ric-3 produced a heteromeric nAChR activated by spinosyn A, spinetorum, acetylcholine and nicotine (Watson et al., 2010). The neonicotinoid imidacloprid did not activate this receptor, indicating that neonicotinoids bind to alternative subunits (Watson et al., 2010).

Within the nAChR α6 subunit there is considerable protein diversity arising from alternative splicing and RNA-editing (Grauso et al., 2002, Jin et al., 2007). Most insects have two variants of exon 3 which are alternatively spliced in a mutually exclusive manner (Grauso et al., 2002, Jin et al., 2007). *B. mori* produces transcripts containing both exon 3a and 3b, and the frequency of these transcripts increases with developmental stage (Jin et al., 2007, Shao et al., 2007). Additionally, insects have between two and four versions of exon 8 which undergo mutually exclusive splicing (Grauso et al., 2002, Jin et al., 2007, Rinkevich and Scott, 2009). Eighteen different isoforms produced by alternative splicing were observed in *T. castaneum* (Rinkevich and Scott, 2009). RNA-editing is also common in the nAChR α6 subunits of insects. This was first found in *D. melanogaster*, where seven A-to-I editing sites were observed in exons 5 and 6 of the α6, which encodes loop E of the acetylcholinesterase binding site (Grauso et al., 2002). There are ten RNA-editing sites exist in the α6 subunit of *B. mori*, of which seven are located in exon 5 (Jin et al., 2007). Some RNA-editing sites in α6 are evolutionarily conserved among insects from four different orders (Jin et al., 2007).

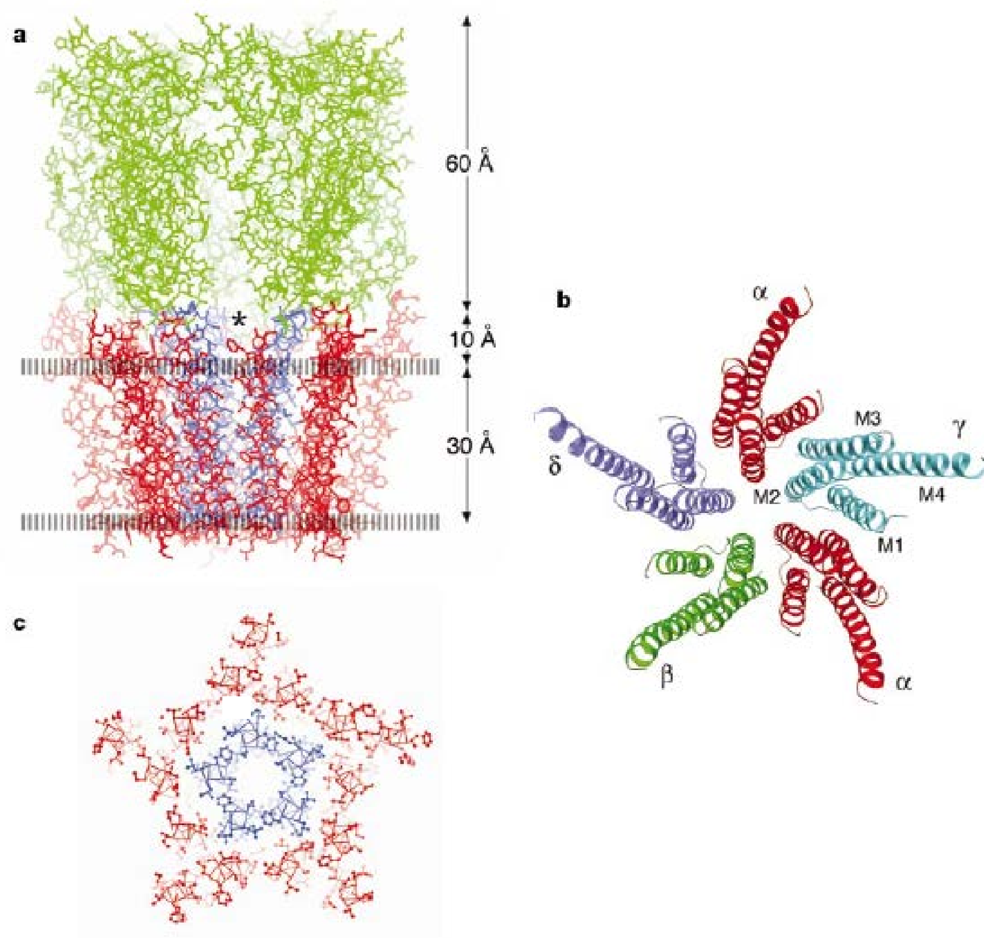

Figure 1.5. Structure of the *Torpedo marmorata* nAChR. a) View showing the receptor in relation to the membrane surface (broken lines). Blue = pore facing helices; red = lipid-facing helices, green = β-sheet structure comprising the ligand binding domain. b) Stereo view of the pore, as seen from the synaptic cleft. The five subunits are shown in different colours (α = red; β = green; γ = cyan; δ= blue). c) Cross-sectional view through the pentamer at the middle of the membrane. Blue = pore facing helices; red = lipid-facing helices. From (Miyazawa et al., 2003) reprinted with permission from AAAS.

1.4 Regulation of Splicing

Splicing of precursor mRNA is an important regulatory step of gene expression. During this step introns are removed and exons are joined together, creating mature RNA. Alternative splicing, the inclusion of different exons in mRNA, generates different isoforms from a single gene. This means a single gene can encode multiple distinct protein products (Smith and Valcárcel, 2000, Keren et al., 2010). Alternative splicing can also act as an on–off gene expression switch by the introduction of premature stop codons (Smith and Valcárcel, 2000). There are a number of types of alternative splicing. These include intron retention, exon skipping, alternative splice site selection and mutually exclusive exons (Figure 1.6), (Keren et al., 2010).

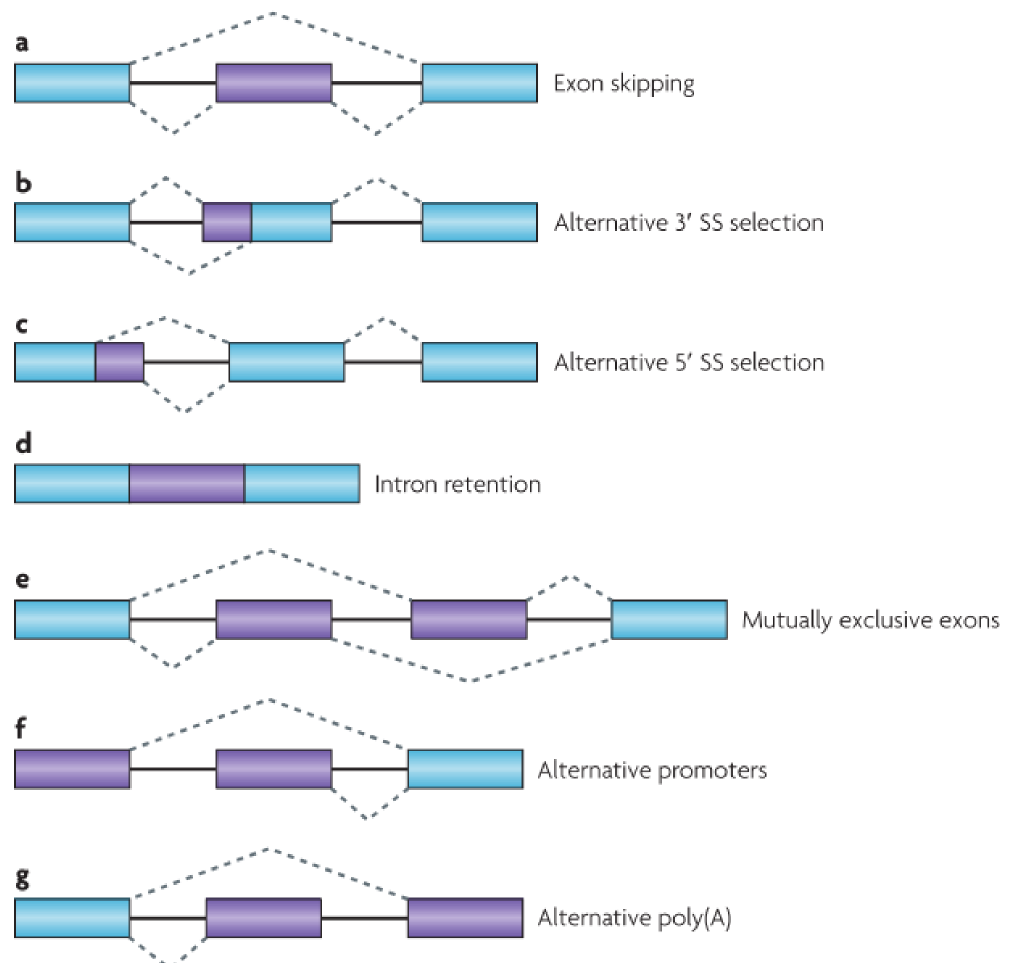

Figure 1.6. Types of alternative splicing. From (Keren et al., 2010) reprinted with permission from AAAS.

Splicing depends on the recognition of introns and exons by the spliceosome, a complex composed of five small nuclear ribonucleoprotein particles (snRNPs) and between 50 and 100 polypeptides (Smith and Valcárcel, 2000). Four motifs are required in pre-mRNA for the spliceosome to function (Figure 1.7): the 5' and 3' splice sites at the exon-intron junctions, a branch site sequence in the intron upstream of the 3' splice site and the polypyrimidine tract which is between the branch site and the 3' splice site (Keren et al., 2010, Smith and Valcárcel, 2000). U1 snRNP binds to the 5' splice site and the two subunits of U2 snRNP bind to the polypyrimidine tract and 3' splice site, whilst splicing factor 1 binds to the branch site (Smith and Valcárcel, 2000).

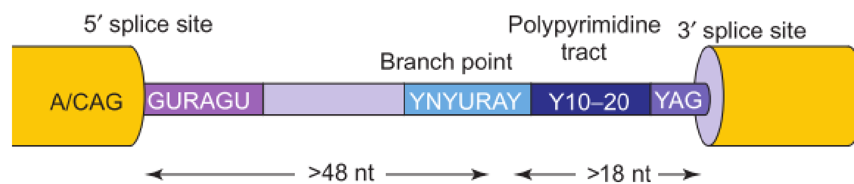

Figure 1.7. Splice-site elements in a typical metazoan intron. Y = pyrimidine; R = purine; N = any nucleotide. From (Smith and Valcárcel, 2000).

Alternative splicing is regulated by *cis*-acting RNA sequence motifs (in introns and exons) which provide binding sites for *trans*-acting proteins. The *cis*-acting RNA elements are intronic splicing enhancers, exonic splicing enhancers, intronic splicing silencers and exonic splicing silencers (Keren et al., 2010, Lee et al., 2012). Alternative splicing can be caused by mutations in intron or exon sequence or by changes in *trans*-acting proteins (Keren et al., 2010, Baxter et al., 2010).

Splice-site selection is influenced by Serine/Arginine rich proteins such as alternative splicing factor (ASF) and Protein suppressor of white apricot (SWAP). Higher concentrations of ASF favour the selection of the intron-proximal splice site whilst limiting concentrations promote the use of the strongest splice site even if it is further away (Eperon et al., 2000). Protein suppressor of white apricot (SWAP) has been shown to sometimes have the opposite effect of ASF, for example in Fibronectin, SWAP increases the skipping of an internal exon, whilst ASF promotes the inclusion of this exon

(Sarkissian et al., 1996). However both ASF and SWAP were shown to increase exon skipping in the protein CD45 (Sarkissian et al., 1996).

Splicing is also regulated by other *trans*-acting proteins including polypyrimidine tract binding proteins, heterogenous nuclear ribonucleoproteins, the TIA1 RNA-binding protein, Fox proteins and Nova proteins (Tang et al., 2011, Eperon et al., 2000, Del Gato-Konczak et al., 2000, Ule et al., 2005, Wang et al., 2008).

1.5 Mechanisms of insecticide resistance

Insects can become resistant to insecticides by genetic changes that either reduce the dose of the insecticide they are exposed to, or alter the insecticide target site so that it binds less effectively to the chemical. Insects can reduce the dose of insecticide through behavioural changes, reduced penetration, absorption/sequestration of the insecticide or by detoxification (Feyereisen, 1995). There are a number of molecular mechanisms which can cause these genetic changes including point mutations in genes, changes in gene copy number or changes in the expression of genes (Feyereisen, 1995).

1.5.1 Reduced penetration

Insects can evolve a thicker cuticle or alter the composition of the cuticle to prevent the uptake of insecticides. In the aphid *M. persicae*, synergist assays of a resistant clones with an overexpressed P450, did not completely eradicate the resistance, suggesting that an additional mechanism was involved. *In vivo* penetration assays showed significantly reduced penetration of imidacloprid through the cuticle and 32 transcripts encoding cuticular proteins were up-regulated, showing that this is likely to be a second mechanism of neonicotinoid resistance (Puinean et al., 2010). Slower penetration of insecticide has also been observed in the cotton bollworm, *Helicoverpa armigera.* The penetration of deltamethrin into insects with 330-670 fold resistance was significantly slower for up to 24 hours after exposure. After 1 hour 50% of the deltamethrin had penetrated the cuticle of susceptible larvae but only 20-30% had passed through the cuticle of the resistant larvae (Ahmad et al., 2006).

1.5.2 Metabolic Resistance

Metabolic insecticide resistance involves the enhanced detoxification of insecticides, mediated by the increased production of metabolic enzymes or a mutation in a key enzyme that changes or enhances its ability to break down a particular insecticide (Li et al., 2007). The three main classes of metabolic enzymes involved are glutathione-*S*-trasferases (GSTs), esterases and cytochrome P450 monoxygenases (P450s),(Li et al., 2007). GSTs act by catalysing the conjugation of reduced glutathione to xenobiotics including insecticides, herbicides and plant defence compounds, allowing more rapid excretion (Milligan et al., 2001, Li et al., 2007). Resistance to organophosphates, organochlorines and pyrethroids can be mediated by GSTs (Huang et al., 1998, Lumjuan et al., 2005). Esterases have been implicated in insecticide resistance to organophosphates, carbamates and pyrethroids through amplification of the genes or mutations within the genes (Devonshire and Moores, 1982, Campbell et al., 1998, Field et al., 1988). Cytochrome P450s have been shown to metabolise pyrethroids, DDT, neonicotinoids and carbamates (Wheelock and Scott, 1992, Joussen et al., 2008, Edi et al., 2014).

Some metabolic enzymes are able to metabolise a wide range of chemicals, so cross-resistance between different classes of insecticides can occur. For example, overexpression of a single P450 Cyp6g1 in *D. melanogaster* confers resistance to both DDT and imidacloprid (Daborn et al., 2001, Daborn et al., 2002) and the amplification of a single esterase gene in *M. persicae* gives resistance to pyrethroids, carbamates and organophosphates (Devonshire and Moores, 1982, Field et al., 1988). On the other hand some enzymes are very specific, for example a strain of *Culex pipiens quinquefasciatus* with P450-mediated resistance was 1300 fold resistant to permethrin but only 1.5 fold resistant to bifenthrin despite both of these insecticides having the same mode of action (Hardstone et al., 2007). Genomic studies have found variation in the number of metabolic enzymes present in different species of insect (Table 1.2). For example, the number of P450 genes in insect genomes sequenced to date ranges between 37 in the body louse, *Pediculus humanus*, to 180 in the

mosquito *C. quinquefasciatus* (Lee et al., 2010, Arensburger et al., 2010, Feyereisen, 2011).

Table 1.2. Number of potential insecticide detoxification genes in selected insects. P450= cytochrome P450; GST = glutathione-*S*-transferase, CCEs = carboxyl/choline esterases.

Species	P450s	GSTs	CCEs	Reference(s)
P. humanus	37	13	17	(Lee et al., 2010)
A. pisum	58	20	29	(IAGC, 2010)
B. mori	84	76	23	(Yu et al., 2008, Yu et al., 2009, Ai et al., 2011)
P. xylostella	90	36	63	(You et al., 2013)
A. aegypti	160	26	30	(Strode et al., 2008)
C. quinquefasciatus	170	37	47	(Arensburger et al., 2010)

1.5.3 Target-site resistance

The first report of a point mutation in an insecticide target site which gave more than 10-fold resistance to cycodienes in *D. melanogaster*, was an Ala to Ser substitution in the GABA receptor (ffrench-Constant et al., 1993). Knock-down resistance (*kdr*) which gives 10-30 fold resistance to pyrethroids was also found to be caused by a point mutation in the target site gene, the voltage-gated sodium channel. *Kdr* was first characterised in the housefly *Musca domestica* and the German cockroach *Blattella germanica* where a single nucleotide polymorphism caused a leucine to a phenylalanine substitution at position 1014 (in IIS6) (Williamson et al., 1996, Miyazaki et al., 1996). Since then L1014F has been found in more than 16 species of insect and alternative substitutions at the same site, L1014H and L1014S, have been reported (Davies et al., 2007). Another sodium channel substitution M918T, which gives up to 500-fold resistance to type II pyrethroids was found in combination with L1014F in house flies (Williamson et al., 1996). An alternative *skdr* T929I was discovered in *P. xylostella* (Schuler et al., 1998). Resistant strains which had L1014F + T929I displayed resistance ratios of up to 5000-fold to type I pyrethroids and up to 10000-fold to type II pyrethroids (Schuler et al., 1998).

To date, over 30 different pyrethroid resistance associated-mutations or combinations of mutations have been found in more than one arthropod pest species (Rinkevich et al., 2013). Twenty-four of these have been shown to decrease the sensitivity of the sodium channel to pyrethroids in *Xenopus* oocytes (Figure 1.8).

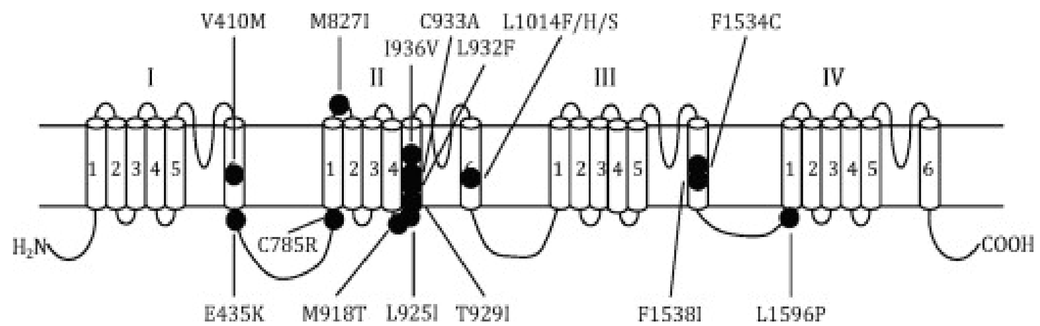

Figure 1.8. Sodium channel substitutions that confer pyrethroid insensitivity in channels expressed in *Xenopus* oocytes. From (Rinkevich et al., 2013). Reprinted with permission from AAAS.

Resistance to organophosphates and carbamates has been linked to two target-site mechanisms, point mutations in the acetylcholinesterase-1 (*ace-1*) gene and overexpression, commonly as a result of gene amplification, of carboxylesterase enzymes. In *C. pipiens*, a mosquito which carries West Nile virus, a single amino acid substitution G119S in the *ace-1* gene was found in strains displaying high carbamate and organophosphate resistance (Weill et al., 2003). This substitution was predicted to be located in the active gorge of the acetylcholinesterase enzyme, near the catalytic site. *In vitro* expression of recombinant ace-1 with and without the G119S alteration was carried out in *D. melanogaster* cells. In cells transfected with the wild-type *ace-1*, acetylcholinesterase activity reduced substantially after incubation with the carbamate propoxur. In contrast, cells expressing enzyme with the G119S substitution maintained normal acetylcholinesterase activity (Weill et al., 2003). However, G119S carries a fitness cost, as it is less efficient at degrading acetylcholine in the absence of insecticide. In order to overcome this, gene duplication arose in *C. pipiens*. Individuals with two resistance genes had low

survival and fertility. In contrast, mosquitoes which had one resistant and one susceptible gene had fitness as high as wild-type in non-treated areas (Labbé et al., 2007). The malaria mosquito, *Anopheles gambiae*, also has the G119S change and multiple copies of the resistant allele 119S were found in *A. gambiae* from Ghana, whilst the susceptible allele was always single-copy (Weetman et al., 2015).

The target site of spinosad, the α6 subunit of the nAChR, is unusual as insects without functional α6 are still viable (Watson et al., 2010). A lab strain of *D. melanogaster* with a non-functional α6 subunit displayed over 1000-fold resistance to spinosad and had an inversion mutation which disrupted α6 after exon 8b, so that TM3, the cytoplasmic loop, TM4 and extracellular C-terminal tail domains were missing (Perry et al., 2007). In insects from the field mis-spliced transcripts of α6, with premature stop codons, have been found in spinosad-resistant *P. xylostella* and *Bactrocera dorsalis* (Baxter et al., 2010, Hsu et al., 2012, Rinkevich et al., 2010). A point mutation in exon 9, conferring a G275E change was found in two species of spinosad-resistant thrips, *Frankliniella occidentalis and T. palmi* (Bao et al., 2014, Puinean et al., 2012). Neonicotinoids target different subunits of the nAChR so target-site modifications would not be expected to give cross-resistance between spinosyns and neonicotinoids (Lansdell and Millar, 2004, Watson et al., 2010). Mutations in α1 and α3 subunits of *Nilaparvata lugens* and mutations in the β1 subunit of *M. persicae* have been associated with neonicotinoid resistance (Liu et al., 2005, Bass et al., 2011).

In addition to developing resistance to chemical insecticides, insects can become resistant to biopesticides including Cry toxins produced by the bacteria *Bacillus thuringiensis* (Bt). For example in *P. xylostella* down-regulation of the ABC transporter gene *Pxwhite* in the midgut was correlated with resistance to Cry1AC. Silencing of this gene caused a significant increase in resistance to the Cry1AC toxin, which implies that *Pxwhite* may encode a receptor for Cry toxins (Guo et al., 2015).

1.6 Resistance management

Attempting to prevent the development of resistance is important both in crop protection (resistance to insecticides, fungicides and herbicides) and in healthcare (drug resistance). Resistance management involves using pesticides or medicines in such a way as to slow down the development of resistance as much as possible. In some cases, modelling can be used to work out the best management strategy. In medicine, evolution of anti-biotic resistance is faster when multiple drugs are used at the same time, if there is synergy between the drugs. In contrast combining antagonistic drugs can slow down the development of resistance (Hegreness et al., 2008). A study modelling herbicide resistance found that using two herbicides in combination was more effective than alternating the herbicides (Diggle et al., 2003). In addition, resistance was far less likely to develop if the area of weeds is small, so the isolation of weed populations is an important strategy (Diggle et al., 2003). Insecticides are split into different groups by their mode of action, and the insecticide resistance management committee (IRAC) recommends rotating between three different insecticide groups with a gap of at least two insect generations between applications of the same insecticide group (IRAC, 2011). However, in practise this is often difficult to achieve due to lack of available active ingredients.

1.7 Objectives

In 2011 (the start of this project), no studies of the mechanisms of insecticide resistance in *T. absoluta* had been published and understanding the mechanisms was predicted to be an important part of effective monitoring of resistance and informing resistance management strategies. Furthermore, most previous studies of insecticide efficacy in *T. absoluta* only used insects collected in South America. Therefore, I investigated the mechanisms underlying resistance to two insecticide classes, pyrethroids and spinosyns and included samples collected from the new range of *T. absoluta* populations. Additionally, since no DNA or amino acid sequences for insecticide target sites or metabolic enzymes in *T. absoluta* had been published, methods for obtaining these sequences were needed.

The specific objectives for this thesis were:

1) To assess the frequency and world-wide distribution of pyrethroid target-site resistance mutations in *T. absoluta* (Chapter 3)

2) To assess the susceptibility of five European populations of *T. absoluta* to spinosad (Chapter 4)

3) To select a population of *T. absoluta* for spinosad resistance (Chapter 4) and determine the mechanism(s) underlying this resistance (Chapter 6).

4) To sequence the transcriptome of insecticide-susceptible and insecticide -resistant populations of *T. absoluta* and annotate cytochrome P450 transcripts and insecticide target sites (Chapter 5).

2. General Materials and Methods

2.1 *T. absoluta* populations

2.1.1. Live insect populations

Populations of *T. absoluta* were reared in a controlled environment of 26 °C and 16 hr light; 8 hr dark in the insectary at Rothamsted Research. This was under quarantine conditions with permission from the Department for the Environment, Food and Rural Affairs (licence to import, move and keep prohibited invertebrates, No.112593/209885-4). Mesh cages were set up, each containing a potted tomato plant (var. *Money Maker*) in a tray with three layers of felt for water absorption. A bug vacuum (Backyard Safari, UK) was used to release 50-100 adults into each cage. Fresh tomato plants were added to cages when 80% of leaves on the old plant had been 'mined'. The origins of the populations are given in Table 2.1.

Table 2.1. Origin of live populations of *T. absoluta*

Population	Date of collection	Origin	Supplier
TA1	31-01-2010	Spain	Pablo Bielza
TA2	07-05-2010	Spain	Pablo Bielza
TA3	06-07-2010	Italy	Pablo Bielza
TA4	15-09-2010	Portugal	Pablo Bielza
GA	2008	Brazil	Bayer
Spin	23-01-2012	Portugal	Rob Jacobson

2.1.2. Preserved insect material

Samples of adults and larvae in ethanol were provided by Pablo Bielza and Dina Cifuentes (University of Cartagena, Spain). These originated from 27 different locations in Europe and South America (Figure 2.1). Samples were kept at -20°C.

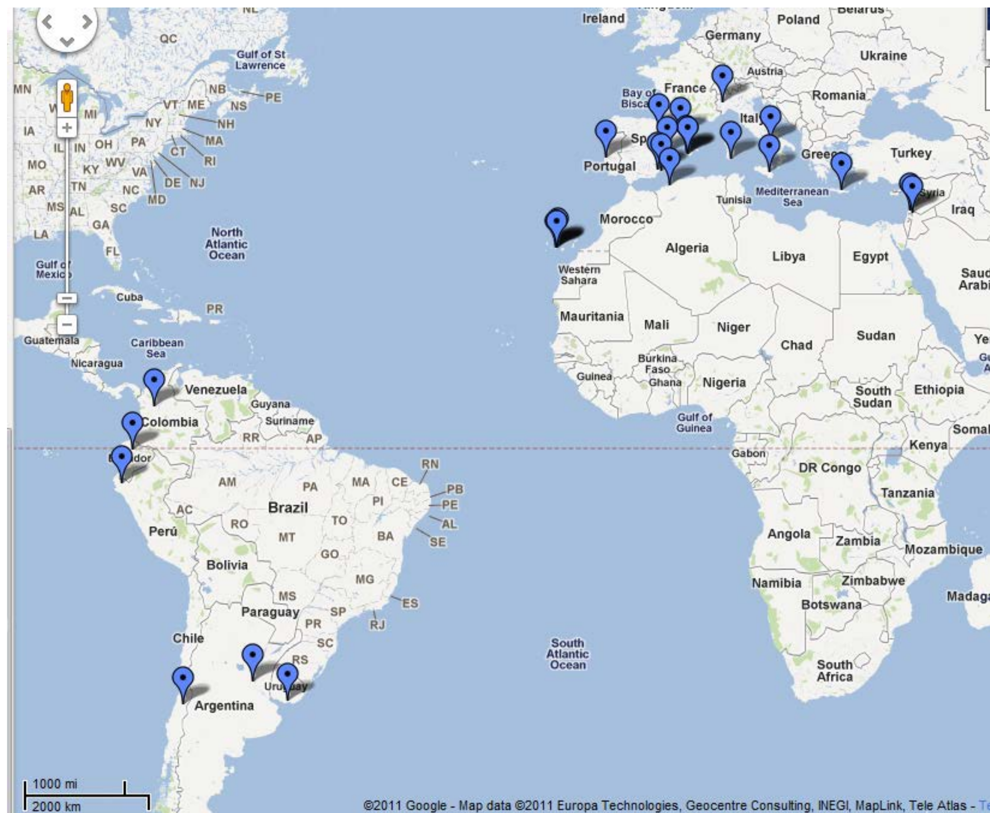

Figure 2.1. Origin of populations of *T. absoluta* from 27 locations

2.2 DNA extraction

Two methods of DNA extraction were used depending on the purity of DNA required. DNAzol® extraction was an economic method which gave high yields of DNA from individual *T. absoluta*. However, the DNA was relatively impure as there was no column filtration and contaminated with RNA as the protocol contains no RNase treatment. Therefore, when pure DNA without RNA contamination was required e.g. when used to PCR-amplify large DNA fragments, the DNeasy Plant mini-kit was used. This method gave lower yields so was more suitable for ‘pools’ of insects.

2.2.1. DNAzol® (Life Technologies, USA)

Isolation of DNA was carried out using DNAzol® Reagent according to the manufacturer’s instructions, but with some modifications (Table 2.2). Individual insects (either adults or larvae) were snap-frozen in liquid nitrogen and homogenized using a pestle prior to adding DNAzol reagent. The resultant

homogenate was centrifuged to remove insoluble tissue fragments and the DNA precipitated using 100% ethanol. The DNA pellet was then washed with 70% ethanol, dried and resuspended in water and stored at -20°C.

2.2.2. DNeasy® Plant Mini Kit (Qiagen, Germany)

Larvae (up to 12 per sample) were frozen in liquid nitrogen and ground with a pestle. DNA was extracted according to the manufacturer's instructions. Briefly, RNase and buffer were mixed with each sample, followed by incubation at 65°C for 10 min to remove RNA. The samples were incubated on ice for 5 min (to stop the reaction) and then filtered through a QIAshredder spin column. The flow through was reapplied to a second column, and two wash steps were performed. Samples were eluted in 100 µl of nuclease-free water and stored at -20°C.

Table 2.2. Modifications to DNAzol® protocol

Step	Manufacturer's instructions	Amended Protocol
1. Homogenisation	In 1 ml DNAzol	In 200 µl DNAzol
2. Centrifugation	10 min at 10,000 x g	15 min at 16,000 x g
3a. DNA precipitation	Add 0.5 ml 100% ethanol	Add 100 µl 100% ethanol
3b. DNA precipitation	Remove precipitate by spooling with pipette tip	Pellet DNA by centrifugation, 30 min at 15°C and 16,000 x *g*
4a. DNA wash	Wash the precipitate with 0.8 -1 ml 75% ethanol	Wash the precipitate with 200 µl 75% ethanol
4b. DNA wash	Store tubes vertically for 1 min to allow DNA to settle	Centrifuge for 5 min at 15°C and 16,000 x *g*,
5a. DNA solubilisation	Air-dry for 5-15 sec	Dry in Speed vacuum for 2 min
5b. DNA solubilisation	Solubilize in 8 mM NaOH and adjust pH using HEPES	Dissolve pellet in 20-40 µl water

2.3 RNA extraction and cDNA synthesis

Eggs, larvae, pupae and adult *T. absoluta* were snap frozen in liquid nitrogen and stored at -80°C prior to RNA extraction. Then RNA was extracted using

the Isolate II RNA mini kit (Bioline Reagents Ltd., UK), according to the manufacturer's instructions. Up to 20 mg frozen tissue was ground using a pestle in 350 µl of Lysis buffer. The lysate was filtered and 350 µl of 70% ethanol was added to the filtrate. The filtrate was transferred to a new column and centrifuged at 11 000 x *g*, binding the RNA to the membrane of the column, and the filtrate discarded. Membrane desalting buffer (350 µl) was used to desalt the silica membrane. The membrane was then treated with DNase1 for 15 min to digest any DNA. Three wash steps were performed, prior to RNA elution in 60 µl of DEPC treated water. Concentrations of RNA were calculated using a spectrophotometer (NanoDrop®, USA) and the integrity of the RNA was checked by incubating a sample for 5 min at 65°C with gel loading buffer II, (Ambion®, UK) and running on an Ethidium Bromide (EtBr) gel (Figure 2.2). RNA was stored at -80°C. RNA was used to synthesise cDNA using the Superscript III reverse transcriptase kit (Invitrogen™, USA). 2-5 µg total RNA was mixed with 1 µl random hexamer primers and 1 µl 10 mM dNTP mix and made up to 13 µl with DEPC treated water. Samples were incubated at 65°C for 5 min and then on ice for 1 min. 4 µl 5x first strand buffer, 1 µl 0.1M DTT, 1 µl RNase inhibitor and 1 µl superscript III reverse transcriptase were added and mixed by pipetting. Two incubation steps (25°C 5 min, 50°C 45 min) were performed. Heating to 70°C for 15 min then inactivated the reaction. The cDNA was stored at -20°C.

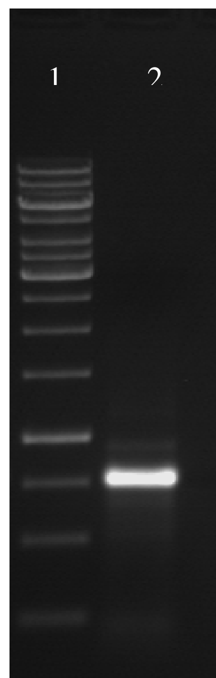

Figure 2.2. Gel electrophoresis of RNA. Track 1, 1 kb DNA Ladder (GeneRuler, Thermo Scientific, UK). Track 2, TA1 RNA (1μg).

2.4 Polymerase Chain Reactions (PCRs)

2.4.1 Standard PCR

Reactions (20 μl) contained 2 μl genomic DNA or cDNA, 10 μl DreamTaq™ Green PCR Master Mix (2X) (Thermo Fisher Scientific, Lithuania), 6 μl sterile distilled water, 1 μl of forward primer (10 μM) and 1 μl of reverse primer (10 μM). An initial denaturation step of 94°C 2 min was performed, followed by 35 cycles of 94°C 30 sec, primer Tm -5°C for 30 sec, 72°C for 1 min/kb, and then a final extension of 5 min at 72°C. A negative control without DNA was run at the same time to check for contamination. Primers were 18-25 nucleotides in length, and normally had a salt-adjusted Tm of approx. 58°C, and GC content of 40-60% as confirmed by OligoCalc (Kibbe, 2007). Primers were designed using Primer3 (Untergasser et al., 2012).

2.4.2 Long PCR

When amplifying fragments over 6 kb, Long PCR Enzyme Mix (A mix of Fermentas Taq DNA Polymerase and a thermostable DNA polymerase with proofreading activity; Thermo Scientific, Lithuania.) was used. Primers were

25-31 nucleotides, with a GC content of 40-60% and a salt-adjusted Tm of 62-73°C and if possible, 1-3 C or G nucleotides were included at the 3' end. PCR reactions contained: 2.5 µl of 10X Long PCR buffer with 15 mM $MgCl_2$, 1 µl dNTP mix (10mM), 18 µl nuclease-free water, 1 µl of forward primer (10 µM), 1 µl of reverse primer (10 µM), 0.5 µl of Long PCR Enzyme Mix per reaction and 50 ng of genomic DNA. A 16 hr programme (94°C 2 min, 35 cycles of: 94°C 10 sec, 50°C 20 sec, 68°C 25 min, with a final extension of 68°C 20 min) was performed.

2.5 Purification of PCR Products

The Wizard® SV Gel and PCR Clean-Up System (Promega, USA) was used to purify PCR products. If, after running the PCR product on an EtBr gel, more than one band was present, the desired DNA fragment(s) was excised from the gel, using a razor blade and dissolved in membrane-binding solution at 65°C, (10 µl solution per 10mg of agarose gel slice). The dissolved gel mixture, or single PCR products were purified using a column-based system, according to the manufacturer's instructions. Two wash steps were performed, and the PCR fragment eluted in 50µl of nuclease-free water. Concentrations of fragments were determined using a spectrophotometer (NanoDrop®, USA).

2.6 Cloning of PCR fragments

The StrataClone PCR cloning kit was used for cloning of PCR fragments, following the manufacturer's instructions. Purified PCR products were ligated into the StrataClone PCR cloning vector pSC-A-amp/kan for 5 min at room temperature. The ligation mix (1 µl) was then used to transform StrataClone SoloPack Competent Cells. The transformation mixture was incubated for 20 min on ice followed by heat shock at 37°C for 45 sec. Cells were allowed to recover for 1.5 hrs in LB broth ((10 g NaCl, 10 g tryptone, 5 g yeast extract per litre) at 37°C in a shaking incubator. LB-ampicillin plates were prepared and 40 µl of X-gal was spread on the plates. IPTG was not added as the competent cells contained the lacZΔM15 mutation, which supports blue-white screening with plasmid pSC-A-amp/kan, containing the lacZ′ α-complementation cassette.

The recovered cells (100 µl) were plated and incubated at 37°C for 16 hrs. White colonies were selected. Half of the colony was streaked onto a fresh plate to be used for plasmid preparation. The other half of the colony was then placed in 20 µl of sterile distilled water and a 2 µl aliquot used as the template for a colony PCR, containing 10 µl Dreamtaq green PCR mix, 0.5 µl M13F (10µM) , 0.5 µl M13R (10µM) and 7 µl sterile distilled water. The M13 primer sequences and position in the vector are shown in Figure 2.3. The colony PCR conditions were 95°C 5 min, 35 cycles of: 94°C 30 sec, 50°C 30 sec, 72°C 1 min. The samples from the colony PCR were subsequently run on an agarose gel to check that the cloning was successful.

Samples of bacteria containing the correct plasmids were then used to isolate the plasmids. A pipette tip was used to pick up the streaked bacterial colonies and place them in 15 ml tubes containing 2.5 ml LB with ampicillin (10 g NaCl, 10 g tryptone, 5 g yeast extract per litre, 0.25 mg ampicillin). These were left overnight on a 37°C shaking incubator. The following day, plasmids were purified using the Genejet plasmid miniprep kit (Thermo Scientific, Lithuania) following the manufacturer's instructions.

TTGTAAAACGACGGCCAGTGAGCGCGCGTAATACGACTCACTATAG
GGCGAATTGGAGCTCCCGCGGTGCGGCCGCTCTAGAACTAGTGGAT
CCCCCGGGCTGCAGCCCAATGTGGAATTCGCCCTT[PCR_product]AG
GGCGAATTCCACAGTGGATATCAAGCTTATCGATACCGTCGACCTC
GAGGGGGGGCCCGGTACCCAGCTTTTGTTCCCTTTAGTGAGGGTTA
ATTGCGCGCTTGGCGTAATCATGGTCATAGCTGTTTCC

Figure 2.3. Vector sequence flanking the cloning site for the StrataClone PCR Cloning Vector used in the StrataClone cloning kit. The positions of primers are underlined and coloured (M13, red; T7, purple; T3, pink; M13R, blue). The position of the PCR product is shown in yellow highlight highlighted.

2.7 Sequencing of PCR fragments and plasmids

Purified PCR fragments or fragments cloned in plasmids were sent to Eurofins Genomics, Germany for sequencing, using their 'value read' service. Purified

PCR fragments were diluted with nuclease-free water to a concentration of 10ng / µl in 15 µl (150 ng total), with 2 µl primer (10 µM) added to a total volume of 17 µl. Purified plasmid DNA (1500 ng in 15 µl) was sequenced with the T3 and T7 primers (Figure 2.3). When the sequences were obtained, Geneious Version 8.1, Biomatters Ltd. was used to view, align and annotate the data.

2.8 Rapid Amplification of cDNA ends (RACE)

The SMARTer™ RACE cDNA Amplification kit (Clontech Laboratories, Inc., USA) was used to obtain sequences of the ends of cDNAs. Specialised first-strand cDNA synthesis using 5'-CDS Primer and SMARTer IIA oligo was carried out according to the manufacturer's instructions. The cDNA was amplified using Universal Primer mix (Long, 0.4µM, 5'-ctaatacgactcactatagggcaagcagtggtatcaacgcagagt-3'; Short, 2µM, 5'-ctaatacgactcactatagggc-3') as the forward primer. Genome-specific reverse primers were designed with 23-28 nucleotides; Tm 65°C -80°C and GC content 50-70% (Chapter 6)

5' RACE was performed according to the manufacturer's instructions, except half the recommended volumes of all reagents were used. Two negative controls were included, the first with all reagents except the genome-specific primer and the second with all reagents except the universal primer mix. The PCR program was 5 cycles of 94°C 30 sec, 72°C 3 min then 5 cycles of 94°C 30 sec, 70°C 30 sec, 72°C 3 min and finally 30 cycles of 94°C 30 sec, 68°C 30 sec, 72°C 3 min. This was as recommended by the manufacturer apart from an increase in the number of cycles in the third step from 25 to 30 because the target gene was a low copy number transcript. The PCR products were sequenced using the genome-specific primer.

2.9 Genome Walking

The Universal GenomeWalker™ 2.0 kit (Clontech Laboratories Inc., USA) was used to obtain the sequence of introns which failed to amplify using traditional or long-range PCR. Genomic DNA was digested with four different

restriction enzymes (*Dra*1, *Eco*RV, *Pvu*II and *Stu*I) to create DNA libraries. These DNA libraries were purified and then ligated to GenomeWalker adaptors (Figure 2.4). PCR was then performed using a genome specific primer (see Chapter 6) and an adaptor primer (Figure 2.4) using Advantage 2 polymerase mix, according to the manufacturer's instructions. A Master mix was prepared with 19.5 µl nuclease-free water, 2.5 µl Advantage 2 PCR buffer (10X), 0.5 µl dNTPs (10mM each), 0.5µl Adaptor Primer 1 (10 µM) and 0.5µl Advantage 2 polymerase (50X) per sample. Master mix (23.5 µl), genome-specific primer (0.5 µl; 10 µM) and ligated DNA (1µl) were added to each tube. A negative control was included with no DNA. The parameters for thermal cycling were 7 cycles of 94°C 25 sec, 72°C 3 min then 32 cycles of 94°C 25 sec, 67°C 3 min, with a final extension step of 67°C for 7 min.

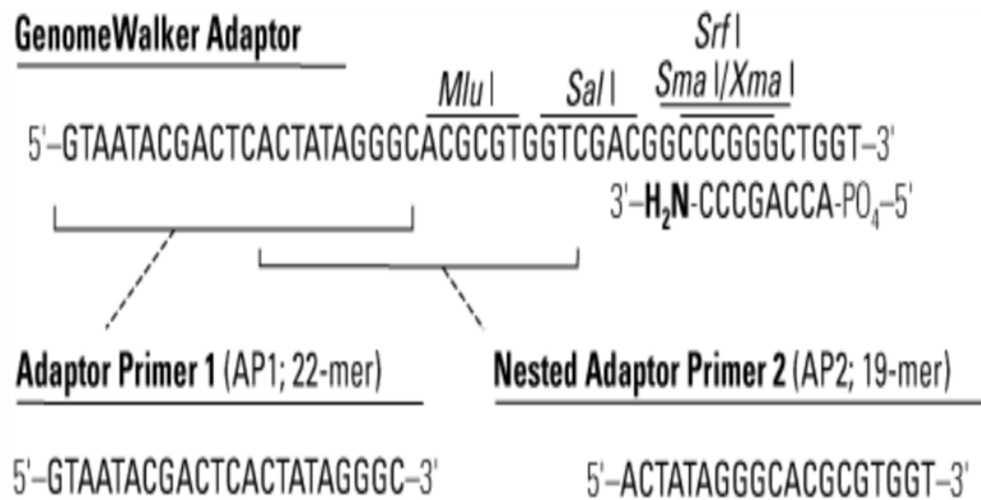

Figure 2.4. GenomeWalker Adaptor and Adaptor Primer sequences. Universal GenomeWalker™ 2.0 User Manual, Clontech laboratories Inc

2.10 Quantitative PCR (qPCR)

For the target sequences at least two pairs of primers were designed in different areas of the gene sequence using Primer3, primer size 18-23 bp with an optimal Tm of 60 °C a product size of 100-150 bp and a GC content of 40-60-% (Untergasser et al., 2012). Control reactions used primers that target housekeeping genes and were selected on the basis that they were stably expressed in an RNA-seq experiment (see Chapter 5). The primers were tested to ensure they were specific for the desired product using melt curve analysis.

Reactions were 15 µl containing 7.5 µl SYBR® Green JumpStart Taq ReadyMix (Sigma-Aldrich, UK), 0.5 µl of forward primer (10µM), 0.5 µl of reverse primer, 1.5 µl cDNA (10ng/ µl), and 5 µl nuclease-free water . The following programme was used to test the primers: 95°C 2 min, then 40 cycles of 95°C 10 sec, 57°C 15 sec, 72°C 20 sec, with a final melt step at 95°C. Primers, which displayed a single smooth peak in the melt curve analysis, were selected (Figure 2.5).

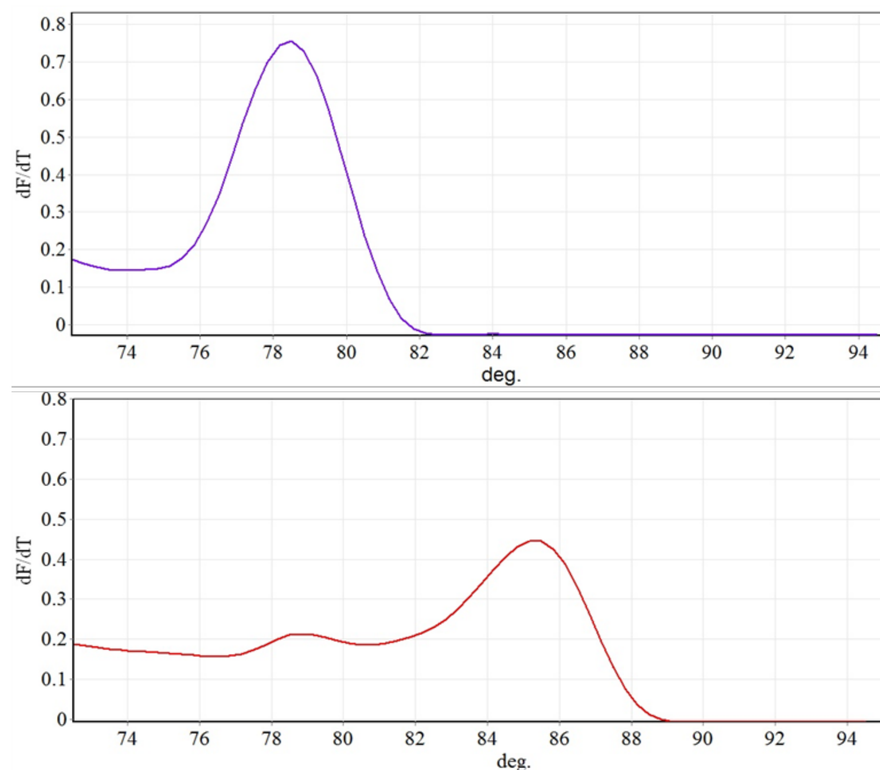

Figure 2.5. Testing of qPCR primers. A) Primers amplify a single product B) Primers amplify more than one product.

Quantitative PCR by relative quantification requires validation experiments to show that the efficiencies of the target and endogenous control amplifications are approximately equal. Efficiency testing was done by creating a standard curve for each primer pair. Reactions were as above, but with five different concentrations of cDNA between 100ng/µl and 0.01ng/ µl. Three or four technical replicates were made for each concentration. The PCR program was the same as that used for the primer testing, but without the final melt step.

Finally, the expression of candidate genes was compared between two insect populations, with two control housekeeping genes tested alongside the gene of interest. Primers for housekeeping genes were designed using transcripts from the *T. absoluta* transcriptome (chapter 5); primer sequences are shown in table 2.3. Four biological replicates per population and two technical replicates were performed. A liquid-handling robot CAS 1200 (Corbett Research Ltd, UK) was used to set up reactions. Samples were run on a Rotor-Gene 6000™ (Corbett Research Ltd , UK), and relative gene expression was analysed using $2^{-\Delta\Delta}$ C_T method outlined in (Livak and Schmittgen 2001), using Microsoft Excel. This method is based on the principal that in an ideal PCR reaction the quantity of PCR product doubles at each cycle. Briefly, a threshold cycle (C_T) was defined as the cycle number at which the amplified gene reaches a fixed threshold. The C_T values of the technical replicates were averaged, and the geometric mean C_T of the two housekeeping genes was calculated. $2^{-\Delta\Delta}$ Ct was calculated for each biological replicate separately using the equations below:

$\Delta C_T = (C_{T,}$ gene of interest - C_T, housekeeping gene)

$\Delta\Delta C_T = \Delta C_T$ – mean $\Delta C_{t,}$ susceptible population

The standard deviation and mean $2^{-\Delta\Delta}$ Ct value for each population were then calculated. Confidence intervals for the mean $2^{-\Delta\Delta}$ Ct of each population were determined using a normal distribution, with a P-value of 0.05, using the standard deviation and a sample size of four (biological replicates). A gene was considered to be differentially expressed if the 95% confidence intervals for each population did not overlap.

Table 2.3. Housekeeping gene primers 5'-3'. EF = elongation factor, EIF = Eukaryotic translation initiation factor.

Primer Name	Sequence
TA_EFdeltaF	AGCAGCAATATCCACGCTCT
TA_EFdeltaR	GGAATCCACAAGCTGCAAAT
TA_EIF5F2	CAGCGCATGGATTTGTTCTA
TA_EIF5R2	CGGGGCTTTGGATTTTACTT

3. Resistance to pyrethroids

The results detailed in this chapter have been published in *Insect Biochemistry and Molecular Biology* (Appendix1), and *Pesticide Biochemistry and Physiology* (Appendix 2).

Haddi K, **Berger M**, Bielza P, Cifuentes D, Field LM, Gorman K, Rapisarda C, Williamson MS and Bass C (2012). Identification of mutations associated with pyrethroid resistance in the voltage-gated sodium channel of the tomato leaf miner (*Tuta absoluta*). *Insect Biochemistry and Molecular Biology* 42, 506-513.

Silva WM, **Berger M**, Bass C, Balbino V. Q, Amaral MHP, Campos, MR and Siqueira HAA(2015). Status of pyrethroid resistance and mechanisms in Brazilian populations of *Tuta absoluta*. *Pesticide Biochemistry and Physiology* 122, 8-14.

Note: Some of the data in this chapter were obtained by a PhD student Khalid Haddi, a PhD student Wellington Marques da Silva and an A-level summer student, Nicole Newman. Table headings and figure legends indicate these contributions.

3.1 Introduction

Pyrethroids are neurotoxic insecticides which make up approximately 17% of the world insecticide market (Sparks, 2013). They are synthetic analogues of natural pyrethrins found in *Chrysanthemum* flowers and extracts of dried *Chrysanthemum* flowers are thought to have been used as insecticides since 100 AD. However, the low photo-stability of natural pyrethrins makes them inefficient for agricultural use, whereas the synthetic pyrethroids are more photo-stable, have a higher insecticidal toxicity and a low toxicity towards mammals (Davies et al., 2007). Pyrethroids can be divided into two groups, type II which have a cyano group, and type I which do not (Vais et al., 2001). The target of pyrethroids is the insect sodium channel (Williamson et al., 1993)

and when the pyrethroids bind they cause incapacitation of the insect, known as ‘knock-down’. Insects exposed to lethal doses of pyrethroids display symptoms of uncoordinated movement, followed by paralysis and then death (Davies et al., 2007). Over time, insects have evolved resistance to pyrethroids, often as a result of mutations in genes encoding the sodium channel. The majority of sodium channel mutations confirmed to give pyrethroid resistance are clustered in regions IIS4, IIS6 and IIIS6 of the channel (Rinkevich et al., 2013).

Resistance to a number of pyrethroid insecticides has been reported previously for *T. absoluta*, including to deltamethrin, bifenthrin and permethrin (Siqueira et al., 2000b, Lietti et al., 2005, Silva et al., 2011). However, as of 2011, the molecular mechanisms underlying pyrethroid resistance in *T. absoluta* had not been characterised. Thus in the study reported here leaf dip bioassays were used to evaluate the susceptibility of five field populations of *T. absoluta* to two pyrethroid insecticides, λ cyhalothrin and tau-fluvalinate and to investigate whether resistance was mediated by changes in the *para*-type sodium channel, the IIS4–IIS6 region of the *para* gene was cloned and sequenced.

Knowledge of the frequency of pyrethroid resistance and its distribution in world-wide *T. absoluta* populations is essential for effective control of this pest and to minimise the wasteful application of ineffective chemistry. Therefore, diagnostic tools were developed to allow sensitive detection of mutations in individual *T. absoluta* larvae and adults. These new diagnostic tools were then used to screen field-collected samples of diverse geographic origin to examine the frequency and distribution of the mutations in global populations of this pest species.

3.2 Specific Methods

3.2.1 Pyrethroid Bioassays

Five populations of *T. absoluta* (TA1, TA2, TA3, TA4 and GA; see Table 2.1) were tested for susceptibility to the pyrethroids lambda-cyhalothrin (Syngenta) and tau-fluvalinate (Makhteshim) using a leaf dip bioassay (see 4.2.2). Six

concentrations of each pyrethroid were tested with three replicates of 15-20 larvae per concentration. Mortality was assessed after 48 hours and GenStat 13th edition was used to estimate the LC50s (see 4.2.5).

3.2.2 Cloning and sequencing of regions encoding domain II of the *T. absoluta* sodium channel

Total RNA was extracted from pools of 15- 20 individuals of each population using Trizol and following the manufacturer's instructions. Genomic DNA was removed by DNase I digestion using DNA-free DNase treatment and removal reagent (Ambion). A quantity of 4 mg of RNA sample was then used for cDNA synthesis using the Superscript III reverse transcriptase kit (Invitrogen™, USA) according to the manufacturer's instructions. Degenerate primers were designed to conserved motifs of the sodium channel gene as described previously (Martinez-Torres et al., 1997). Nested PCRs were done using primers DgN1 and DgN3 in a primary PCR and primers DgN2 and DgN3 in a secondary reaction (Table 3.1).

Specific primers were designed for PCRs on genomic DNA (Table 3.1). Genomic DNA was extracted from ten individuals of each of the five lab strains of *T. absoluta* using DNAzol® reagent (see 2.2). Additionally, DNA was extracted from pools of twenty insects from the lab strains. To determine the positions and sizes of two introns within this region of the gene, nested PCR was done using primers TAF1 and TAR4 followed by TAF2 and TAR3 for the first intron and primers TAF5 and TAR1 followed by TAF4 and TAR2 for the second intron (Table 3.1). To genotype the lab strains, the primers TAF2 and TARouter were used to amplify the DNA region encoding amino acids 918 to 929. To amplify the region coding for amino acid 1014 a nested PCR was undertaken, using the primers TaF3 and TaR1 in the first reaction and primers TaF4 and TaR2 in the second reaction (Table 3.1). PCR products were either sequenced directly or cloned using the StrataClone PCR Cloning kit (see 2.6). Plasmid DNA and PCR products were sent to Eurofins Genomics, Germany for sequencing (see 2.7).

Table 3.1. Primers used for amplification of the *T. absoluta* para-type sodium channel and TaqMan assays.

Name of Primer	Sequence (5'-3')
DgN1	GCNAARTCNTGGCCNACNYT
DgN2	GCNAARTCNTGGCCNAC
DgN3	YTTRTTNGTNTCRTTRTCRGC
TAF2	GGCCGACGTTTAATTTACTC
TARouter	TGTTTCAACAGAATGACGATACTA
TAF4	GTATGTGGGACTGTATGTTGG
TAR1	GGTGTCGTTATCGGCAGTAG
TAF3	AGAATGGATTGAGAGTATGTGG
TAR2	GTTATCGGCAGTAGGTGTCGA
TAkdr_F	CTTCTTAGCCACCGTCGTCATT
TAkdr_R	CGCTTTTACTGGTATATTGCAATAAAAAGCT
TAkdr VIC	AACCACAAGATTACC
TAkdr FAM	ACCACAAAATTACC
TAT929I_F	ACGATGGGTGCCTTGGG
TAT929I_R	TGCATACCCATCACGGCAAATAT
TAT929IVIC	CACAATACGAAGGTCAGGTT
TAT929IFAM	CACAATACGAAGATCAGGTT
TAM918T_F	TGGCCGACGTTTAATTTACTCATCT
TAM918T_R	TGCCCAAGGCACCCATC
TAM918TVIC	TCCTACCCATAATCG
TAM918TFAM	TCCTACCCGTAATCG

3.2.3 TaqMan® PCR

TaqMan® assays were used to assess the frequency of three *para* mutations in field-collected insects from 35 locations. DNA was extracted from over 200 individual insects using DNAzol® reagent (see 2.2). Primers and probes (Table 3.1) were designed using the Custom TaqMan Assay Design Tool (Applied Biosystems, USA). The first probe, labelled with VIC® reporter dye at the 5' end, was for detection of the wild-type allele, whilst the second probe, labelled with FAM® reporter dye, was for detection of the mutant allele. Each probe also had a 3' non-fluorescent quencher. PCR reactions contained 2 µl genomic DNA extracted from individual insects using DNAzol reagent, 7.5 µl of SensiMix DNA kit (Quantace, UK), 800nM of each primer and 200nM of each probe. Samples were run on a Rotor-Gene 6000™ (Corbett Research, UK) using the temperature cycling conditions of: 10 minutes at 95°C followed by 40 cycles of 95°C for 10 s and 60°C for 45 s. The increase in fluorescence of the two probes VIC and FAM was monitored in real time by acquiring each

cycle on the yellow (530 nm excitation and 555 nm emission) and green channel (470 nm excitation and 510 emission) of the Rotor-Gene respectively. Genomic DNA templates of known genotype were used to optimise the assay. In all runs, at least one control for each genotype (mutant homozygous, heterozygous and wild-type homozygous) was included. To facilitate genotyping, a scatter plot comparing VIC and FAM fluorescence values at the end of the 40 cycles was created using the Rotor-Gene analysis option. DNA sequencing was used to confirm results for samples with an ambiguous TaqMan trace.

3.3 Results and Discussion

3.3.1 Susceptibility of five laboratory populations of *T. absoluta* to pyrethroids

The susceptibility of five populations of *T. absoluta* to two pyrethroids was assessed by determing the LC_{50} values (Table 3.2). This showed that there was a wide variation between the populations. The highest LC_{50} for λ-cyhalothrin was 1514 mg L^{-1} for GA and the lowest was 85 mg L^{-1} for TA1, an 18-fold difference. The LC_{50}s for tau-fluvalinate ranged from 821 mg L^{-1} (TA3) to 9259 mg L^{-1} (GA), a 12- fold difference. The recommended field rates are 25 mg L^{-1} and 50 mg L^{-1} for λ-cyhalothrin and tau fluvalinate respectively. Therefore, control failure would be expected in all of the populations tested.

Table 3.2. Susceptibility of five populations of *T. absoluta* to pyrethroids. Data obtained by K. Haddi.

Insecticide	Population	LC_{50} mg L^{-1}	5% CI	95% CI
λ-cyhalothrin	TA1	85	37	159
	TA2	351	176	507
	TA3	631	456	837
	TA4	700	472	957
	GA	1514	1137	2106
Tau fluvalinate	TA1	2047	1235	4483
	TA2	1952	1365	2841
	TA3	821	544	1177
	TA4	3716	1443	29,334
	GA	9259	1403	61,099

3.3.2 Cloning and sequencing of regions of the *T. absoluta* sodium channel gene

The sequences of the *T. absoluta* para-type sodium channel gene encoding the segments 4-6 of the domain II region were obtained by amplification of cDNA using degenerate primers. This region was highly conserved between *T. absoluta* and other Lepidoptera including the silkworm *Bombyx mori* (Shao et al., 2009), the diamond back moth *Plutella xylostella* (Sonoda et al., 2008b) and the corn earworm, *Helicoverpa zea* (Hopkins and Pietrantonio, 2010) (Figure 3.1). This region encompasses a 'mutation hot-spot' where many mutations conferring resistance to pyrethroids have been identified in a diverse range of insects (Rinkevich et al., 2013). Specific primers for *T. absoluta* were designed to characterise the introns in this region and the position and sequence of the two introns is shown in Figure 3.2.

```
T. absoluta var 1    SWPTFNLLISITGRTMGALGNLTFVLCIIIFIFAVMGMQLFGKNYVD
T. absoluta var 2    SWPTFNLLISIMGRTMGALGNLIFVLCIIIFIFAVMGMQLFGKNYVD
P. xylostella        SWPALNLIISIMGRTVGALGNLTFVLCIIIFIFAVMGMQLFGKNYTD
B. mori              SWPTLNLLISIMGRTMGALGNLTFVLCIIIFIFAVMGMQLFGKNYVD
H. zea               SWPTLNLLISIMGRTMGALGNLTFVLCIIIFIFAVMGMQLFGKNYVD

T. absoluta var 1    NVDRFPDGDLPRWNFTDFMHSFMIVFRVLCGEWIESMWDCMLVGDVS
T. absoluta var 2    NVDRFPDGDLPRWNFTDFMHSFMIVFRVLCGEWIESMWDCMLVGDVS
P. xylostella        HVDRFPDGDLPRWNFTDFMHSFMIVFRVLCGEWIESMWDCMLVGDVS
B. mori              YVDRFPDGDLPRWNFTDFMHSFMIVFRVLCGEWIESMWDCMLVGDVS
H. zea               YVDRFPDGDLPRWNFTDFMHSFMIVFRVLCGEWIESMWDCMLVGDVS

T. absoluta var 1    CIPFFLATVVIGNFVVLNLFLALLLSNFGSSSLSTPTADNDTNKI
T. absoluta var 2    CIPFFLATVVIGNFVVLNLFLALLLSNFGSSSLSTPTADNDTNKI
P. xylostella        CIPFFLATVVIGNLVVLNLFLALLLSNFGSSSLSTPTADQETNKI
B. mori              CIPFFLATVVIGNLVVLNLFLALLLSNFGSSSLSTPTADQDTNKI
H. zea               CIPFFLATVVIGNLVVLNLFLALLLSNFGSSSLSTPTADQDTNKI
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Figure 3.1. Amino acid alignment of domains IIS4-IIS6 of the *T. absoluta* sodium channel with the corresponding sequence of *Plutella xylostella* (BAF37093.2), *Bombyx mori* (NP_001136084.1) and *Helicoverpa zea* (ADF80418.1).The positions of the L1014F, M918T and T929I are shown in red boxes. Data obtained by K. Haddi.

Figure 3.2. Sequence of domain IIS4-S6 of the *T. absoluta para*-type sodium channel with primer and probe locations and positions of known *kdr/ skdr* mutations shown with boxes. Exons are shown in upper case and introns in lower case letters. Data obtained by K. Haddi.

Sequencing of pooled samples of the five lab strains revealed the presence of mutations conferring three amino acid substitutions known to give a resistant phenotype: M918T, T929I and L1014F. Sequencing of individuals found a high frequency of the mutations in all of the lab strains (Table 3.3). All ten individuals of all five strains were homozygous for L1014F (*kdr*). The two super-*kdr* (s*kdr*) changes, M918T and T929I, were only found in combination in individual larvae when both were in the heterozygous form. To test if the two mutations were on separate alleles or on the same allele, several individuals of this genotype were cloned and sequenced. Sequencing of ten colonies derived from the genes of each individual showed that M918T and T929I were only ever present on separate alleles.

The frequency of M918T, T929I and L1014F did not fully explain the variation in susceptibility between the populations found in the bioassays. This suggests that there may be other factors which affect the resistant phenotype. Metabolic enzymes have been shown to confer pyrethroid resistance in a number of insect species. For example, P450-mediated detoxification was found to be the primary mechanism in a deltamethrin-resistant strain of *Tribolium castaneum* (Zhu et al., 2010) and increased expression of P450s was the main mechanism in the malaria vector *Anopheles funestus* (Wondji et al., 2009, Riveron et al., 2013).

Table 3.3. Frequency of three mutations in 10 individuals of each of five laboratory populations of *T. absoluta*. Data obtained by K. Haddi

Population	Genotype Frequency		
	M918T	T929I	L1014F
TA1	0.2	0.8	1
TA2	0.5	0.5	1
TA3	0.35	0.65	1
TA4	0.45	0.45	1
GA	0.2	0.8	1

3.3.3 TaqMan assays to determine frequency of L1014F, M918T and T929I in *T. absoluta* populations

Three novel high-throughput assays were designed to test for the presence of M918T, T929I and L1014F in field populations of *T. absoluta* from 27 diverse geographical locations. The TaqMan assay could be used to test up to 72 individuals at a time, taking 90 minutes to complete. As *T. absoluta* is a diploid species, there were three possible genotypes: R/R (two copies of the resistant allele), R/S (heterozygous) and S/S (two copies of the susceptible allele). At the end of the assay, samples were scored by comparing the green and yellow channels. R/R samples gave a high signal in the green channel and low in the yellow; S/S gave a high signal in the yellow channel and low in the green and R/S gave an intermediate signal in both channels. DNA extracted from the lab strains was used as a control for each genotype. Since all of the lab strains were homozygous for F1014, L1014 homozygotes were not available as controls. An intermediate signal in both channels was seen for some of the individuals originating from Columbia and these were sequenced to confirm they were heterozygous (one copy 1014F and one copy 1014L) and then used as heterozygous controls in subsequent assays.

The TaqMan assays worked well for most individuals and the three genotypes could be distinguished easily by looking at the real-time signal in the two channels (Figure 3.3) or by using the scatter plot of the end-points of the assays (Figure 3.4). Of the 200 samples analysed, three (1.5%) had failed reactions and 17 (8.5%) needed confirmation by sequencing. This is in line with a TaqMan assay for *kdr* in the mosquito *Anopheles gambiae*, which had the lowest number of failed reactions and the best accuracy in scoring genotypes when compared to five alternative techniques (Bass et al., 2007). In this study, out of 96 samples, only five reactions failed and all of the samples were scored correctly. The authors proposed that DNA degradation could have been responsible for the failure of some samples to amplify a product (Bass et al., 2007).

In the present study the homozygous *kdr* mutation L1014F was fixed in all the *T. absoluta* populations analysed apart from the one from Colombia where some individuals had the mutation in the heterozygous form (Table 3.4). *Kdr* was first characterised in the housefly *Musca domestica* and the German cockroach *Blattella germanica* (Williamson et al., 1996, Miyazaki et al., 1996) but since then L1014F has been found in more than 20 insect species and alternative substitutions at the same site, L1014C/H/S/W, have been reported (Rinkevich et al., 2013). A study of the effect of *cis*-methrin on *M. domestica* sodium channels expressed in an oocyte system showed that L1014F conferred resistance to pyrethroids in two ways. Firstly, it reduced the sensitivity of the channels to *cis*-methrin by at least 10-fold and secondly, it decreased the duration of the open-state of *cis*-methrin modified channels (Smith et al., 1997). L1014 is situated close to the glycine hinge of IIS6 and substitution with a phenylalanine or histidine, may affect the ability of the IIS6 helix to bend thereby impeding movement of the IIS1-S4 voltage sensing domain. Reduced sensitivity to voltage means that the channels are harder to activate, so a lower proportion are open (Burton et al., 2011) and this reduces the effect of pyrethroids which preferentially bind to open channels (Davies et al., 2007).

All *T. absoluta* populations tested in the current project had T929I and most had M918T (Table 3.4). The *skdr* substitution M918T (domain II S4-S5 loop), which gives up to 500-fold resistance to type II pyrethroids was first found in combination with L1014F in house flies (Williamson et al., 1996). It has since been reported in many different arthropods including aphids, thrips and mites (Eleftherianos et al., 2008, Toda and Morishita, 2009, Nyoni et al., 2011). Pyrethroid-resistant tomato red spider mites (*Tetranychus evansi*) have M918T in the absence of L1014F (Nyoni et al., 2011). An alternative *skdr* substitution T929I was first identified diamond back moth, *Plutella xylostella,* where resistant populations with L1014F and T929I had resistance ratios of up to 5,000-fold to type I pyrethroids and up to 10,000-fold to type II pyrethroids (Schuler et al., 1998). T929I has also been found to correlate with pyrethroid resistance in the absence of L1014F in the maize weevil, *Sitophilus zeamais* (Araujo et al., 2011). It has been proposed that *skdr* mutations may have a

synergistic effect when combined with *kdr,* as sodium channels with T929I alone gave a 10-fold reduction in sensitivity to deltamethrin, but when combined with *kdr* the reduction in sensitivity was 10,000-fold (Vais et al., 2001). M918T (in the IIS4-IIS5 linker) and T929I (IIS5 helix) promote closed-state inactivation and additionally increase the rate of dissociation of pyrethroids (Davies et al., 2007).

The overall frequency of the two *skdr* mutations in the *T. absoluta* field samples tested was 0.35 for M918T and 0.6 for T929I suggesting that the latter might be being preferentially selected. M918T and T929I were never present in the same allele but many individuals had one M918T allele and one T929I allele. These insects having have half of their para-type sodium channels with M918T and half with T929I would have no wild-type channels. *Kdr* and *skdr* mutations have been shown to be recessive in house flies (Williamson et al., 1993) but aphids heterozygous for L1014F and M918T were more resistant than aphids homozygous for L1014F on its own, suggesting that *kdr /skdr* mutations are not completely recessive in all cases (Eleftherianos et al., 2008). Either way, *T. absoluta* which are homozygous for L1014F and heterozygous for both M918T and T929I would be expected to be strongly resistant to pyrethroids.

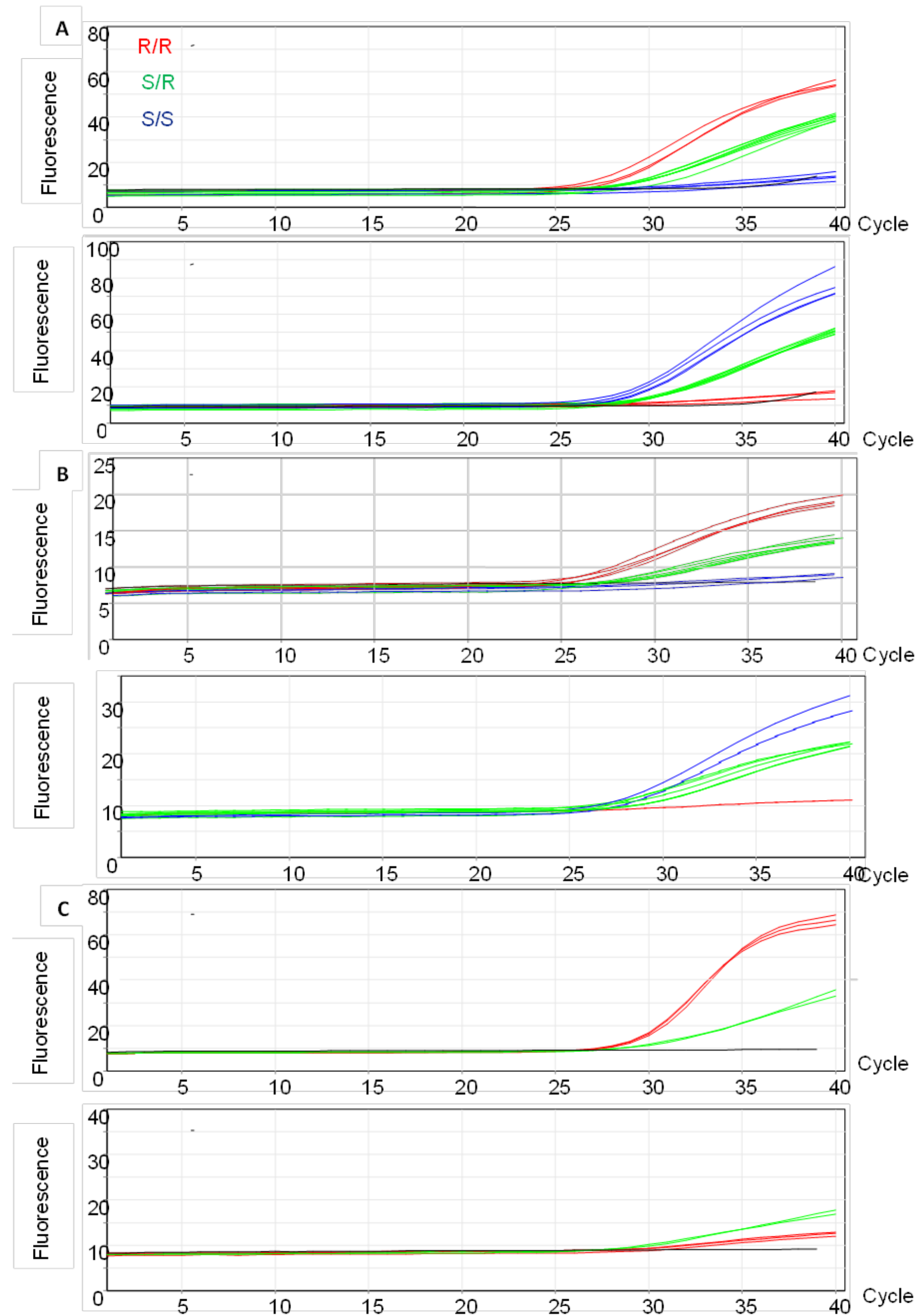


Figure 3.3. Real time TaqMan detection of mutations in *T. absoluta*. (A) M918T. (B) T929I. (C) L1014F. For each pair of graphs, the top graph shows increase of FAM-labelled probe specific for the mutant allele, and the bottom graph shows increase of VIC-labelled probe specific to the wild type allele. Blue= homozygous wild type, Green = heterozygous, Red = homozygous mutant. Data obtained by M. Berger

Table 3.4. *Kdr/skdr* mutation frequencies in *T. absoluta* populations collected from different geographical origins. N = number of individuals. (p) = grown in protected environments. Data obtained by M. Berger

Sample No.	Country	Location	Sampled from	N	Frequency		
					L1014F	M918T	T929I
1	Canary Is.	Tejina	Tomato	12	1.00	0.54	0.46
2	Canary Is.	Guía de Isora	Tomato	10	1.00	0.35	0.60
3	Canary Is.	Granadilla	Tomato	10	1.00	0.25	0.60
4	Canary Is.	Arico	Tomato	8	1.00	0.25	0.69
5	Baleares Is.	Teulera	Tomato	3	1.00	0.33	0.67
6	Baleares Is.	San Fangos	Tomato	3	1.00	0.33	0.67
7&8	Algeria	Mostaganem	Tomato(p)	9	1.00	0.17	0.83
9	Italy	Turín	Unknown	3	1.00	0.33	0.50
10	Ecuador	La Tola	Tomato	11	1.00	0.55	0.41
11	Spain	Tudela	Tomato	8	1.00	0.44	0.44
12	Argentina	Barrancas	Tomato(p)	7	1.00	0.00	1.00
13	Argentina	LaPrimavera	Tomato	8	1.00	0.13	0.75
14	Argentina	La Plata, Bs.As	Tomato(p)	3	1.00	0.00	1.00
15	Italy	Cagliari	Tomato(p)	5	1.00	0.20	0.80
16	Canary Is.	La Palma	Unknown	7	1.00	0.36	0.64
17	Italy	Sicilia	Aubergine	14	1.00	0.43	0.54
18	Spain	Ramonete/Lorca	Tomato	6	1.00	0.25	0.75
20	Colombia	Antioquía/Rione	Tomato	10	0.60	0.50	0.45
21	Crete	Heraklion	Wild plants	3	1.00	0.50	0.50
22	Spain	Mazarrón	Tomato	16	1.00	0.13	0.81
24	Peru	Chulacanas	Tomato	7	1.00	0.43	0.50
25	Spain	Valencia	Unknown	9	1.00	0.44	0.44
26	Spain	Maresme	Tomato	12	1.00	0.50	0.50
27	Portugal	Silveira	Tomato	5	1.00	0.20	0.80
28	Italy	Sele	Tomato	17	1.00	0.44	0.41
29a	Israel	Beit Hashita	Unknown	5	1.00	0.40	0.50
29b	Israel	Ein Hmifraz	Unknown	6	1.00	0.50	0.50
Total				217	0.98	0.35	0.60

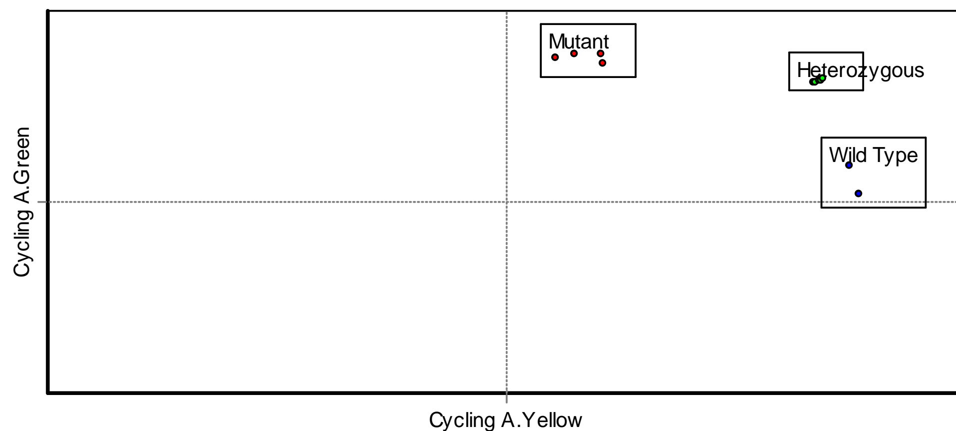


Figure 3.4. Scatter plot to facilitate genotyping of *T. absoluta* for T929I . The x axis shows the fluorescence emitted by the FAM-labelled probe specific for the Mutant allele (929I) after 40 cycles, and the y axis shows fluorescence emitted by the VIC-labelled probe specific to the Wild type allele (929T) after 40 cycles. Data obtained by M. Berger

3.3.4 Geographical distribution of the three pyrethroid-resistance mutations in *T. absoluta*.

The frequencies of *kdr/skdr* in countries where we were able to test at least six insects were mapped (Figure 3.5), showing all of the mutations were present in all of the countries and no strong geographical trend was observed. A genetic study of *T. absoluta* revealed high genetic homogeneity in *T. absoluta* populations from the Mediterranean Basin and South America with a single genetic type identified (Cifuentes et al., 2011). The authors proposed that a single genetically uniform and invasive population has been able to spread through South America, and then to the Mediterranean Basin. Since we found all three *kdr* mutations in all the regions we tested, our findings would suggest that *T. absoluta* arrived in Europe already with pyrethroid resistance.

Figure 3.5. Map of *kdr/skdr* frequencies in *T. absoluta* populations from countries where the sample size was >6. The red pie charts show the frequency of L1014F, blue pie charts the frequency of M918T and green pie charts the frequency of T929I. Data obtained by M. Berger.

3.3.5 Detection of *kdr/skdr* in field populations of *T. absoluta* from Brazil

The original study did not include field populations from Brazil, and since Brazil is the ninth biggest producer of tomatoes (FAOSTAT, 2012), data on the prevalence of *kdr* was needed . TaqMan assays were used on insects collected from eight regions of Brazil in 2010-2011; showing that L1014F was present at 100% frequency across all regions (Table 3.3). M918T and T929I were also found in all locations surveyed but T929I was more common than M918T with 49% of insects homozygous I929 compared with 19% of insects homozygous for T918 (Table 3.5). As before, I929 and T918 were never present on the same allele. Resistance to pyrethroids in Brazilian populations of *T. absoluta* was first described for permethrin in 2000 (Siqueira et al., 2000b), and has since been reported for bifenthrin (Silva et al., 2011, Gontijo et al., 2012). Our

molecular data suggests that resistance to pyrethroids is now present throughout Brazil.

Table 3.5. Frequency of L1014F, M918T and T929I in eight *T. absoluta* populations from Brazil determined by TaqMan assays. S = susceptible allele, R = resistant allele. Data obtained by WM Silva and M Berger.

Population	L1014F			M918T			T929I		
	RR (%)	RS (%)	SS (%)	RR (%)	RS (%)	SS (%)	RR (%)	RS (%)	SS (%)
ANP - GO	100	0	0	20	30	50	60	40	20
GCB - CE	100	0	0	10	50	40	20	50	30
IRA-BA	100	0	0	30	20	50	40	20	40
PLN- SP	100	0	0	10	40	50	60	40	0
PLT-RS	100	0	0	70	20	10	10	30	60
SUM-SP	100	0	0	0	70	30	70	30	0
TNG- CE	100	0	0	10	50	40	40	50	10
VDN- ES	100	0	0	0	10	90	90	10	0
Total	100	0	0	18.75	36.25	45	48.75	33.75	20

3.3.6 Detection of a fourth novel mutation in *T. absoluta*.

A limitation of TaqMan assays is that they are specific for known mutations, and cannot detect additional SNPs. So conventional PCR/sequencing was used on 17 samples which had given an ambiguous trace in either M918T or T929I TaqMan assays (see 3.3.3). Additionally, direct sequencing was done on 50 individuals from five lab populations. This revealed the presence of a fourth mutation in *T. absoluta* conferring a L925M substitution in the sodium channel. In the lab populations, 2 out of 50 samples (4%) had L925M in the

heterozygous form and for the 17 field samples which gave an ambiguous TaqMan trace, 4 (24%) had L925M. The presence of L925M would be expected to have caused interference with the T929I and M918T TaqMan assays as it is positioned within the primer binding sites for both assays. The close proximity of the three mutations meant that it was not possible to design a fourth TaqMan assay for L925M. Therefore, direct sequencing was used on a further 50 field samples to assess the frequency. Seven samples (14%) had L925M in the heterozygous form (Table 3.6). These samples were homozygous T929 and heterozygous for M918T or homozygous M918 and heterozygous for T929I. Samples which were homozygous for T918 or I929 were not observed with M925. This implies that T918, I929 and M925 are each found on a unique allele. L925M is a novel mutation that has not been described previously, however, an alternative substitution, L925I has been found in pyrethroid-resistant whiteflies, bed bugs and cattle ticks (Karatolos et al., 2012a, Morin et al., 2002, Morgan et al., 2009, Rinkevich et al., 2013, Yoon et al., 2008). Therefore L925M would also be expected to confer resistance to pyrethroids in *T. absoluta.*

Table 3.6. Genotypes of 50 samples of *T. absoluta* at positions 918, 925 and 929. Samples with the L925M are highlighted. Data obtained by N. Newman under the supervision of M. Berger.

Sample	Origin	Mutation frequency		
		M918T	L925M	T929I
Ba1	Boavista, Portugal	S/S	S/S	R/R
Ba2	Boavista, Portugal	S/S	S/S	R/R
Ba3	Boavista, Portugal	R/S	S/S	R/S
Ba4	Boavista, Portugal	S/S	S/S	R/R
Ba5	Boavista, Portugal	S/S	R/S	R/S
Ba8	Boavista, Portugal	S/S	S/S	R/R
Ba10	Boavista, Portugal	S/S	R/S	R/S
Ba11	Boavista, Portugal	R/R	S/S	S/S
Ca.G10	Canada de Gallego, Spain	R/S	R/S	S/S
Ca.G11	Canada de Gallego, Spain	R/R	S/S	S/S
Ca.G12	Canada de Gallego, Spain	S/S	R/S	R/S
Ca.G1	Canada de Gallego, Spain	R/S	S/S	R/S
Ca.G3	Canada de Gallego, Spain	S/S	S/S	R/R
Ca.G4	Canada de Gallego, Spain	R/S	S/S	R/S
Ca.G5	Canada de Gallego, Spain	S/S	S/S	R/R
Ca.G6	Canada de Gallego, Spain	S/S	S/S	R/R
Ca.G7	Canada de Gallego, Spain	S/S	S/S	R/R
Ca.G8	Canada de Gallego, Spain	R/S	S/S	R/S
Ca.G9	Canada de Gallego, Spain	R/S	S/S	R/S
Ca10	Canada de Gallego, Spain	S/S	R/S	R/S
Ca11	Canada de Gallego, Spain	S/S	S/S	R/R
Ca12	Canada de Gallego, Spain	R/S	S/S	R/S
Ca13	Canada de Gallego, Spain	R/S	S/S	R/S
Ca1	Canary islands	R/S	S/S	R/S
Ca2	Canary islands	R/R	S/S	S/S
Ca3	Canary islands	R/S	S/S	R/S
Ca4	Canary islands	R/S	S/S	R/S
Ca5	Canary islands	R/S	S/S	R/S
Ca9	Canary islands	R/S	S/S	R/S
CH1	Charneca, Portugal	R/R	S/S	S/S
CH2	Charneca, Portugal	S/S	S/S	R/R
CH3	Charneca, Portugal	R/S	S/S	R/S
CH5	Charneca, Portugal	R/R	S/S	S/S
CH7	Charneca, Portugal	S/S	R/S	R/S
CH8	Charneca, Portugal	R/S	S/S	R/S
CH9	Charneca, Portugal	R/S	S/S	R/S
CH10	Charneca, Portugal	S/S	R/S	R/S
CH11	Charneca, Portugal	R/S	S/S	R/S
CH12	Charneca, Portugal	R/S	S/S	R/S
Ni1	Nijar, Spain	S/S	S/S	R/R
Ni2	Nijar, Spain	S/S	S/S	R/R
Ni3	Nijar, Spain	S/S	S/S	R/R
Ni4	Nijar, Spain	S/S	S/S	R/R
Ni5	Nijar, Spain	S/S	S/S	R/R
Ni6	Nijar, Spain	S/S	S/S	R/R
Ni7	Nijar, Spain	R/S	S/S	R/S
Ni8	Nijar, Spain	S/S	S/S	R/R
Ni9	Nijar, Spain	S/S	S/S	R/R
Ni10	Nijar, Spain	R/R	S/S	S/S
Ni11	Nijar, Spain	R/S	S/S	R/S

3.4 Conclusions

Five laboratory populations of *T. absoluta* originating from Brazil and Europe showed high levels of resistance to two different pyrethroids in bioassays. Whilst there was considerable variation between populations, even the least resistant had LC_{50} values above the recommended field rate for these insecticides. This data is in agreement with previous studies of pyrethroid resistance in *T. absoluta* (Siqueira et al., 2000b, Silva et al., 2011, Lietti et al., 2005). To elucidate the mechanisms underlying this resistance, a section of the target site known to have mutations in other species was cloned and sequenced. This revealed three mutations conferring substitutions known to be involved in resistance, M918T, T929I and L1014F.

The frequency and distribution of L1014F, M918T and T929I mutations in *T. absoluta* from 35 different geographic locations showed that they were at high frequency throughout the regions surveyed. No individual was found that did not have at least two of the three mutations. All of the European strains were homozygous for L1014F. Whilst L1014F, T929I and M918T have been identified previously in other insects, *T. absoluta* is only the second species to be found with all three, the other being *Thrips tabaci* (Toda and Morishita, 2009). Furthermore, this is the first report of individuals with all three mutations (one allele with L1014F + M918T and the other with L1014F+ T929I). The rapid expansion of *T. absoluta* over the last eight years may have been in part mediated by the resistance of this pest to insecticides including pyrethroids. L1014F, M918T and T929I have been functionally expressed in *Xenopus* oocytes and shown to reduce the sensitivity of insect sodium channels to pyrethroids (Vais et al., 2000, Vais et al., 2001).

In this study, I developed novel real-time TaqMan PCR assay that can accurately genotype large numbers of individual larvae or adults for three *kdr/ skdr* mutations conferring L1014F, M918T and T929I. The assays worked well, giving unambiguous results for 180 out of the 200 samples analysed. One disadvantage of TaqMan PCR is that it is specific for a given mutation, so it will not pick up alternative mutation in the same region. I discovered that a small number of insects had L925M in heterozygous form. Whilst L925M has

not been described, L925I at the same site has been linked to pyrethroid resistance in other species.

Clearly these findings have major implications for the control of *T. absoluta* with pyrethroid insecticides. Both M918T and T929I in combination with L1014F are known to give strong resistance across the entire class of synthetic pyrethroids. The fact that one or other of these combinations are found within all of the field samples tested suggests that pyrethroids are likely to be ineffective at controlling *T. absoluta* across its range. Alternative insecticides with different modes of action should be used instead. In the next chapter, I undertake bioassays to determine the susceptibility of *T. absoluta* to an alternative insecticide spinosad. Spinosad is a newer insecticide in the chemical class spinosyns and targets a different part of the insect nervous system to pyrethroids.

4. Bioassays to determine sensitivity of *T. absoluta* to Spinosad

4.1 Introduction

Spinosad is an insecticide which combines spinosyns A and D, originally discovered as secondary metabolites of the soil bacteria *Saccharopolyspora spinosa*. Spinosyns target the nicotinic acetylcholine receptors of the insect nervous system (Perry et al., 2007, Watson et al., 2010). An insect which has ingested or come into contact with spinosad will stop feeding, display tremors and involuntary muscle contractions followed by paralysis and death. Insects in the orders Lepidoptera, Diptera and Thysanoptera are the most sensitive to spinosad. Spinosad has low toxicity to mammals and birds and a short half-life, making it more environmentally friendly than many alternative insecticides (Thompson et al., 2000).

A recent study of the efficacy of ten insecticides used against *T. absoluta* in Brazil found that spinosad was one of just two insecticides (the other being abamectin), which gave 100% mortality at the recommended field rate in all seven regions tested (Silva et al., 2011). However, resistance to spinosad has been reported in *T. absoluta* populations in Chile, where biochemical investigation has revealed that the activity of three classes of metabolic enzymes, P450s, glutathione-s-transferases and esterases, were significantly different in the resistant field populations compared with a laboratory susceptible population (Reyes et al., 2012).

In 2012, there was a report of control failure to spinosad in a population of *T. absoluta* in Portugal (personal communication, Rob Jacobson). To elucidate the level of resistance in this population, larvae, named as 'Spin' were sent to Rothamsted Research to be reared and tested in the insectary. Leaf-dip bioassays were conducted to calculate the LC_{50} and compare it with susceptible populations. Selection with Spinosad was used to increase the resistance of this population. Bioassays with the synergist Piperonyl butoxide (PBO) were conducted to investigate if metabolic enzymes were involved in resistance and a novel bioassay was developed to test adults for spinosad resistance.

4.2 Specific Methods

4.2.1 Insect material

T. absoluta populations collected from Spain (TA1), Italy (TA3), Portugal (TA4) and Brazil (GA) between 2008 and 2010 and one population collected from a field in Portugal in 2012 after reported control failure using spinosad (Spin-Parent), were reared on tomato under conditions of 26 °C temperature and 16hr light in the insectary at Rothamsted Research.

4.2.2 Selection of the Spin-Parent population to give the SpinSel strain

The SpinSel strain was selected from the Spin-Parent population by placing larvae (n >100) on spinosad coated tomato leaves for 3 days, and taking the survivors to the next generation. The concentration of spinosad used was increased during selection at certain generations (Table 4.1). Conserve (spinosad 11.6% w/v) was diluted with water containing 0.01% agral to make final concentrations between 20 and 120 mg L^{-1} and applied to both the upper and lower leaf surfaces. Initially, the selection of SpinSel was carried out by treating the leaves of a whole plant with spinosad. A problem with this is that as the plant grows new leaves, the larvae could move to these. To overcome this, a selection chamber (Fig 4.1) was built to select larvae using detached leaves. This was used for the final selection step (F19).

Table 4.1. Selection of Spin population

Selection	Generation (of larvae) since field collection	Concentration of Spinosad (mg L^{-1})
1st	F5	20
2nd	F6	40
3rd	F8	40
4th	F9	80
5th	F10	120
6th	F11	120
7th	F12	120
8th	F13	120
9th	F15	120
10th	F16	120
11th	F19	120

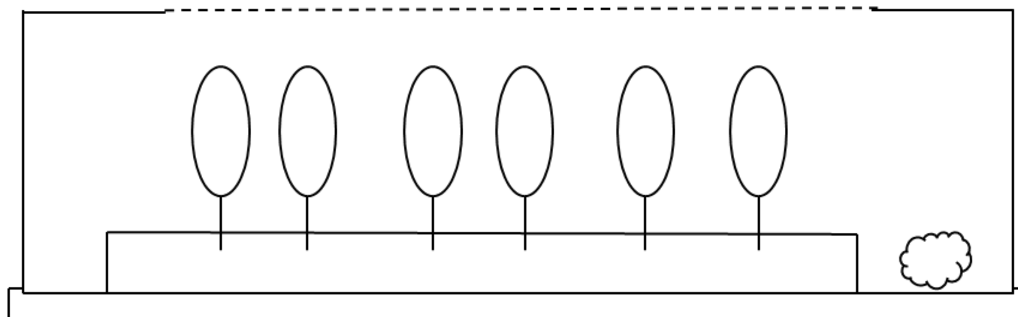


Figure 4.1. Selection chamber. A rectangle was cut from a plastic container and fine mesh was glued on top. Leaves were immersed in insecticide, left to dry and then put in moist oasis in the chamber. At least 100 second instar larvae were added to 12 leaves (8-10 larvae per leaf), and a piece of cotton wool placed in the chamber for pupation. After 72 hrs the cotton wool and leaves (containing the surviving insects) were placed in a cage on an untreated plant.

4.2.3 Bioassays of *T. absoluta* larvae

4.2.3.1 Leaf-dip bioassays

Leaf-dip bioassays were performed as described in the Insecticide Resistance Action Committee (IRAC) susceptibility test method 22. Conserve (spinosad 11.6% w/v) was diluted with water containing 0.01% agral to final concentrations between 1 and 1000 mg L^{-1}.

Preliminary bioassays were performed to determine 6 concentrations to use in the full bioassay to give low mortality at the lowest concentration, intermediate mortality at middle concentrations and 100% mortality at the highest concentration. Tomato leaves were dipped in insecticide for 10 seconds and then left to dry on paper towels for 30 minutes. Leaves dipped in 0.01% agral were used to determine the control mortality. Each leaf was placed in a petri dish on top of a piece of filter paper moistened with 200 µl water, and moistened cotton wool was wrapped around the base of the petiole as described previously (Roditakis et al., 2013b). Eight second-instar larvae were placed on each leaf using a fine paintbrush, with three to six biological replicates per concentration. The petri dishes were left in a controlled environment of 26 °C, 16 hr light; 8hr dark ligh and after 72 hrs, the numbers of live and dead larvae were counted using a lamp and dissecting microscope.

4.2.3.2 Synergist Assays

PBO was diluted with acetone to a concentration of 75 mg L^{-1}, chosen after testing a range of concentrations in a lab reference population (TA4), and choosing the highest concentration which did not cause control mortality. Topical application using a micro-syringe was deemed unsuitable because the smallest possible droplet (0.25 µl) was bigger than the diameter of second-instar larvae. Instead, larvae were exposed through contact with coated vials. Glass vials were coated in PBO by pipetting 500 µl of the diluted solution into each vial using a displacement pipette and then placing the glass vials on their side without lids on a rotating machine until dry. Control vials were set up with 500 µl acetone. Eight second instar larvae were then placed into each coated vial and after two hours the larvae were transferred to insecticide coated leaves for leaf dip bioassays described (4.2.3.1).

4.2.4 Bioassays of *T. absoluta* adults

An IRAC method has only been developed for the larval stage of *T. absoluta,* so in order to test the sensitivity of adult moths to spinosad, a novel method was developed. One practical difference between adults and juvenile Lepidoptera is that adults are able to fly. Therefore, a way of temporarily knocking out the adults was required and this was achieved by exposure to CO_2 for 40 seconds, with adults making a full recovery a few minutes afterwards. Three different methods of bioassay were tested: leaf-dip, topical and feeding.

4.2.4.1 Leaf-dip bioassays

Leaf-dip bioassays were performed on adults using the same method as described for larvae (4.2.2). Three petri-dishes containing ten insects were tested for each insecticide concentration. Moths were knocked out with $CO_2$and then placed on top of insecticide-coated leaves using a paint brush. The petri dishes were placed in a controlled environment of 26 °C, 16 hr light; 8hr dark light. Mortality was assessed after 72 hrs.

4.2.4.2 Topical bioassays

A micro-syringe was used to apply 0.5 µl of insecticide (diluted with acetone) directly onto the thorax of adults. Three reps of ten insects were tested for each insecticide concentration with 100% acetone used to assess control mortality. Treated insects were kept in a controlled environment of 26 °C, 16 hr light; 8hr dark light and mortality was recorded after 72 hrs.

4.2.4.3 Feeding bioassays

Adult moths were knocked out with CO_2 and then placed in 50ml polypropylene tubes (Greiner Bio-One Ltd., UK) using a paintbrush. Cotton wool balls were dipped in a mixture of insecticide and sugar solution (final concentration 10% sugar), wrung out to prevent dripping and used to plug the top of the tubes. Ten insects were put in each tube, and three reps of six insecticide concentrations were tested. The tubes were left a controlled environment of 26 °C, 16 hr light; 8hr dark light and mortality was assessed after 72 hrs.

4.2.5 Statistical analysis

GenStat 17th Edition (Payne et al., 2011) was used to calculate LC_{50}s (the concentration expected to kill 50% of individuals) and Probit analysis (Finney, 1947) was performed with control mortality estimated. The transformation link for the proportion of insects dead was logit. Logs to base 10 were taken of the dose. When comparing two populations, tested on the same day, the same slope was used to allow comparison of LC_{50}s. If 95% confidence intervals did not overlap populations were considered to be significantly different. Resistance ratios were calculated from:

$$RR = \frac{LC_{50}\ \text{resistant population}}{LC_{50}\ \text{susceptible or unselected population}}$$

4.3 Results and Discussion

4.3.1 Susceptibility of *T. absoluta* populations

T. absoluta populations TA1, TA3 TA4 and GA were tested for sensitivity to spinosad using leaf-dip bioassay to estimate LC_{50} values. The LC_{50}s ranged between 1.8 and 6.1 mg L^{-1} (Table 4.2). Since the recommended field rate for application of spinosad is 80-120mg L^{-1}, it would be expected to give good control of these populations.

Table 4.2. *T. absoluta* LC_{50}s for Spinosad (mg L^{-1}). CI = Confidence Interval

Population	LC_{50}	5% CI	95% CI
TA1	5.2	3.1	7.3
TA3	1.8	1	2.8
TA4	6.1	3.7	9.6
GA	3	1.8	4.4
Spin-Parent (F3-F4)	14.9	8.3	23.5
Spin (F11-F12)	8.9	3.4	18.7
Spin (F20-F21)	3.1	1.3	5.3
SpinSel (F11-F12; 5 selections)	25.2	10.8	50
SpinSel (F20-F21; 11 selections)	498.6	259.3	1105.8

Other studies have tested the susceptibility of field populations of *T. absoluta* to spinosad. A study of insecticide toxicity on six populations collected in Greece in 2010-2011 found LC_{50}s in the range of 0.08-0.26 mg L-1, with an estimated mortality of 100% for all populations treated at the field rate (Roditakis et al., 2013a). Similarly, a study of seven field populations collected from Brazil found low resistance to spinosad (LC_{50}s 0.46 – 2.26 mg L-1) with 100% mortality expected at the field dose (Silva et al., 2011). The LC_{50}s obtained in the present study were slightly higher on average, possibly due to slight differences in experimental method, for example the formulation of Spinosad and the adjuvant used.

4.3.2 Initial susceptibility of the *T. absoluta* Spin-Parent population

Larvae were collected from a field in Portugal where control failure with spinosad had been reported. This population 'Spin-Parent' was reared on insecticide-free tomato plants until numbers were sufficient to conduct a bioassay, which gave an LC_{50} of 14.9 mg L^{-1} (Table 4.2). This was the highest recorded LC_{50} of all the populations tested and significantly higher than the TA1, TA3 and GA populations (Table 4.2). The confidence intervals overlapped with those of TA4, also from Portugal, and this might suggest that Portugal has a higher risk of resistance development. However, all LC_{50}s were still well below the recommended field rate of 120 mg L^{-1} applied on crops in Portugal.

4.3.3 Selection of the Spin-Parent population to give the SpinSel strain

The Spin-Parent population was split between two cages and then one had no exposure to insecticide (Spin) and the other was selected with spinosad (SpinSel). After five selections with spinosad the two populations were compared by leaf dip bioassay. The LC_{50} of the selected population was 25.2 mg L^{-1}, whilst that of the unselected population was 8.9 mg $L\text{-}^{1}$. The confidence intervals of the two populations still overlapped at this stage. After 11 selections the two populations were tested again and the LC_{50} for SpinSel was 498.6 mg L^{-1}, compared to 3.1 mg L^{-1} for the unselected Spin giving a resistance ratio of 161 (LC_{50} of the selected population divided by the LC_{50} of the unselected population). Furthermore differences between the two populations in terms of the physical damage to spinosad-coated tomato leaves could now be seen (Figure 4.2).

In comparison with other strains the LC_{50} of SpinSel was 276-fold higher than the TA1 strain and 5000-fold higher than the LC_{50} of 0.07 reported in a laboratory susceptible population established from an area of crops that had never been sprayed (Reyes et al., 2012).

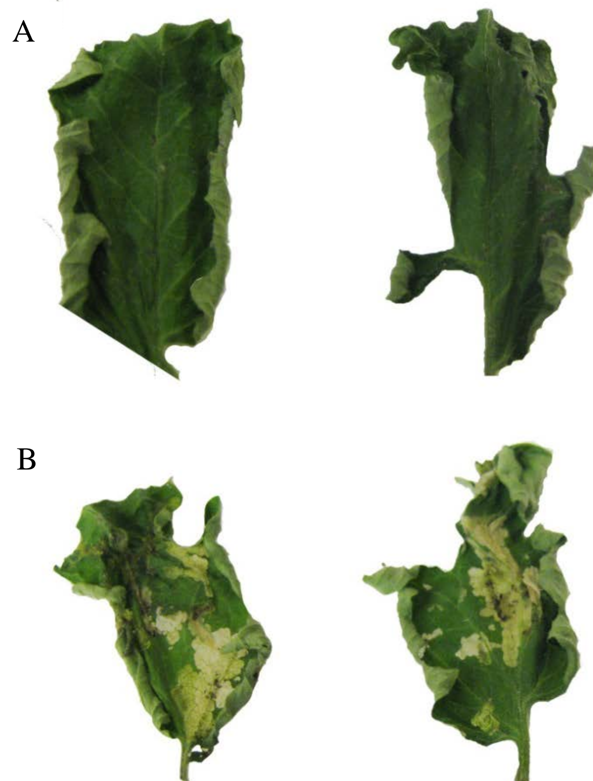


Figure 4.2. Spinosad lead dip bioassay. Spinosad-dipped leaves mined by A) Spin and B) SpinSel *Tuta absoluta* populations. Eight larvae per leaf; 72 hr incubation; 300 mg L^{-1} spinosad.

A population of *T. absoluta* in Brazil selected in the laboratory with Spinosad for 12 generations has been reported to reach an even higher LC_{50} of 3706 mg L^{-1}. However, this was found to be unstable, reverting to levels similar to the susceptible strain after 8 generations with no selection (Campos et al., 2014b). This matched the pattern observed in the unselected Spin population which displayed a 5-fold reduction in resistance after 17 generations without selection (Table 4.2). The lack of stability of the resistance observed is likely due to a fitness cost associated with the resistant phenotype. Fitness costs associated with insecticide resistance have been described in many species including *Culex pipiens, Nilaparvata lugens,* and *Myzus persicae* (Hardstone et al., 2009, Liu and Han, 2006, Foster et al., 2000). Fitness costs associated with spinosad resistance in *Helicoverpa armigera* included longer development time, lower fecundity and reduced pupal survival (Wang et al., 2010a).

The selection of SpinSel was initially carried out by treating the leaves of a whole plant with spinosad. A problem with this is that as the plant grows new leaves, the larvae could move to these. Although spinosad sometimes has systemic properties when applied to roots, it does not spread between treated and untreated leaves (van Leeuwen et al., 2005). Therefore a selection chamber with detached treated leaves was used for the final selection step. It is possible that the resistance observed would have occurred in fewer generations if the selection chamber had been used from the beginning. It is also hard to predict how long selection might take in the field as this would be affected by many environmental factors that are not present in the laboratory. Furthermore in the selection experiments described here the concentration of spinosad was increased during the selection, whereas in a field-scenario they would be likely to remain constant, i.e. the label rate. However, the diamond back moth, *Plutella xylostella*, became highly resistant after just two years of exposure to spinosad in the field (Zhao et al., 2002)

4.3.4 Synergist assays

The chemical PBO has been shown to inhibit both P450s and esterase (Young et al., 2005, Jones, 1998), so PBO assays are a useful tool for determining whether resistance involves these metabolic enzymes (Jones, 1998). Therefore to determine whether the mechanisms underlying resistance in the SpinSel strain are mediated by P450s/esterases, a bioassay using PBO was done. This showed that the LC_{50}s were not significantly different between larvae treated with PBO and untreated controls (Figure 4.3), showing that enhanced activity/expression of metabolic enzymes is not the main mechanism of resistance in this strain. This is in contrast to a previous study which found higher metabolic activity in spinosad-resistant field populations of *T. absoluta* in Chile (Reyes et al., 2012). However, the laboratory selected population in Brazil showed no increase in esterase or P450-dependent O-demethylase activity with spinosad selection (Campos et al., 2014b).

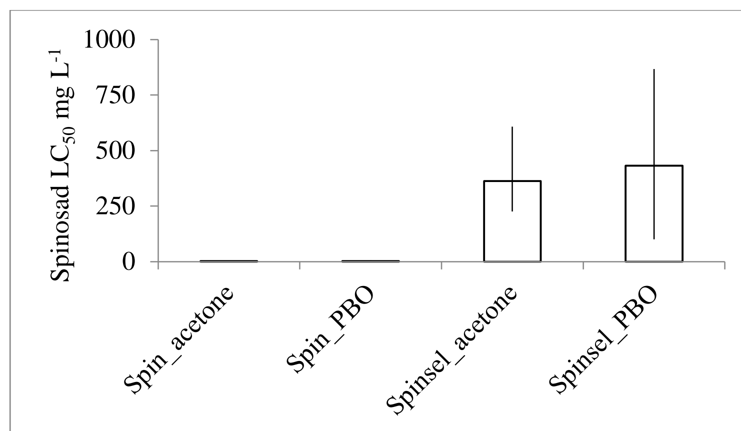


Figure 4.3. Results of Piperonyl butoxide (PBO) bioassays. *Tuta absoluta* larvae treated with acetone or 75mg L^{-1} PBO for two hours prior to insecticide treatment. Error bars show 5% and 95% confidence intervals.

4.3.5 Adult bioassays

Novel bioassays were developed to test the sensitivity of adult moths to spinosad. A preliminary bioassay used the leaf-dip method but only the larval stage feeds on leaves so adults would only be exposed through direct contact when resting or ovipositing on the leaves. However, this gave very high control mortality (>50%) so was not suitable. A second approach used a micro-syringe to apply insecticide, diluted with acetone, directly on to the insect. This had previously been described to test larvae of *T. absoluta* (Lietti et al., 2005) but it was difficult to carry out as the moths tended to twitch when touched, even when knocked out by CO_2 and the moths were very delicate. Control insects which had acetone applied topically showed high mortality after 72 hrs (>50%). Therefore, it was decided to perform feeding bioassays using insecticide mixed with sugar solution. Sugar solution is routinely used as an energy source for laboratory-reared adult *T. absoluta* (Roditakis, 2011) and preliminary experiments showed low control mortality (< 20%) with this method.

The mortality rate of Spin and SpinSel adults exposed to spinosad through the feeding assay was assessed after 72hrs. The LC50 of Spin adults was 3.9 mg L^{-1} compared with 170.5 mg $L^{-1}$for SpinSel adults (Table 4.3). This represents a resistance ratio of 43.7 and this difference was significant, as the confidence

intervals of the populations did not overlap. However, the resistance ratio in the SpinSel adults was significantly lower than the 161 found for larvae. This might result from the different bioassay methods used. One disadvantage of feeding assays to measure resistance is that the exact amount of insecticide ingested is not known so the adults may not have ingested the same as the larvae. A study comparing ingestion and contact bioassays in *Bactrocera oleae* found that spinosad was more toxic when ingested, with the LC_{50} from ingestion approx. 1/15 of that from contact. However the resistance ratios were consistent between the two methods. Therefore the two methods were equally valuable for comparing populations (Kakani et al., 2010). An alternative explanation for the difference in resistance ratios in the present study is that resistance may be stage-specific. Stage-specific resistance to Bt-toxins has been found in the Colorado potato beetle (Wierenga et al., 1996) and expression of metabolic enzymes in neonicotinoid-resistant *Bemisia tabaci* was found to correlate with developmental stage (Jones et al., 2011).

Table 4.3. Relative toxicity of Spinosad to *T. absoluta* adults. CI, confidence interval.

Population	LC_{50} (mg L^{-1})	5% CI	95% CI
Spin	3.9	2.0	6.8
SpinSel	170.5	92.1	257.3

4.4 Conclusions

In this chapter susceptibility to spinosad was tested in four populations of *T. absoluta,* giving LC_{50}s between 1.8 and 6.1 mg L^{-1}. A population from a field in Portugal with reported control failure had a higher LC_{50} of 15 mg L^{-1} which rose to approx. 500 mg L^{-1} after selection in the laboratory. A novel bioassay was developed to test the adults of this population and they also displayed high resistance, with an LC_{50} of 170 mg L^{-1}. Since the licensed field rate is 60mg L^{-1} in Brazil, 120mg L-1 in Portugal and 87mg L^{-1} in the UK, control failures would be expected in populations displaying this level of resistance. However, LC50s above the field rate have not yet been published for natural populations.

Tests on *T. absoluta* in Brazil, Turkey and Greece collected in 2010-2011 all gave very low LC_{50}s (Table 4.4).

Table 4.4. Summary of reported LC_{50}s for spinosad from four studies of *T. absoluta* in South America and Europe

Population	Date of	Origin	LC50	Reference
GR-IER5	2011	Greece	0.08	Roditakis 2013a
GR-PEL2	2010	Greece	0.16	Roditakis 2013a
GR-TYMP1	2011	Greece	0.18	Roditakis 2013a
GR-PEL3	2010	Greece	0.18	Roditakis 2013a
GR-IER3	2010	Greece	0.18	Roditakis 2013a
GR-IER4	2010	Greece	0.26	Roditakis 2013a
GBN	2010	Brazil	0.17	Campos 2014a
VDN	2011	Brazil	0.31	Campos 2014a
TNG	2010	Brazil	0.13	Campos 2014a
PLN	2010	Brazil	0.007	Campos 2014a
PLT	2011	Brazil	0.17	Campos 2014a
SMR	2011	Brazil	0.63	Campos 2014a
IRQ	2011	Brazil	0.41	Campos 2014a
ANP	2011	Brazil	0.15	Campos 2014a
Bodrum	2009	Turkey	1.6	Dagli 2012
Demre	2011	Turkey	0.6	Dagli 2012
Fethiye	2011	Turkey	0.8	Dagli 2012
Alanya	2011	Turkey	0.6	Dagli 2012
Kumluca	2011	Turkey	0.3	Dagli 2012
Kampus	2011	Turkey	0.3	Dagli 2012
Izmir	2011	Turkey	0.6	Dagli 2012
TA1	2010	Spain	5.2	This study
TA3	2010	Italy	1.8	This study
TA4	2010	Portugal	6.1	This study
GA	2008	Brazil	3.0	This study
Spin-Parent	2012	Portugal	14.9	This study

More recently, there have been reports from growers of spinosad resistance in populations of *T. absoluta* in Denmark and the UK (Rob Jacobson, Personal Communication). A positive correlation between number of spinosad applications and resistance ratios in field populations of Olive fruit fly has also been observed (Kakani et al., 2010) and a similar trend may be becoming apparent in *T. absoluta.* Spinosad is a particularly important insecticide for the

control of *T. absoluta* because it is derived from soil bacterium and is therefore classed as a 'natural' insecticide and one of the few compounds that can be used in organic tomato production. Secondly, for non-organic growers, spinosad is compatible with several Integrated Pest Management (IPM) strategies and is used for early season control while the numbers of natural enemies, such as *Macrolophus pygmaeus*, used for control increase in number (Rob Jacobson, personal communication). These predators are not harmed by spinosad but are killed by many alternative synthetic insecticides.

Ideally, to avoid further resistance to spinosad in *T. absoluta*, different classes of insecticide should be rotated. However, this is difficult in practise because *T. absoluta* already exhibits resistance to many chemical classes. Pyrethroids are not recommended for control (see Chapter 3), and there have been recent reports of resistance to diamides (Roditakis et al., 2015). Alternative methods of control such as mating disruption (Cocco et al., 2013) or use of biological enemies (Urbaneja et al., 2012) could be used to reduce the number of insecticide sprays. However, judicious use of insecticides forms an important part of many IPM strategies and are used to augment biological control which often does not provide sufficient levels of control. Furthermore whilst biological control works well in protected cropping (such as in glasshouses) it provides much less effective control in open field settings.

Elucidating the mechanism responsible for the observed resistance to spinosad would help control strategies by allowing diagnostics to be developed for monitoring the frequency and distribution of resistance and so inform effective control strategies. A synergist bioassay did not find a significant reduction in resistance after exposure to PBO, suggesting that metabolic resistance is not mediated by P450s/esterases. A bioassay on adults found resistance, but not as high as in the larvae. This could suggest multiple mechanisms of resistance, not all of which are present in every life stage. The next chapter describes next generation sequencing to assemble transcriptomes for Spin and SpinSel and compare gene expression. Chapter six describes studies of the target site of spinosad and comparisons of resistant and susceptible populations.

5. Generation of *T. absoluta* transcriptome

5.1 Introduction

A transcriptome is the total transcripts in any organism/tissue at the time when the mRNA is isolated. As such it allows the identification of genes being expressed and this has proven useful in identifying genes/proteins involved in insecticide resistance. The transcriptomes of insect pests have been used to identify genes linked to insecticide resistance including cytochrome P450s, cuticular proteins, ABC transporters, carboxylesterases, heat shock proteins and cathepsins (Silva et al., 2012, Mamidala et al., 2012, Keeling et al., 2012). Additionally, transcriptomics has been used to assess the viability of biological control, by annotating genes involved in immune response to microbial pathogens (Pascual et al., 2012). On the other hand for genes encoding insecticide target sites and the mutations associated with resistance, next generation sequencing has allowed the identification of full length genes, which are often long and complex (Karatolos et al., 2011). Several genomes and transcriptomes have been published for some Lepidoptera (ISGC, 2008, Zhan et al., 2011), but neither have been reported for *T. absoluta* and indeed only a few gene sequences have been deposited in GenBank. Therefore, we decided to create a reference transcriptome for *T. absoluta*, allowing the annotation of cytochrome P450s and insecticide target sites.

5.2 Specific Methods

5.2.1 454 sequencing

RNA was extracted from 10 pooled TA1 *T. absoluta* larvae using the Bioline Isolate II RNA mini kit (see 2.3). Genomic DNA was removed by DNase I digestion using DNA-free DNase Treatment and Removal Reagent (Ambion ®, USA). Double-stranded cDNA was synthesised using Clontech SMARTer kits (to increase the proportion of full-length cDNAs) and normalised using the Evrogen Trimmer kit (to improve representation of transcripts across the library). The Liverpool Centre for Genomic Research then prepared a 454 fragment library from the normalised cDNA, and pyro-sequenced one plate.

5.2.2 Illumina sequencing

5.2.2.1 Sequencing of *T. absoluta* strain TA1

Total RNA was extracted from all four life stages (eggs n=50; larvae L2-L3 n=16; pupae n=10; Adults n=10) of the TA1 population using the Bioline Isolate II RNA mini kit (see 2.3). The RNA was pooled and sent to Eurofins Genomics, Germany for preparation of a random-primed normalised cDNA library (with an insert size of 150-450 bp), and sequencing on the Illumina HiSeq 2000 by paired-end 100 bp reads. FastQC was used to check the quality of the raw reads obtained.

5.2.2.2 Sequencing of *T. absoluta* strains Spin and SpinSel

RNA was extracted from 3 replicates of 10 larvae from the Spin and SpinSel populations, using the Bioline Isolate II RNA mini kit (see 2.3). The six samples were sent to The Genome Analysis Centre ™ (TGAC) for Illumina TruSeq RNA library preparation (insert size between 254 and 281bp) and multiplexed for sequencing on one lane of an Illumina HiSeq 2000 using 100bp paired-end sequencing. FastQC was used to check the quality of the raw reads.

5.2.3 Newbler *de-novo* assembly

454 Life Sciences Corporation Newbler Version 2.6 was used for *de-novo* assembly of the 454 sequence reads by the Liverpool Centre for Genomic Research. Newbler compares all reads to all other reads to identify possible overlaps. Unitigs, groups of reads with consistent overlaps between each other, are produced (Margulies et al., 2005) and these act as high-confidence contigs to seed the rest of the assembly (Miller et al., 2010). The unitigs are compared to other unitigs and any overlapping unitigs are combined to create larger contigs. Next, regions are identified where contig sequences diverge and contigs are broken at these boundaries. If a read spans two contig ends the contigs are re-joined. A quality control step is performed where reads are mapped to the contig sequences and contigs are broken if there are less than four reads spanning the contig. A final consensus regeneration step is performed to obtain the final contigs (Margulies et al., 2005). This was named assembly 1.

5.2.4 Trinity *de-novo* assembly

The Illumina sequences were assembled using Trinity (Grabherr et al., 2011) a programme designed for *de-novo* transcriptome assembly of organisms without a reference genome. Trinity consists of three steps. First 'Inchworm' assembles the reads into unique linear transcripts by constructing a catalogue of 25bp *k*-mers from the raw reads. Inchworm uses the most abundant *k*-mer to initiate the assembly of a contig and then extends it in each direction by finding the highest occurring *k*-mer with a 24bp overlap to the end of the contig. The extension continues until the contig cannot be extended any more. This is then repeated until all the k-mers have been used. K-mers that only appear once in the raw data are not used, in order to reduce the impact of sequencing errors on the assembly. Next 'Chrysalis' connects contigs together and constructs de Bruijn graphs and then 'Butterfly' simplifies and trims the graphs and looks for the most plausible paths through the graphs, where each path represents a possible transcript. Butterfly then extracts linear sequence for all plausible transcripts (Grabherr et al., 2011). The programme PuTTY was used to send UNIX commands to a Linux server from a windows PC. The Trinity parameters were paired mode, with 2 CPUs used for Inchworm and 32 CPUs for Butterfly. Five assemblies were performed: TA1 alone (assembly 2), Spin alone (assembly 3), SpinSel alone (assembly 4), Spin plus SpinSel (assembly 5) and Spin, SpinSel plus TA1 (assembly 6).

5.2.5 Annotation

A tera-blastx search against the non-redundant protein database (NCBI 22/10/14), with an e-value of 1 $\times 10^{-3}$ and minimum score of 50, was performed within Galaxy (Goecks et al., 2010) for each of the six assembled transcriptomes. The resulting XML files were imported into Blast2GO (Conesa et al., 2005) for further analysis and annotation. Expressed sequence tags (ESTs) with hits against insecticide target sites were imported into Geneious (Biomatters Ltd.) and assembled to create consensus sequences. The consensus sequences were translated and aligned with corresponding *B. mori* proteins using MUSCLE (Edgar, 2004) .

5.3 Results and Discussion

5.3.1 Transcriptome assemblies

454-based pyrosequencing of *T. absoluta* normalised cDNA (TA1) gave a data set of expressed sequence tags, comprising 771,332 reads with an average read length of 434bp. These reads were assembled into 37,422 contigs, with an N50 of 734bp (Table 5.1, assembly 1).

Table 5.1. Comparison of transcriptome assemblies. Blast searches of translated nucleic acid (tera-blastx) against the non-redundant protein NCBI database (22/10/14) with an E-value of 0.001.

Assembly ID	1	2	3	4	5	6
Population(s)	TA1	TA1	Spin	SpinSel	Spin plus SpinSel	Spin plus SpinSel plus TA1
Sequencing	454	Illumina	Illumina	Illumina	Illumina	llumina
Raw reads	771,332	218,811,874	92,659,054	87,745,672	360,809,452	579,621,326
Assembler	Newbler	Trinity	Trinity	Trinity	Trinity	Trinity
Contigs	37,422	130,056	134,639	128,437	162,474	207,300
Trinity 'genes'	n/a	61,926	65,646	64,420	80,714	103,980
GC content	36%	41%	40%	40%	40%	39%
Min contig length	1	201	201	201`	201	201
Max contig length	6215	30,329	29,084	29,102	29,110	30,845
Mean contig length	472	1,006	1,079	1,081	1,059	1,011
Median contig length	392	585	573	582	537	478
N50	734	1,717	1,952	1943	1967	1,979
Contigs with at least one blast hit	11,281	59,463	53,041	51,945	57,160	66,755
Proportion of contigs with at least one blast hit	30%	46%	39%	40%	35%	32%

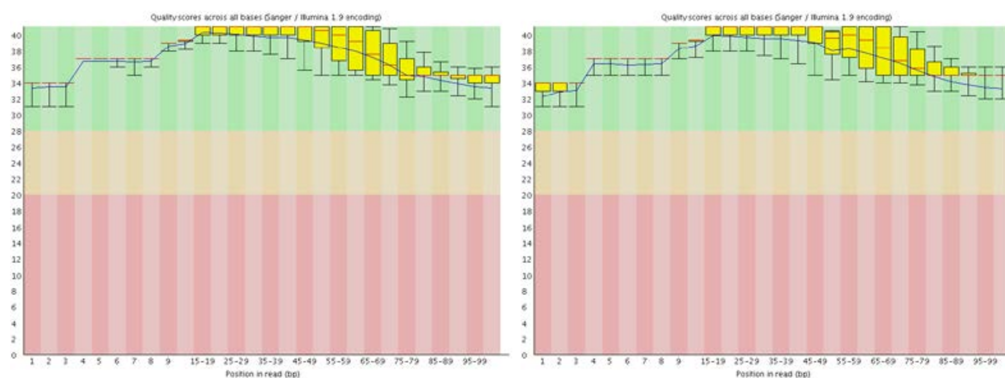
Sequences for known insecticide target sites were missing from the assembly including the voltage-gated sodium channel, GABA-gated chloride channel and the nicotinic acetylcholine receptor. This suggested that the list of contigs was incomplete. In order to obtain a more complete reference transcriptome, one lane of normalised paired-end 100bp Illumina sequencing was performed. This gave approximately 109 million paired-end reads, with a mean quality score of 37/40. Additionally, 97% of the DNA bases had a quality score above 30. The FastQC quality tool in galaxy was used to further analyse the raw reads and they were found to be high quality across the full 100bp length so did not require trimming (Figure 5.1). The raw reads were *de-novo* assembled using Trinity. One hundred and thirty thousand contigs were produced with a mean contig length of 1006 bp and an N50 of 1717bp (Table 5.1, assembly 2). Trinity estimated 61,926 genes for *T. absoluta*, which is much higher than the predicted 14,623 – 16,329 genes in *B. mori* and the 16,866 protein-coding genes estimated for *Danaus plexippus* (Table 5.2). The median length of the *T. absoluta* contigs was 585 base pairs, whereas the predicted median CDS size in *B. mori* was 768-867 base pairs (ISGC, 2008). The GC content for *T. absoluta* was 41%, similar to that of *B. mori* (38%), but higher than that of *D. plexippus* (32%) (Zhan et al., 2011).

Table 5.2. Number of predicted genes in some insect genomes.

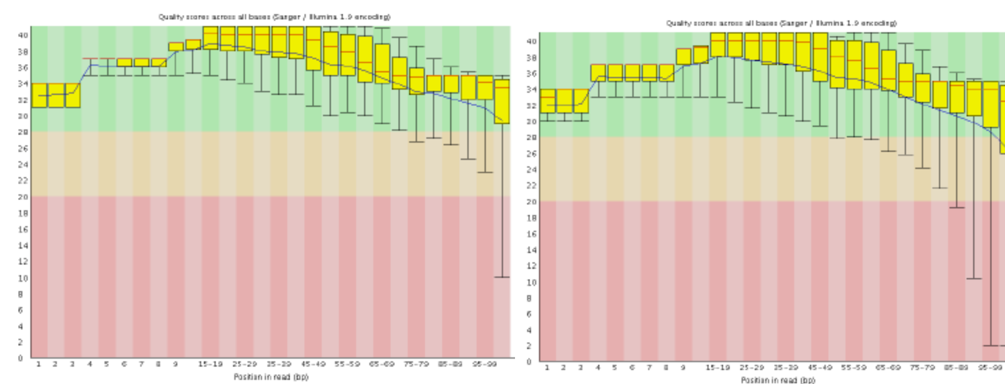
Species	Order	Date published	Predicted genes	Reference
D. melanogaster	Diptera	2000	13,601	(Adams et al.,
A. gambiae	Diptera	2002	13,683	(Holt et al., 2002)
T. castaneum	Coleoptera	2008	16,404	(TGSC, 2008)
B. mori	Lepidoptera	2008	14,623	(ISGC, 2008)
D. plexippus	Lepidoptera	2011	16,866	(Zhan et al., 2011)
H. melpomene	Lepidoptera	2012	12,669	(THGC, 2012)
P. xylostella	Lepidoptera	2013	18,071	(You et al., 2013)

An additional lane of Illumina sequencing was performed with 3 biological replicates of *T. absoluta* Spinosad-susceptible (Spin) and resistant (SpinSel) RNA. FastQC analysis found that the sequences were high quality with the majority of reads having a quality score over 30 (slightly lower than for TA1) (Figure 5.1). Four assemblies were produced using Trinity, Spin alone (assembly 3), SpinSel alone (assembly 4), Spin plus SpinSel (assembly 5) and Spin, SpinSel plus TA1 (assembly 6). Assembly 5 gave 162,474 contigs with a mean length of 1,059bp. The combined assembly 6 had the highest number of contigs, 207,300 and a similar mean length (1,011 bp; Table 5.1).

TA1



Spin



SpinSel

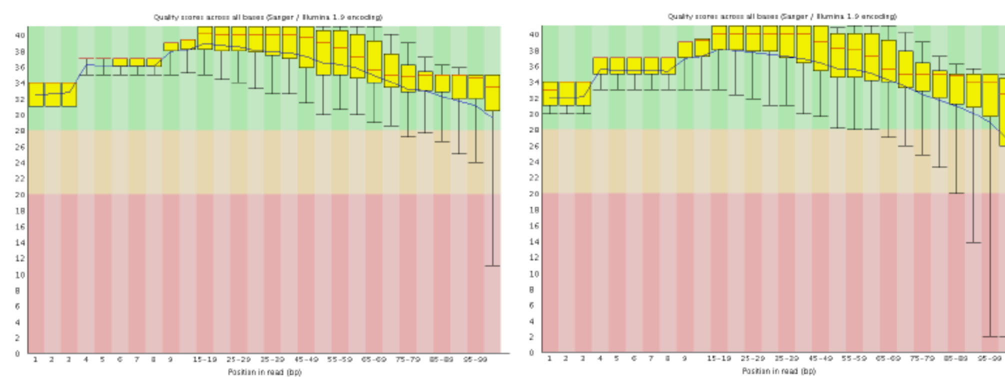


Figure 5.1. Sequence quality of Illumina paired end reads (FastQC analysis). Y axis is quality score and x axis is position in read (base 0-base 100). Green section is quality score over 30 (considered high quality). Left hand panels shows left paired reads, right hand panel shows right paired reads.

5.3.2 Blast analysis of transcriptomes

A blast search for the *T. absoluta* TA1 sequences against the non-redundant protein database, found at least one hit for 46% of contigs in the Illumina assembly. This is comparable to other *de-novo* transcriptomes for example 43% of *Cimex lectularius* and 37% of *Trialuerodes vaporarium* contigs had at least one hit against non-redundant proteins (Karatolos et al., 2011, Mamidala et al., 2012) . Contigs without a blast hit are expected to be either novel genes or genes with unassigned biological functions (Mamidala et al., 2012). The combined assembly of TA1, Spin plus SpinSel only had hits for 32% of contigs. However, this assembly had a much greater total number of contigs, so the number of contigs with a blast hit was still high (66,755).

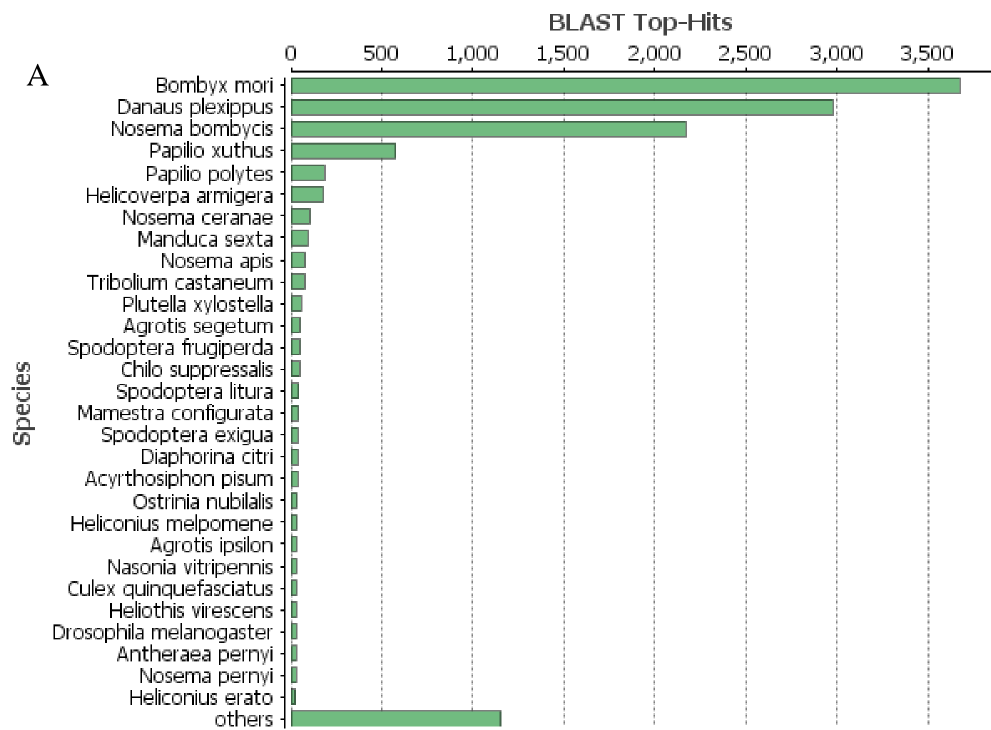
The most common top hits for all assemblies of *T. absoluta* were against *B. mori*, and *D. plexippus*, (Figure 5.2). The first lepidopteran genome to be published was for *B. mori* where two draft genomes were obtained independently by whole genome shotgun sequencing in 2004 (Mita et al., 2004, Xia et al., 2004) and these were merged and assembled to create a final 342 Mb genome in 2008 (ISGC, 2008). The genome of *D. plexippus* was published in 2011 (Zhan et al., 2011)and this was followed in 2012 by the genome of a second butterfly, *Heliconius melpomene* (THGC, 2012) and in 2013 by the diamond back moth, *P. xylostella* (You et al., 2013). Hits against *H. melpomene and P. xylostella* were less common in the *T. absoluta* transcriptomes.

Interestingly, the third most common top-hit species for *T. absoluta* was *Nosema bombycis* in the TA1, Spin and SpinSel transcriptomes (Figure 5.2) *N, bombycis* is a species within microsporidia which can infect a broad range of lepidopteran hosts (Pan et al., 2013), so this suggests that the *T. absoluta* were parasitised either by *Nosema spp*., or a related organism. *N. bombycis* infection causes the disease pébrine in *B. mori*, the symptoms being lethargic and slow developing larvae which become covered in black spots and eventually die (Pan et al., 2013). None of these symptoms were observed in the populations of *T. absoluta* reared in the Rothamsted insectary but there are over 1000 species within microsporidia (Pan et al., 2013) and it is possible that the *T. absoluta*

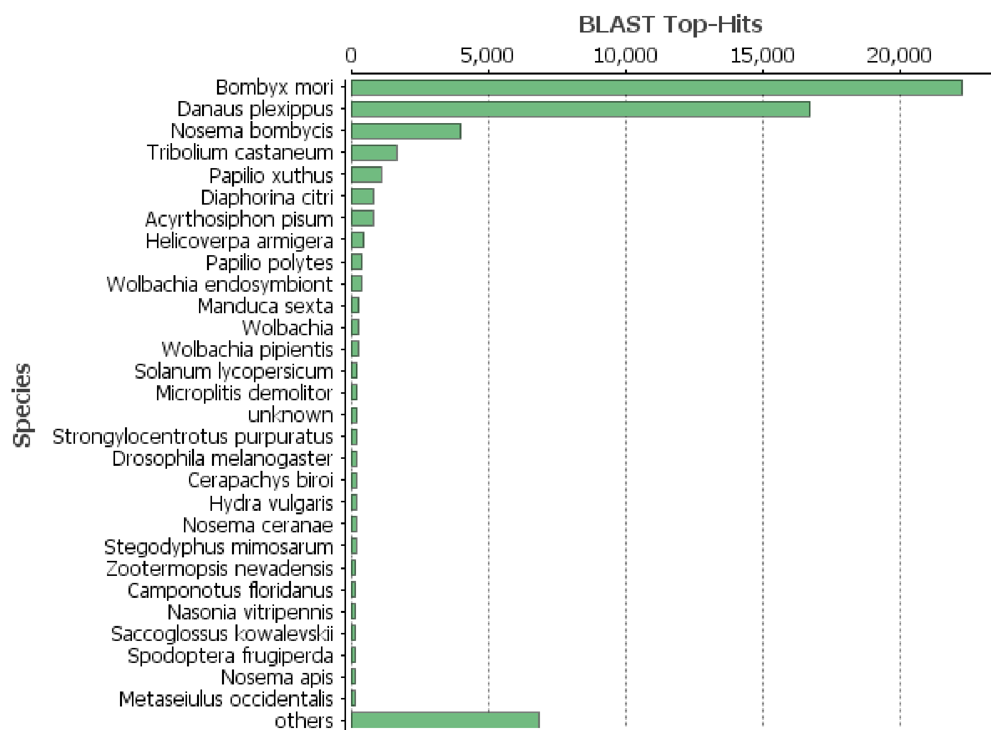
were infected with a species which is rare or not economically significant, and therefore is not represented in the non-redundant protein database. Knowledge of pathogens with the ability to infect *T. absoluta* could be useful for biological control strategies.

The bacterial parasite *Wolbachia* is also within the top 10 top-hit annotations of the *T. absoluta* TA1 transcriptome Assembly 2, with 1.5% of annotations. This transcriptome contained eggs, larvae, pupae and adults of TA1. In contrast, *Wolbachia* hits were negligible in the TA1, Spin and SpinSel larval transcriptomes (less than 0.001% of hits). *Wolbachia* is normally transmitted vertically from mother to daughter, and can change its host's biology to favour this vertical transmission through cytoplasmic incompatibility (preventing uninfected females from producing offspring with infected males), male-killing, parthenogenesis and feminisation of males, (Goodacre and Martin, 2012). However, Wolbachia infection can also have positive effects on host insects, for example by making the insect more resistant to viruses (Hedges et al., 2008). It is estimated that 40% of terrestrial arthropods are infected with Wolbachia (Zug and Hammerstein, 2012).

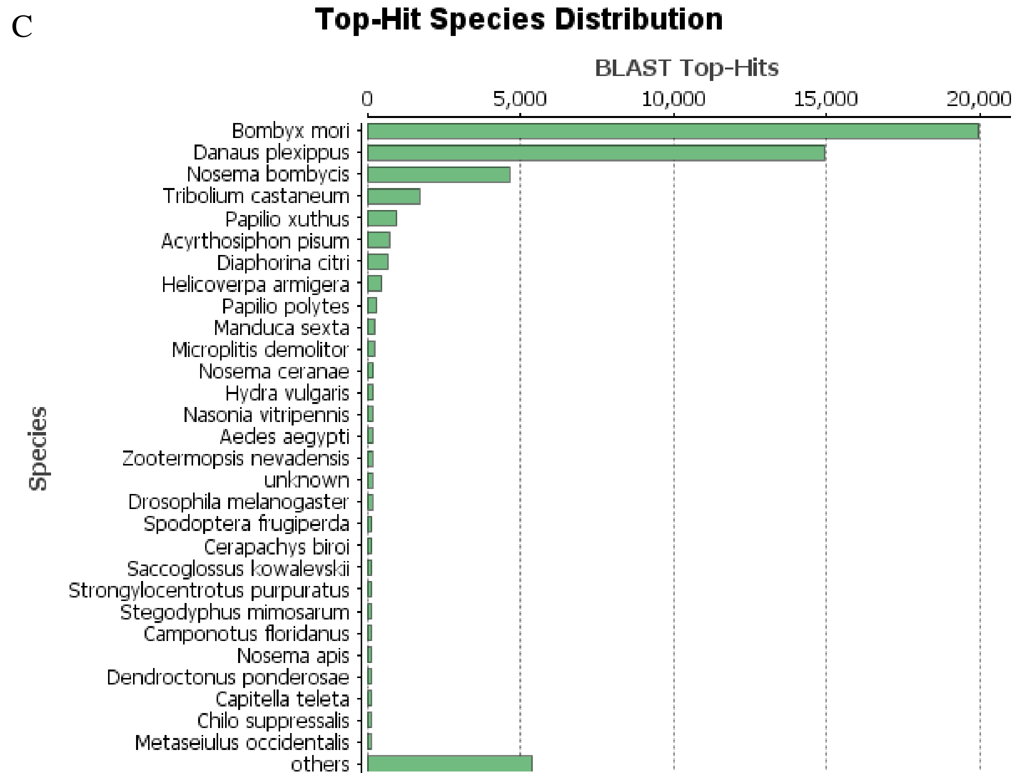
A



B



C



D

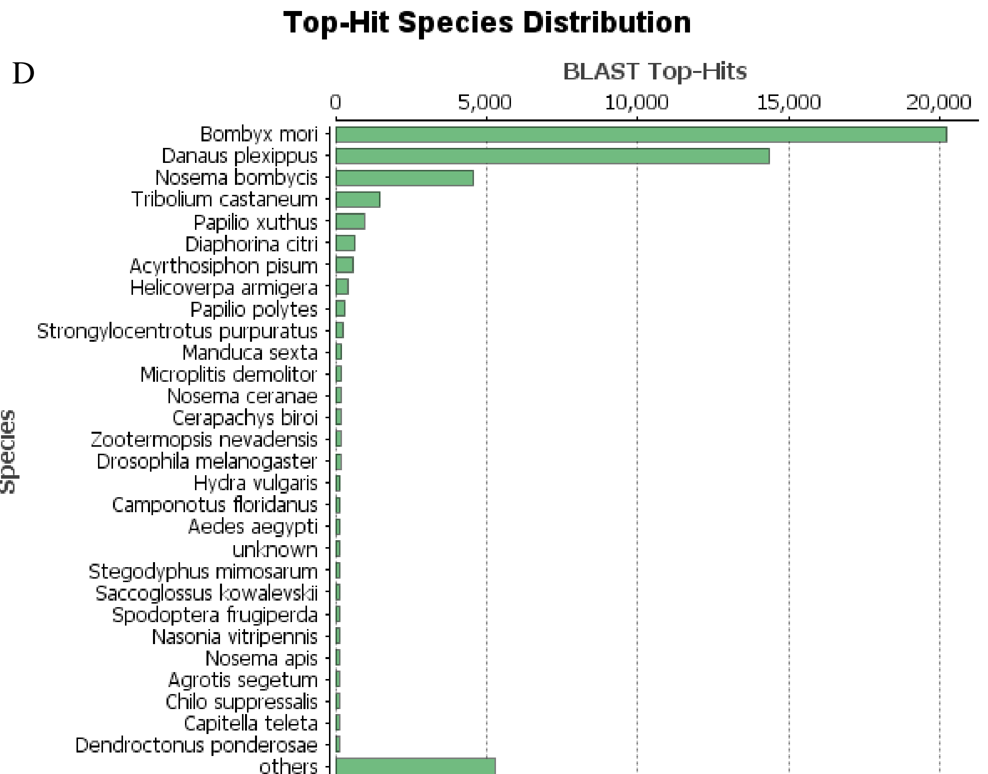


Figure 5.2. Species distribution of top-hit annotations for *T. absoluta* transcriptome. A = TA1 larvae (assembly 1) TA1 eggs, larvae, pupae & adults (assembly 2); B = Spin larvae (assembly 3); C = SpinSel larvae (assembly 4)

5.3.3 Transcripts encoding cytochrome P450s and insecticide target sites

An analysis of the 454 data (assembly 1), found more than sixty transcripts annotated as encoding cytochrome P450s, whilst the Illumina assembly (assembly 2) had over 200 transcripts with hits against P450s (details and annotation of all transcripts encoding P450s in these two assemblies, including the closest BLAST hit is provided in Appendix 3). However, a significant number of these transcripts do not encode full length P450s and it is therefore likely that the total number of unique genes they represent is <200. Additional next-generation sequencing or extension by random amplification of cDNA ends (RACE) would be required to resolve this. As a guide, the number of P450 genes in insect genomes sequenced to date ranges between 37 in the body louse, *Pediculus humanus*, to 180 in the mosquito *Culex pipiens* (Feyereisen, 2011, Lee et al., 2010). In *B. mori*, 84 P450-like sequences were identified, of which 78 were functional and six were pseudogenes (Ai et al., 2011). We were interested in identifying transcripts encoding *T. absoluta* P450s because of their importance in conferring resistance to a wide variety of insecticides in crop pests and disease vectors. For example, constitutive overexpression of a single P450 gene, *CYP6CM1*, is correlated with imidacloprid-resistance in *Bemisia tabaci* and mutations within this gene could be used to distinguish resistant and susceptible individuals (Karunker et al., 2008). Overexpression of *CYP4G61* is associated with resistance to the juvenile hormone analogue, pyriproxyfen, in *Trialeurodes vaporariorum* (Karatolos et al., 2012b) and in *Anopheles gambiae*, expression of *CYP6CM2*, correlates with resistance to DDT, pyrethroids and carbamates whilst expression of *CYP6P3* confers pyrethroid and carbamate resistance. CYP6P3 was shown to metabolise the carbamate bendiocarb, whilst CYP6M2 did not, suggesting that CYP6M2 may cause bendiocarb resistance through an indirect mechanism such as the breakdown of secondary metabolites (Edi et al., 2014).

T. absoluta sequences with hits against insecticide target sites were also identified in the transcriptomes. The 454 data only had transcripts for two known target sites, the ryanodine receptor and the ecdysone receptor, whereas

the Illumina assemblies contained sequences encoding seven target sites (Table 5.3).

Table 5.3. Annotation of insecticide-target sites in *T. absoluta* transcriptomes

Gene	Complete CDS?	CDS (AAs)	Pairwise identity with *B. mori*	*B. mori* accession
Acetylcholinesterase-1	Missing start	674	83%	NP_001037380.1
Acetylcholinesterase-2	Yes	638	92%	AAZ91685.1
Ecdysone receptor	Yes	547	80%	BAA07890.1
GABA-gated chloride channel	Yes	494	90%	XP_012548871.1
Glutamate-gated chloride channel	Yes	424	90%	BAO58781.1
nAChR α1	Yes	534	96%	NP_001103388.1
nAChR α2	Yes	545	96%	NP_001103397.1
nAChR α3	Missing start and end	338	99%	ABV45513.1
nAChR α4	Missing end	403	94%	NP_001103389.1
nAChR α5	Missing start and end	119	98%	ABV45516.1
nAChR α6	Missing start	485	93%	ABL67934.1
nAChR α7	Yes	507	95%	ABV45520.2
nAChR α8	Yes	531	91%	NP_001166817.1
nAChR α9	Yes	420	64%	NP_001103399.1
nAChR β1	Yes	518	97%	ABV45508.1
nAChR "β3"	Yes	378	21%	ABV45510.1
Ryanodine receptor	Yes	5121	91%	XP_012544748.1
Voltage-gated sodium channel	Missing end	2038	92%	NP_001136084.1

Multiple transcripts encoding the voltage-gated sodium channel of *T. absoluta* were obtained including those with and without the T929I mutation conferring resistance to pyrethroids as reported and discussed in chapter 3 (Figure 5.3). in addition the Illumina data gave contigs with homology to nAChR subunits, complete coding sequences for α1, α2, α7, α8, α9, β1 and β3 and partial sequences for α3, α4, α5 α6 (Appendix 4). These had 21-99% identity with *B. mori* protein sequences (Table 5.3). In total 11 subunits were identified which is comparable to the 10-12 subunits typically found in insects (Table 5.4), where the nAChR encodes the target site for three classes of insecticides, spinosyns, neonicotinoids and nereistoxin analogues. Mutations in the nAChR have been shown to confer resistance to multiple classes of insecticides. For example mutations in the genes encoding the α1 and α3 subunits of *N. lugens* and the β1 subunit of *M. persicae* have been associated with neonicotinoid resistance (Liu et al., 2005, Bass et al., 2011). A point mutation, conferring the G275E substitution, in the α6 subunit was associated with spinosad resistance in thrips (Puinean et al., 2012, Bao et al., 2014). Because the nAChR α6 subunit appears to represent the spinosad target-site its identification is highly relevant to this PhD project and full characterisation of this receptor subunit is described in chapter six.

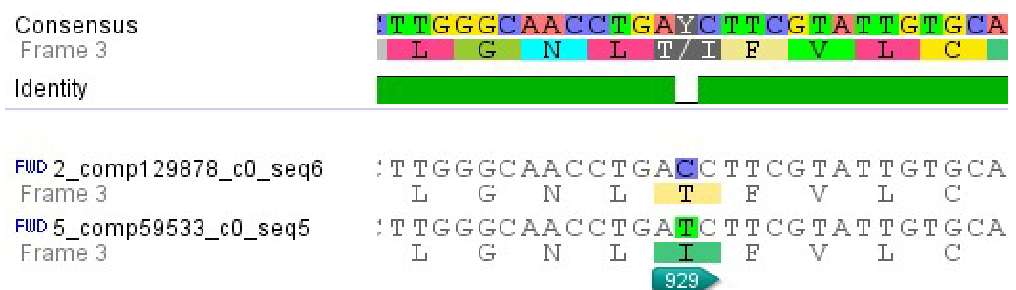


Figure 5.3. Transcripts encoding the voltage-gated sodium channel with and without the T929I pyrethroid-resistance mutation.

Table 5.4. Comparison of nAChR subunits across different species

Species	α subunits	β subunits	δ subunits	γ subunits	ε subunits	Total	Reference
Mammal	9	4	1	1	1	16	(Millar, 2003)
Chicken	10	4	1	1	1	17	(Millar, 2003)
D. melanogaster	7	3	0	0	0	10	(Sattelle et al., 2005)
A. gambiae	9	1	0	0	0	10	(Jones et al., 2005)
A. mellifera	9	2	0	0	0	11	(Jones et al., 2006)
B. mori	8	4	0	0	0	12	(Shao et al., 2007)
T. castaneum	11	1	0	0	0	12	(Rinkevich and Scott, 2009)
T. absoluta	9	2	0	0	0	11	This study

Other targets found in *T. absoluta* were acetylcholinesterase, the ecdysone receptor, the glutamate-gated chloride channel and the ryanodine receptor. *T. absoluta* acetylcholinesterase-1 and acetylcholinesterase-2 had 83% and 92% similarity to the corresponding *B. mori,* genes (Table 5.3). Acetylcholinesterase is the target site of organophosphates and carbamates so mutations in these genes might be expected to be involved in resistance in *T. absoluta* to these compounds. Two organophosphates, chlorpyrifos and methamidophos are registered for use against *T. absoluta* (IRAC, 2011). In *D. melanogaster* a single acetylcholinesterase gene is present, however most insects have two and the *ace-1* gene, which is paralogous to that of *D. melanogaster* is the location of insecticide-resistance mutations (Kono and Tomita, 2006). In *P. xylostella,* 3-D modelling has predicted that two mutations in *Ace-1*, A298S and G324A,

will cause prothiofos resistance (Lee et al., 2007) and RNAi of *ace-1* and *ace-2* showed that si-pxace1 significantly increased sensitivity to chlorpyrifos whilst si-pxace2 was not significantly different (He et al., 2012). So far mutations in Ace genes of *T. absoluta* have not been reported. The complete coding sequence of the *T. absoluta* ecdysone receptor, the target of diacylhydrazines, was also obtained but although diacylhydrazines are also used to manage *T. absoluta* (IRAC, 2011), there have been no reports of resistance . The sequence of the *T. absoluta* glutamate-gated chloride channel was annotated with 90% similarity to *B. mori* (Figure 5.4). Avermectins, including abamectin and emmamectin benzoate, target this channel, and provide good control of *T. absoluta* at the field rate (Silva et al., 2011). The full sequence coding the ryanodine receptor, a very long gene of 5121 amino acids, was also present. This provides a useful resource for characterising resistance to diamides, which was recently reported in *T. absoluta* from Greece (Roditakis et al., 2015).

```
                 1         10        20         29
1. T. absoluta   MELRLPSCASISLLLLCLLQLTQCMNA-KINFRE
2. B. mori       ..FPRRP..LLV.I.SYFT..AI...SG......

                      39        49        59
1. T. absoluta   KEKQILDQILGPGRYDARIRPSGINGT-DGPAVV
2. B. mori       ...........................G.A.TL.

                  68        78        88        98
1. T. absoluta   SVNIFVRSISKIDDVTMEYSVQLTFREQWLDERL
2. B. mori       R..MYL........YK..................

                        108       118       128
1. T. absoluta   KFNNLGGRLKYLTLTEANRVWMPDLFFSNEKEGH
2. B. mori       ..................................

                    138       148       158       168
1. T. absoluta   FHNIIMPNVYIRIFPNGNVLYSIRISLTLSCPMN
2. B. mori       .................T................

                          178       188       198
1. T. absoluta   LKLYPLDKQTCSLRMASYGWTTDDLVFLWKEGDP
2. B. mori       ..................................

                      208       218       228
1. T. absoluta   VQVVKNLHLPRFTLEKFLTDYCNSKTNTGEYSCL
2. B. mori       .................R................

                  238       248       258       268
1. T. absoluta   KVDLLFKREFSYYLIQIYIPCCMLVIVSWVSFWL
2. B. mori       ..................................

                        278       288       298
1. T. absoluta   DQGAVPARVSLGVTTLLTMATQSSGINASLPPVS
2. B. mori       ..................................

                    308       318       328       338
1. T. absoluta   YTKAIDVWTGVCLTFVFGALLEFALVNYASRSDM
2. B. mori       ..................................

                          348       358
1. T. absoluta   HRENMKKTRREMEAAAQMDAASDLLDTDSNATFA
2. B. mori       ...............S-.................

                 373         374 377       387
1. T. absoluta   M-----------MRQCEIHISPPRKNCCRLWMSK
2. B. mori       .KPLVRGGVDTK.........Q............

                  397       407             423
1. T. absoluta   FPTRSKRIDVISRITFPLVFALFNLAYW
2. B. mori       .....................I......
```

Figure 5.4. The glutamate-gated chloride channel coding sequence in *T. absoluta* and *B. mori* (accession BAO58781.1).

5.4 Conclusions

In this study, several *de-novo* transcriptome assemblies of *T. absoluta* were completed. These were annotated using the non-redundant protein database, and the most common top-hit species were *B. mori* and *D. plexippus*. Interestingly, there were also a high number of hits against the parasitic organisms, *N. bombycis* and *Wolbachia*. The assemblies of 454 and Illumina data identified over 200 transcripts matching cytochrome P450s which can be used in future studies to assess the ability of *T. absoluta* to develop metabolic resistance to insecticides. Further analysis of is required to assess how many of the transcripts encode unique genes, and how many are alternative isoforms of the same gene. The transcriptome was also mined for insecticide target sites. Not all of the insecticide target sites could be found in the 454 assembly, so additional Illumina sequencing was done on multiple populations and life stages of *T. absoluta*. Sequences with homology to seven insecticide target sites were annotated including acetylcholinesterase-1, the voltage-gated sodium channel, ryanodine receptor, the glutamate-gated chloride channel and multiple subunits of the nAChR. This will provide a useful resource for scientists studying insecticide resistance mechanisms. In the next chapter, the target site of spinosad, the α6 subunit of the nAChR, will be analysed in more detail. Additionally, the transcriptomes produced in this chapter will be used for analysing differences in expression between *T. absoluta* strains before and after selection with spinosad.

6. Analysis of the nAChR α6 subunit of *T. absoluta*

6.1 Introduction

In chapter 4, selection of *T. absoluta* in the laboratory gave rise to a population, SpinSel, with a high level of resistance to spinosad. However, the molecular basis of the resistance was unknown. The target site of spinosad is the nAChR, a ligand-gated ion channel vital for the functioning of the central nervous system. The receptor is a pentamer formed from a combination of α, β, δ, γ and ε subunits as heteromers or homomers (Millar, 2003). In vertebrates, different subunit combinations are found in muscle, neuronal tissues and sensory epithelia. Each subunit contains hydrophilic extracellular domains with a binding site for acetylcholine-like neurotransmitters, and four hydrophobic trans-membrane domains (TM1-4). The TM2 domain of each subunit forms the lumen of the ion channel (Tomizawa and Casida, 2001). Nicotine, a natural compound produced by plants in the genus *Nicotiana*, binds to nAChRs and has been used in crop protection for many years. Modern insecticides, including spinosad and neonicotinoids also bind to nAChRs but bind to different types of nAChRs. Spinosad targets the α6 subunit of nAChRs, whilst nAChRs which contain α5/α6 subunits have been shown to be insensitive to the neonicotinoid imidacloprid (Lansdell and Millar, 2004, Watson et al., 2010).

The first demonstration of an altered nAChR conferring resistance to spinosad was a Dα6 knockout population of *D. melanogaster.* This population, Df(2L)1402/CyO, had a recessive mutation giving 1180-fold resistance in homozygotes (Perry et al., 2007). More recent studies have elucidated target site resistance to spinosad in agricultural pest species including *F. occidentalis* (western flower thrips), *B. dorsalis* (oriental fruit fly) and the diamond back moth, *P. xylostella* (Puinean et al., 2012, Baxter et al., 2010, Rinkevich et al., 2010, Hsu et al., 2012). To look for potential target site alterations associated with resistance in *T. absoluta*, the nAChR α6 subunits from spinosad resistant (SpinSel) and susceptible populations were amplified and sequenced. Additionally, the level of expression of this subunit was assessed in Spin and SpinSel larvae and adults.

6.2 Specific Methods

6.2.1 Cloning and sequencing of the *T. absoluta* nAChR α6 subunit

Initially, degenerate primers (Table 6.1, lines 1-4) designed for the *Spodoptera* sp. (R. Carvalho, Personal communication) nAChR were used to amplify a fragment from Spin cDNA. A pooled RNA sample was extracted from three Spin 3rd instar larvae (generation F4) and reverse transcribed to make cDNA. The primers Spodα6F3 and Spodα6R1 were used for the primary PCR, with an annealing temperature of 52°C using Dreamtaq green polymerase (Thermo Scientific, USA). A secondary PCR was performed using 1 µl of PCR product diluted 1:10 using the primers Spodα6F3 and Spodα6R4. To obtain the 3' end, an alternative secondary PCR with Spodα6F5 and Spodα6R1 was also done. These two PCR products were sequenced by Eurofins Genomics and assembled in Geneious 7, enabling the design of *T. absoluta* specific primers (Table 6.1, lines 5-25). RACE (see 2.8) was used to obtain the 5' UTR with the reverse primer Tuta_nAchR_267R. Contigs from the *T. absoluta* transcriptome (chapter 5) provided 3' UTR sequence.

RNA was extracted from pools of 12 larvae of each of the five populations TA2, TA3, TA4, GA and Spin (see 2.1.1) using the Bioline RNA isolation kit. Reverse transcription was performed to create cDNA (see 2.3). PCR using kappa HiFi polymerase was carried out with the primers Tuta_nAChR_F1 and Tuta_nAChR_R1 (using the conditions outlined in x with an annealing temperature of x). PCR products were cloned, purified (see 2.6) and sequenced by Eurofins Genomics.

Three pooled samples of 10 larvae were sequenced from the populations Spin (F20) and SpinSel (F20; 11 selections). RNA was extracted and reverse transcribed to make cDNA (see 2.3). Amplification using the F1 and R1 primers described above failed so the α6 subunit was amplified in two pieces. The cDNA was amplified with Dreamtaq green (see 2.4.1) with an annealing temperature of 50°C using the primers Spodα6F3 and Tuta_nAChR_mid_R1,

followed by a secondary PCR with Spodα6F3 and Tuta_nAChR_QR1 to obtain the first half. Additionally, the cDNA was amplified using Tuta_nAChR_midF1 and Tuta_nAChR_R1, followed by Tuta_nAChR_midF2 and Tuta_nAChR_R2 to obtain the second half. PCR products were direct sequenced or cloned and sequenced as described (see 2.6).

Table 6.1. Primers used to amplify and sequence the *T. absoluta* nAChR α6 subunit

Name of Primer	Location	Sequence (5'- 3')
Spoda6F3	Exon 1	TGCCCGTRTCGGAGCAAG
Spoda6F5	Exon 7	TTCTWCTRTCGCTGACGGTGT
Spoda6R4	Exon 11	TGAACACGAACARGCAAAACCT
Spoda6R1	Exon 12	CAYTGCACGATGATRTGCGG
Tuta_nAChR_F1	Exon 2	GCTGCTCAACTCGTACAAC
Tuta_nAChR_77F	Exon 2	GAGCGAGCCWTTAGAGGTCAAGTTCGG
Tuta_nAChR_ex3a_GSP1	Exon 3a	CAAATTAAGCCAGACGTTTGTAGTCAGGAT
Tuta_nAChR_ex3aF	Exon 3a	CAAATCCTGACTACAAACGTCTGG
Tuta_nAchR_267R	Exon 4	ACACCTCCATACTCGCTGTCGTTCC
Tuta_nAChR_296R	Exon 4	GCTTGTTTGGTGTGATGCGAACGTCC
Tuta_nAChR_mid_F1	Exon 6	GGAGGCGATTTATCAGACT
Tuta_nAChR_mid_F2	Exon 6	TGGCGAATGGTATTTGATAGG
Tuta_nAChR_mid_R1	Exon 7	GAGTCTGGTGGCAGTGTA
Tuta_nAChR_QR1	Exon 7	AACACATGGCACGATCAGGT
Tuta_nAChR_R2	Exon 11	ACCTGTCAACAACCATCGC
Tuta_nAChR_R1	Exon 12	AATAGTGTGAACACGAACAGG
Tuta_nAChR_In2_377R	Intron 2	CGTTCTGCGGCTATGAGCTTTCAACCTGA
Tuta_nAChR_InSeqF1	Intron 2	TGTTTGCCAGATGTGGCGT
Tuta_nAChR_InseqF2	Intron 2	CAGACTAGAGATTAAACTTACCT
Tuta_nAChR_InseqR1	Intron 2	TACTCCACAAGGGATATATGTAT
Tuta_nAChR_InseqR2	Intron 2	AGAGTAATGCCTGTAGCTTT
Tuta_nAChR_InF3	Intron 3a	CTCGTAATGTGTCATCCAG
Tuta_nAChR_InR2	Intron 3b	AGAGTAATGCCTGTAGCTTT
Tuta_nAChR_InseqF3	Intron 3b	AGTCCAATATACACGAACTG
Tuta_nAChR_InseqR3	Intron 3b	GTGATATAGGGATTCTAGGT

6.2.2 Sequencing of the genomic *T. absoluta* nAChR α6 subunit

The sequence of the region of the *T. absoluta* gene encoding the nAChR α6 subunit, from exon 2 to exon 4 was obtained by amplifying and sequencing using gDNA extracted with Qiagen or Promega Wizard kits. The introns were too large to amplify with standard PCR, so long PCR was used. Intron 3 was

amplified using the primers Tuta_nAChR_exon3aF and Tuta_nAChR_296R. Intron 2 was too long to be amplified even with long PCR, so genome walking (see 2.9) was used to obtain the 3' end of the intron with genome specific primer Tuta_nAChR_exon3a_GSP1. The primer In2_377R was subsequently designed within the intron sequence, and long PCR using this primer with a forward primer from exon 2 (Tuta_nAChR_77F) was successful. Internal sequencing primers were then used to sequence the introns (Table 6.1).

6.2.3 Analysis of differentially expressed transcripts

Gene expression was compared between six samples, consisting of three biological replicates of two treatments: Spin (not selected with spinosad) and SpinSel (selected with spinosad). The protocol for transcript abundance estimation after *de-novo* assembly described previously in (Haas et al., 2013) was used. In order to quantify the relative expression of transcripts, reads from each of the six samples were re-aligned to assembly 5 and assembly 6 (see chapter 5). First, the software RNA-seq by expectation maximisation (RSEM) uses Bowtie (Langmead et al., 2009) to align the reads to the trinity transcripts; then RSEM estimates the expression level of each transcript (Li and Dewey, 2011) and produces a count matrix with a column for each sample and rows corresponding to each transcript. Two packages were used to detect differentially expressed transcripts: EdgeR (Robinson et al., 2010) and DESeq2 (Love et al., 2014). EdgeR uses a negative binomial distribution to model the count data and estimates dispersion by conditional maximum likelihood. Differential expression of the genes is carried out using a test similar to Fisher’s exact test but modified to take into account over dispersion (Robinson et al., 2010). DeSeq2 also uses a negative binomial distribution to model the read counts. The variability between replicates is estimated with the assumption that genes of comparable average expression will have similar dispersion, which allows a more accurate estimate of dispersion in experiments with low numbers of replicates. DeSeq2 aims to provide a better estimate of fold change than previous algorithms by shrinking the fold change for genes with low counts or high variability between replicates (Love et al., 2014). Once

differentially expressed gens had been identified, qPCR (section 2.10) was used to validate the results using the primers shown in Table 6.2.

Table 6.2. Primers used for qPCR

Primer name	Gene	Sequence (5'- 3')
RNAseq_a6_ex3F	nAChR α6	CCAGACGTTTGTAGTCAGGAT
RNAseq_a6_F1	nAChR α6	GCGAGCCATTAGAGGTCAAG
RNAseq_a6_F2	nAChR α6	CATGCAAGATGGACATCACC
RNAseq_a6_F3	nAChR α6	CTGATTTCCTCCATGGCACT
RNAseq_a6_R1	nAChR α6	ACCTCCATACTCGCTGTCGT
RNAseq_a6_R2	nAChR α6	TCGCCTCCATTTTCATCTTT
RNAseq_a6_R3	nAChR α6	CTCGGCTACCAGGTTCAAAA
47101seq2F	integrator complex subunit 12	GCAGACAGATTGCTGTCCAA
47101seq2R	integrator complex subunit 12	TGCCCATGTCATTGTCACTT
47101seq2F2	integrator complex subunit 12	AAGCGTCGAGCTCTAAAACG
47101seq2R2	integrator complex subunit 12	TAGCAAGTCGAAGGGCAACT
42486seq8F	rna-binding protein 1	GCTACCGTGAATGGGACCTA
42486seq8R	rna-binding protein 1	ATCTTCAGCGTCGCGTATGT
42486seq8F2	rna-binding protein 1	GAATGGGACCTATCCTGCAA
42486seq8R2	rna-binding protein 1	CGCGTATGTTTCCGTATTTG
57733seq7F	integrator complex subunit 4	TGTGAAATTGTTGGGGGATT
57733seq7R	integrator complex subunit 4	GTCGTAGAGTGTCGCGTTGA
57733seq7F2	integrator complex subunit 4	CGCTGGACTTCCTAGTGGAC
57733seq7R2	integrator complex subunit 4	CCTCCAAAGCACCCAAGATA
67725seq2F	u11 u12 small nuclear ribonucleoprotein	TCCAGGCAGTACACAACACC
67725seq2R	u11 u12 small nuclear ribonucleoprotein	CGTCAGCTCCAGGTATCTCC
67725seq2F2	u11 u12 small nuclear ribonucleoprotein	GGGAGGCGTCTGAAACTAGA
67725seq2R2	u11 u12 small nuclear ribonucleoprotein	ATCCCTGTCGTCTCGATGTC
62457seq2F	gem-associated protein 5	AAAGGGGAGGAGCACAAACT
62457seq2R	gem-associated protein 5	GCACGTAGGCCTCCTTGTAG
62457seq2F2	gem-associated protein 5	CCCGCAGTCTCCTTCAAATA
62457seq2R2	gem-associated protein 5	AGTTTGTGCTCCTCCCCTTT
72316seq4F	protein suppressor of white apricot	CTGCCTTACGAATCCAGCTC
72316seq4R	protein suppressor of white apricot	GGCAGTGTGTTGTTGGTCAC
72316seq4F2	protein suppressor of white apricot	ATTTGTTGCTCCCTGACACC
72316seq4R2	protein suppressor of white apricot	TGGAACTGAGGGTTGTCTCC
72316seq4F3	protein suppressor of white apricot	GATTGCGACGACACATCATC
72316seq4R3	protein suppressor of white apricot	ACGTAGTCGGCCATTTTGTC

6.2.4 Comparison of nAChR α6 subunits from different life stages of *T. absoluta*

Larvae (2nd, 3rd and 4th instar), pupae and adults of Spin and SpinSel were frozen in liquid nitrogen. RNA was extracted from pooled samples of 6-12 individuals and cDNA was synthesised (see 2.3). PCR was done with Dreamtaq green and the primers Spodα6F3 and Tuta_nAChR_mid_R1, followed by a secondary PCR with Spodα6F3 and Tuta_nAChR_QR1. PCR products were sent to Eurofins Genomics for sequencing.

6.3 Results and Discussion

6.3.1 Cloning of the nAChR α6 subunit from *T. absoluta*

Degenerate PCR gave fragments of 1376 bp of cDNA sequence for the nAChR α6 subunit from *T. absoluta*. To get the full length of the gene, RACE was used to obtain the missing sequence at the 5' end. The sequences from degenerate PCR, the transcripts from RNA-seq (chapter 5) and the RACE sequencing were then aligned to create a consensus sequence for the full length of the gene. Exons were identified and annotated using the corresponding *P. xylostella* sequence (Baxter, Chen et al. 2010). The consensus sequence with its amino acid translation is shown in Figure 6.1. The translated sequence had high similarity to α6 in *B. mori* and *P. xylostella* (Figure 6.2). Specific primers were subsequently used to amplify cDNA from pooled samples of six populations of *T. absoluta* and 28 clones were sequenced (3-7 per population). Sequences for two variants of exon3 (exons 3a and 3b) and three variants of exon 8 (exon 8a, 8b and 8c) were present (Figure 6.3). There were 40 amino acid positions with variation between clones (Figure 6.4).

A study comparing different insect genomes from four orders of insects found most had two versions of exon 3 and three versions of exon 8 (Jin, Tian et al. 2007). However, *B. mori* had just two version of exon 8 (Jin, Tian et al. 2007). Four versions of exon 8 were present in *T. castaneum*, although only three were found in the RNA (Rinkevich and Scott 2009). *B. mori* was found to be

able to have either exon 3a or 3b or both together, with the relative ratios of these transcripts depending on developmental stage (Jin, Tian et al. 2007)

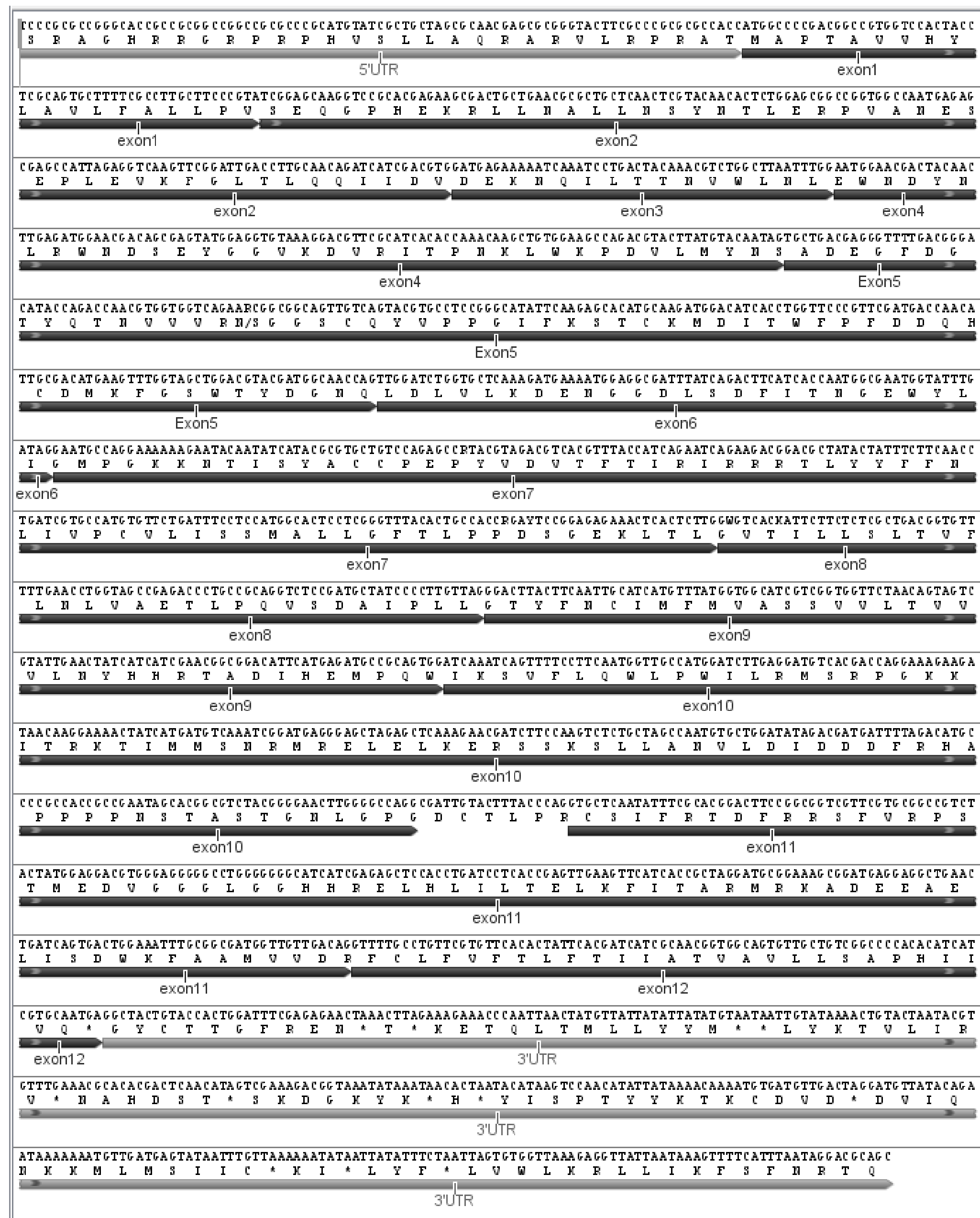


Figure 6.1. cDNA and predicted protein sequence of *T. absoluta* nAChR α6 subunit (with exon3a and 8a).

```
               1        10        20        30        40        50        60
T. absoluta    MAPTAVVHYLAVLFALLPVSEQGPHEKRLLNALLNSYNTLERPVANESEPLEVKFGLTLQQIIDV
B .mori        .V.---ILAAFA.L....................A..................R...........
P. xylostella  .---..LLAA.L.L....................A..................R...........

                   70        80        90       100       110       120       130
T. absoluta    DEKNQILTTNVWLNLEWNDYNLRWNDSEYGGVKDVRITPNKLWKPDVLMYNSADEGFDGTYQTNV
B .mori        .........................E........L..............................
P. xylostella  ............................F.....L..............................

                        140       150       160       170       180       190
T. absoluta    VVR?GGSCQYVPPGIFKSTCKMDITWFPFDDQHCDMKFGSWTYDGNQLDLVLKDENGGDLSDFIT
B .mori        ...S....L...............A.........................I....A.........
P. xylostella  ...SS...L........................................................

                   200       210       220       230       240       250       260
T. absoluta    NGEWYLIGMPGKKNTISYACCPEPYVDVTFTIRIRRRTLYYFFNLIVPCVLISSMALLGFTLPPD
B .mori        ................T...............M...............R................
P. xylostella  ................T...............M................................

                        270       280       290       300       310       320
T. absoluta    SGEKLTLGVTILLSLTVFLNLVAETLPQVSDAIPLLGTYFNCIMFMVASSVVLTVVVLNYHHRTA
B .mori        .................................................................
P. xylostella  .................................................................

                   330       340       350       360       370       380       390
T. absoluta    DIHEMPQWIKSVFLQWLPWILRMSRPGKKITRKTIMMSNRMRELELKERSSKSLLANVLDIDDDF
B .mori        ..........T......................................................
P. xylostella  .................................................................

                        400       410       420       430       440       446
T. absoluta    RHAPPPPNSTASTGNLGPGDCTLPRCSIFRTDFRRSFVRPSTMEDVGGGL----GGHHRELHLIL
B .mori        ...................------.........................----SS.........
P. xylostella  ..V..A.............------.....S...................GGLG...........

                   456       466       476       486       496       510
T. absoluta    TELKFITARMRKADEEAELISDWKFAAMVVDRFCLFVFTLFTIIATVAVLLSAPHIIVQ
B .mori        R..Q......K................................................
P. xylostella  R..Q......K................................................
```

Figure 6.2. Comparison of the *T. absoluta* α6 subunit with *B. mori* (GenBank ABL67934.1) and *P. xylostella* (GenBank GU207835.1). Dots are shown where the amino acid matches *T. absoluta*.

```
           1        10        20        30        40   45
Exon3A     GATGAGAAAAATCAAATCCTGACTACAAACGTCTGGCTTAATTTG
 Frame 1    D  E  K  N  Q  I  L  T  T  N  V  W  L  N  L
Exon3B     GATGAGAAGAATCAACTACTTATAACCAATATATGGCTGTCATTG
 Frame 1    D  E  K  N  Q  L  L  I  T  N  I  W  L  S  L
```

```
             1        10        20        30        40        50        60        70        80     87
1. Exon8A    GGAGTCACTATTCTTCTCTCGCTGACGGTGTTTTTGAACCTGGTAGCCGAGACCCTGCCGCAGGTCTCCGATGCTATCCCCTTGTTA
Frame 1       G  V  T  I  L  L  S  L  T  V  F  L  N  L  V  A  E  T  L  P  Q  V  S  D  A  I  P  L  L
2. Exon8B    GGTGTCACGATAATGTTGTCGATGACTGTGTTTCTCAACCTGGTTGCTGAAAAGATGCCCACTACTTCCGACGCAGTGCCTTTAGTA
Frame 1       G  V  T  I  M  L  S  M  T  V  F  L  N  L  V  A  E  K  M  P  T  T  S  D  A  V  P  L  V
3. Exon8C    GGTGTAACAATTCTACTGTCTCAAACCGTTTTCTCCCTATTGGTGGGGCATGTCATTACAAAAACCTCGGATGCAGTCCCCCTGATA
Frame 1       G  V  T  I  L  L  S  Q  T  V  F  S  L  L  V  G  H  V  I  T  K  T  S  D  A  V  P  L  I
```

Figure 6.3. Alternative exons 3a and 3b and exons 8a, 8b and 8c of the *T. absoluta* nAChR α6 subunit .

		3															4	5			
		66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	134	135	155	171
1	TA1	D	E	K	N	Q	L	L	I	T	N	I	W	L	S	L	E	N	G	T	W
2	TA1	D	E	K	N	Q	I	L	T	T	N	V	W	L	N	L	E	S	G	A	W
3	TA1	D	E	K	N	Q	L	L	I	T	N	I	W	L	S	L	E	N	S	A	W
4	TA3	D	E	K	N	Q	I	L	T	T	N	V	W	L	N	L	E	N	N	T	W
5	TA3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	E	N	S	T	W
6	TA3	D	E	K	N	Q	L	L	I	T	N	I	W	L	S	L	E	S	G	A	W
7	TA3	D	E	K	N	Q	L	L	I	T	N	I	W	L	S	L	E	S	G	T	W
8	TA3	D	E	K	N	Q	I	L	T	T	N	V	W	L	N	L	E	N	G	A	W
9	TA3	D	E	K	N	Q	I	L	T	T	N	V	W	L	N	L	E	N	N	T	W
10	TA4	D	E	K	N	Q	L	L	I	T	N	I	W	L	S	L	E	S	G	A	W
11	TA4	D	E	K	N	Q	L	L	I	T	N	I	W	L	S	L	E	N	N	T	W
12	TA4	D	E	K	N	Q	L	L	I	T	N	I	W	L	S	L	E	S	G	T	L
13	TA4	D	E	K	N	Q	I	L	T	T	N	V	W	L	N	L	E	S	G	A	W
14	TA4	D	E	K	N	Q	I	L	T	T	N	V	W	L	N	L	G	S	S	T	W
15	TA4	D	E	K	N	Q	I	L	T	T	N	V	W	L	N	L	E	S	G	T	W
16	GA	D	E	K	N	Q	L	L	I	T	N	I	W	L	S	L	E	S	G	T	W
17	GA	D	E	K	N	Q	L	L	I	T	N	I	W	L	S	L	E	S	G	A	W
18	GA	D	E	K	N	Q	L	L	I	T	N	I	W	L	S	L	E	N	G	A	W
19	GA	D	E	K	N	Q	I	L	T	T	N	V	W	L	N	L	E	N	S	A	W
20	GA	D	E	K	N	Q	L	L	I	T	N	I	W	L	S	L	E	N	G	T	W
21	GA	D	E	K	N	Q	L	L	I	T	N	I	W	L	S	L	E	N	S	T	W
22	Spin	D	E	K	N	Q	L	L	I	T	N	I	W	L	S	L	E	S	G	A	W
23	Spin	D	E	K	N	Q	I	L	T	T	N	V	W	L	N	L	E	S	G	A	W
24	Spin	D	E	K	N	Q	L	L	I	T	N	I	W	L	S	L	E	S	G	A	W
25	Spin	D	E	K	N	Q	L	L	I	T	N	I	W	L	S	L	E	S	G	T	W
26	Spin	D	E	K	N	Q	I	L	T	T	N	V	W	L	N	L	E	S	S	A	W
27	Spin	D	E	K	N	Q	I	L	T	T	N	V	W	L	N	L	E	S	G	T	W
28	Spin	D	E	K	N	Q	L	L	I	T	N	I	W	L	S	L	E	N	G	T	W
		8													9	10/11 boundary					
		272	275	279	280	283	284	285	286	287	288	289	293	296	319	410	411	412	413	414	415
1	TA1	L	L	L	N	A	E	T	L	P	Q	V	I	L	N	D	C	T	L	P	R
2	TA1	L	L	L	N	A	E	T	L	P	Q	V	I	L	S	D	C	T	L	P	R
3	TA1	L	L	L	N	A	E	T	L	P	Q	V	I	L	N	-	-	-	-	-	-
4	TA3	L	Q	S	L	G	H	V	I	T	K	T	V	I	N	D	C	T	L	P	R
5	TA3	L	L	L	N	A	E	T	L	P	Q	V	I	L	N	-	-	-	-	-	-
6	TA3	L	L	L	N	A	E	T	L	P	Q	V	I	L	N	D	C	T	L	P	R
7	TA3	L	L	L	N	A	E	T	L	P	Q	V	I	L	N	D	C	T	L	P	R
8	TA3	L	L	L	N	A	E	T	L	P	Q	V	I	L	N	D	C	T	L	P	R
9	TA3	L	L	L	N	A	E	T	L	P	Q	V	I	L	N	D	C	T	L	P	R
10	TA4	L	L	L	N	A	E	T	L	P	Q	V	I	L	N	-	-	-	-	-	-
11	TA4	L	L	L	N	A	E	T	L	P	Q	V	I	L	N	D	C	T	L	P	R
12	TA4	L	L	L	N	A	E	T	L	P	Q	V	I	L	N	-	-	-	-	-	-
13	TA4	L	L	L	N	A	E	T	L	P	Q	V	I	L	N	-	-	-	-	-	-
14	TA4	M	M	L	N	A	E	K	M	P	T	T	V	V	N	-	-	-	-	-	-
15	TA4	L	L	L	N	A	E	T	L	P	Q	V	I	L	N	D	C	T	L	P	R
16	GA	L	L	L	N	A	E	T	L	P	Q	V	I	L	N	D	C	T	L	P	R
17	GA	L	L	L	N	A	E	T	L	P	Q	V	I	L	N	-	-	-	-	-	-
18	GA	L	L	L	N	A	E	T	L	P	Q	V	I	L	N	-	-	-	-	-	-
19	GA	L	L	L	N	A	E	T	L	P	Q	V	I	L	N	D	C	T	L	P	R
20	GA	L	L	L	N	A	E	T	L	P	Q	V	I	L	N	-	-	-	-	-	-
21	GA	L	L	L	N	A	E	T	L	P	Q	V	I	L	N	D	C	T	L	P	R
22	Spin	L	L	L	N	A	E	T	L	P	Q	V	I	L	N	-	-	-	-	-	-
23	Spin	L	L	L	N	A	E	T	L	P	Q	V	I	L	N	D	C	T	L	P	R
24	Spin	M	M	L	N	A	E	K	M	P	T	T	V	V	N	-	-	-	-	-	-
25	Spin	L	L	L	N	A	E	T	L	P	Q	V	I	L	N	D	C	T	L	P	R
26	Spin	L	L	L	N	A	E	T	L	P	Q	V	I	L	N	D	C	T	L	P	R
27	Spin	L	L	L	N	A	E	T	L	P	Q	V	I	L	N	-	-	-	-	-	-
28	Spin	L	L	L	N	A	E	T	L	P	Q	V	I	L	S	-	-	-	-	-	-

Figure 6.4. Amino acid differences in nAChR α6 of 28 clones of *T. absoluta* from five populations (TA1, TA3, TA4, GA and Spin). Coloured boxes indicate different exons; amino acid position is listed above.

6.3.2 Comparison of Spin and SpinSel nAChR α6 subunit cDNA sequences

In order to assess if a target site alteration was responsible for the spinosad resistance in the SpinSel population of *T. absoluta*, the α6 subunit from Spin and SpinSel larvae was amplified and sequenced (F20-21). These two populations had significantly different susceptibility to spinosad (chapter 4). Sequencing of the cDNA revealed one difference between the two populations: the larvae from the SpinSel population had a 45 bp deletion, corresponding to exon 3 (Figure 6.5). Exon 3 encodes ligand-binding loop D, part of the acetylcholine binding site (Grauso et al., 2002) so a change here might be predicted to affect spinosad activity. However, proteins missing exon 3 would also be expected to be non-functional and expression of human nAChR α7 (which is equivalent to insect α6) in Xenopus oocytes showed the protein without exon 3 is indeed non-functional (M. Puinean, personal communication).

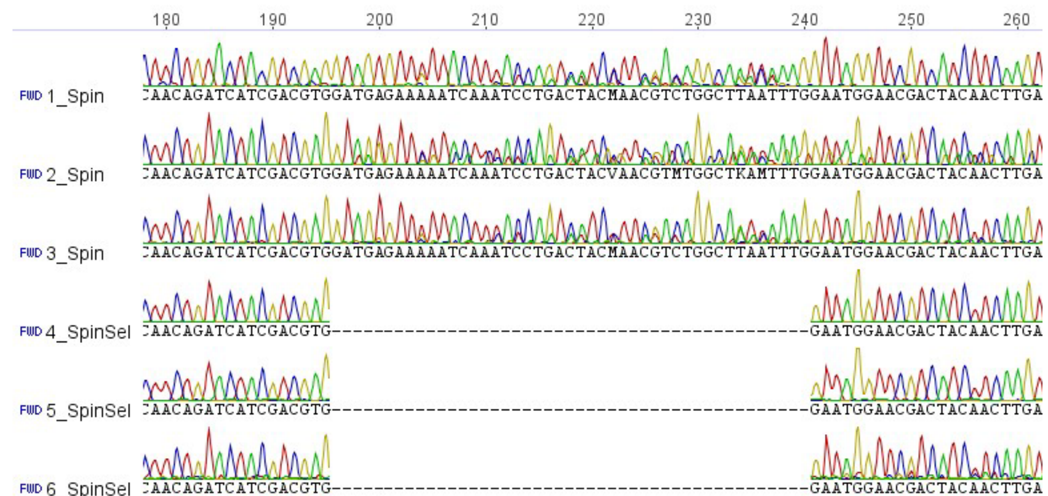


Figure 6.5. Spin and SpinSel cDNA sequence of nAChR α6, pooled samples of 10 larvae per replicate.

A *D. melanogaster* loss of function mutant with an inversion after exon 8b, resulting in a transcript encoding the ligand binding loops and TM1-2 but not TM3-TM4 was over 1181-fold resistant to spinosad (Perry et al., 2012). In

another example of spinosad resistance a strain of *P. xylostella* with 18,000-fold resistance had a variety of isoforms with premature stop codons (Rinkevich et al., 2010). One isoform was the insertion of 40 bases between exons 9 and 10, caused by a point mutation in the 5' splice site (GT to AT) resulting in part of the intron being included in the mRNA (Baxter et al., 2010). This insertion shifted the reading frame and caused a premature stop codon in exon 10, producing a truncated protein without TM4 (Baxter et al., 2010, Rinkevich et al., 2010). Another isoform had an additional seven base pairs inserted after exon 3, causing a premature stop codon in exon 4, encoding a protein with only binding loop D, missing binding loops A, B, C, E and TM1-4 (Rinkevich et al., 2010). In *B. dorsalis* premature stop codons in exon 7 of the α6 subunit were found in spinosad resistant strains which also lacked exons 3-6 or 4-6 (Hsu et al., 2012). A single point mutation in exon 9, resulting in a G275E substitution, was responsible for spinosad resistance in *T. palmi* and *F. occidentalis* (Puinean et al., 2012, Bao et al., 2014), and this mutation is located in TM3. All of these reports support the view that loss of exon 3 in *T.absoluta* could be responsible for the observed spinosad resistance in SpinSel.

To check if any individuals had exon 3 missing in the Spin population, cloning of the pooled PCR products was done. This revealed that 3/18 clones of Spin were missing exon 3 (17%), 6/18 had exon 3b (33%) and 9/18 (50%) had exon 3a. In contrast, all 17 clones of SpinSel were missing exon 3 (Table 6.3). This suggests that a small proportion of Spin-parent field population had exon 3 missing and this difference was selected by the repeated exposure to spinosad during the lab selection.

Table 6.3. Presence or absence of exons 3a and 3b in clones of the *T. absoluta* aα6 subunit from samples of Spin and SpinSel larvae.

Population	Exon 3a	Exon 3b	Neither	Total
Spin	9	6	3	18
SpinSel	0	0	17	17

Interestingly insects missing exon 3 in the α6 subunit have been reported previously, albeit at low levels. For example, in *Tribolium castaneum*, five of 39 (13%) clones of α6 were missing exon 3 (Rinkevich and Scott, 2009). Similarly, in the earlier sequencing of 28 clones from six spinosad susceptible populations of *T. absoluta*, in the present study, one clone of TA3 was missing exon 3 (Figure 6.4). However, this is the first report of an insect population where all transcripts of the α6 subunit are missing this exon, with subsequent exons unaltered, and the first time this genotype has been linked to spinosad resistance.

6.3.3 Sequencing of nAChR α6 subunit gDNA sequences from SpinSel

As the missing exon was observed in the RNA of the nAChR α6 subunit of *T. absoluta*, sequencing of gDNA was required to determine if this resulted from a deletion or exon-skipping. Since both exon3a and 3b were present in SpinSel gDNA the missing exon must result from exon skipping. PCR amplification of the genomic region encompassing exons 2-4 in the SpinSel strain, showed exon 3a is flanked by a large upstream intron of >10kb, the intron between exon 3a and 3b is comparatively smaller at 899 bp and the intron downstream of exon 3b is 4675bp (Figure 6.6). In the spinosad-susceptible populations either exon 3a or 3b is present in the RNA, an example of mutually exclusive exons. (Figure 6.6)

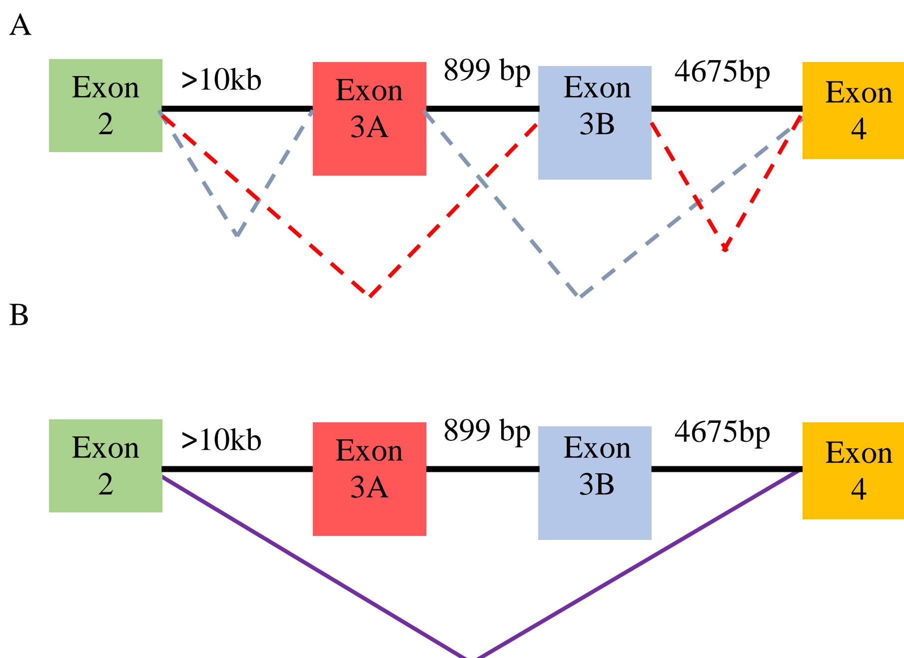


Figure 6.6. Patterns of splicing in the nAChR α6 subunit of *T. absoluta*. A) Spinosad susceptible (mutually exclusive exons). Either exon 3A is included in the mRNA with exon 3B spliced out (blue dashed lines) or Exon 3B is included in the mRNA and exon 3A is spliced out (red dashed lines). B) SpinSel (exon skipping). Both exon 3A and exon 3B are spliced out (purple solid line).

Splicing of precursor mRNA is an important regulatory step in gene expression, during which introns are removed and exons are joined together. Splicing depends on the recognition of introns and exons by the spliceosome, a complex composed of proteins and small nuclear RNAs. Alternative splicing, the inclusion of different exons in mRNA, generates different isoforms from a single gene (Keren et al., 2010) and is regulated by *cis*-acting RNA sequence motifs (in introns and exons) which provide binding sites for *trans*-acting proteins. Disruption in normal patterns of alternative splicing can therefore be caused by mutations in intron or exon sequences (Keren et al., 2010, Baxter et al., 2010). Splice-site recognition is also mediated by *trans*-acting proteins including Serine/Arginine rich (SR) proteins and polypyrimidine tract binding

proteins. For example, in mice the regulation of two mutually exclusive calcium channel exons 8 and 8a was shown to be mediated by a polypyrimidine tract binding protein (Tang et al., 2011).

To look for any alterations in intron or exon sequence which could be responsible for the skipping of exon 3 in *T. absoluta*, over 4000 bp of genomic DNA was sequenced spanning the region containing exons 3a and 3b (Appendix 5). This showed that the coding sequence of both exon 3a and 3b was identical in the genomic sequence of Spin and SpinSel (Figure 6.7), ruling out the possibility of a mutation in an exonic splicing regulator binding site, such as an exon splicing enhancer or silencer which promote exon inclusion or exclusion (Keren et al., 2010, Lee et al., 2012).

In higher eukaryotes, four motifs in the pre-mRNA are required, but not sufficient, for the spliceosome to function: the 5' and 3' splice sites at the exon-intron junctions, a branch site sequence in the intron upstream of the 3' splice site and the polypyrimidine tract which is between the branch site and the 3' splice site (Keren et al., 2010). However, the 5' and 3' splice sites were the same in Spin and SpinSel. The last two nucleotides of exon 2, 3a and 3b were TG and the first two bases of introns 2, 3a and 3b were GT. The last two bases of introns 2, 3a and 3b were AG and the first two bases of exons 3a, 3b and 4 were GA (Table 6.4). These are the highly conserved canonical splice sites which involve the major rather than minor spliceosome in their splicing (Burset et al., 2000).

Table 6.4. Splice sites (SS) in regions from exon 2 to exon 4 of the nAChR α6 subunit of *T. absoluta*

Population	Intron 2		Intron 3A		Intron 3B	
	5' SS	3' SS	5' SS	3' SS	5' SS	3' SS
Spin	TG|GT	AG|GA	TG|GT	AG|GA	TG|GT	AG|GA
SpinSel	TG|GT	AG|GA	TG|GT	AG|GA	TG|GT	AG|GA

.

The Splicing Regulation Online Graphical Engine (SROOGLE) was used to identify splice/branch sites and polypyrimidine tracts flanking exons 3a and 3b using the algorithms developed by (Kol et al., 2005) and (Schwartz et al., 2008). There were no splice or branch sites that differed between Spin and SpinSel, but a single t/c SNP in the predicted polypyrimidine tract upstream of exon 3a was observed in SpinSel compared to Spin (Figure 6.7).

Genetic variation in other parts of the intron sequence could affect intronic splicing enhancers and silencers, which provide binding sites for splice factor proteins (Lee et al., 2012) so intronic sequence upstream and downstream of exons 3a and 3b was compared between Spin and SpinSel. Significant genetic variation, including multiple SNPs and large indels were observed (Figure 6.7; appendix 5). In the case of the unselected Spin population, there was a much greater degree of heterozygosity which required cloning and sequencing to resolve. Because many sequence polymorphisms were observed between Spin and SpinSel it would be difficult to ascertain which, if any, might be responsible for the observed skipping of exons 3a and 3b. Therefore, the region spanning exon 3a and 3b was also sequenced in the populations TA1, TA3, TA4 and GA which were susceptible to spinosad (see Table 4.2). In TA4, the primary haplotype observed was 100% identical in sequence to that of SpinSel over a region spanning more than 4000 bp (Appendix 5), including at the site of the SNP observed between Spin and SpinSel in the polypyrimidine tract (Figure 6.7). Analysis of nAChR transcripts of TA4 found no evidence of exon skipping with 50% of clones containing exon 3a and 50% exon 3b (Figure 6.4). This finding strongly suggests that the intron sequence differences observed between the Spin and SpinSel strain around exons 3a and 3b are unlikely to be responsible for the exon skipping and associated resistance in *T. absoluta*.

```
Spin consensus      GTTTTGTCATAACACGGATAGAGATAAAACATACTATATCGTGTACAGTCAATTCTGAAAT
SpinSel consensus   GTTTTGTGAAAACACAGATAGAGATAAAACATACTATTTCGTGTACAGTCAATTCTGAAAT
TA4 consensus       GTTTTGTGAAAACACAGATAGAGATAAAACATACTATTTCGTGTACAGTCAATTCTGAAAT

Spin consensus      GAATATAATTTTTTCTTTCTTTATTATATCAATAAGATTAAGTGATGGTAATATGGGTAAA
SpinSel consensus   GAATATAACTTTTTCTTTCTTTATCATATCAATAAGATTAAGTGATGGTAATATGGGTAAA
TA4 consensus       GAATATAACTTTTTCTTTCTTTATCATATCAATAAGATTAAGTGATGGTAATATGGGTAAA

Spin consensus      AGCCAGTACAACCCAACAACCTGATGTTTATTATGTTTTTCGTTCCTTTCTTGCGACGCCG
SpinSel consensus   AGCCAGTACAACCCAACAACCTGATGTTTATTATGTTTTCCGTTCCTTTCTTGCGACGCCG
TA4 consensus       AGCCAGTACAACCCAACAACCTGATGTTTATTATGTTTTCCGTTCCTTTCTTGCGACGCCG

Spin consensus      TGCGTTCTATTAACAAAATAAGGATGAGAAAAATCAAATCCTGACTACAAACGTCTGGCTT
SpinSel consensus   TGCGTTCTATTAACAAAATAAGGATGAGAAAAATCAAATCCTGACTACAAACGTCTGGCTT
TA4 consensus       TGCGTTCTATTAACAAAATAAGGATGAGAAAAATCAAATCCTGACTACAAACGTCTGGCTT

Spin consensus      AATTTGGTAAGTGGTAGGTGNCGGGCCCGGCGCCTCGCGTCCCGCCCCCGCTCCCGTCGTC
SpinSel consensus   AATTTGGTAAGTGGTAGGTG-CGGGCCCGGCGCCTCGCGTCCCGCCCCCGCTCCCGTCGTC
TA4 consensus       AATTTGGTAAGTGGTAGGTG-CGGGCCCGGCGCCTCGCGTCCCGCCCCCGCTCCCGTCGTC

Spin consensus      AACTTTCACTGGTAACATCAAGTTTTCCCTAACAAACACTTCGACAGTCAATTTTATCTAT
SpinSel consensus   AACTTTCACTGGTAACATCAAGTTTTCCCTAACAAACACTTCGACAGTCAATTTTATCTAT
TA4 consensus       AACTTTCACTGGTAACATCAAGTTTTCCCTAACAAACACTTCGACAGTCAATTTTATCTAT

Spin consensus      CACCACACAGTACTTTCGACTGCACCCTCTGAATGCGGTTTTGTATCGGCTAGGATGCGAT
SpinSel consensus   CACCACACAGTACTTTCGACTGCACCATCTGAATGCGGTTTTGTATCGGCTAGGATGCGAT
TA4 consensus       CACCACACAGTACTTTCGACTGCACCATCTGAATGCGGTTTTGTATCGGCTAGGATGCGAT

Spin consensus      GGGCGAGGCAAGAATTGGAAAATTGAGATTGACTGAGCGTAAATTGCATTTTGCTAGACAG
SpinSel consensus   GGGCGAGGCAAGAATTGGAAAATTGAGATTGACTGAGCGTAAATTGCATTTTGCTAGACAG
TA4 consensus       GGGCGAGGCAAGAATTGGAAAATTGAGATTGACTGAGCGTAAATTGCATTTTGCTAGACAG

Spin consensus      CACTGCGAATTTACTTATTTCACTGTGTATTGGGAATCATTGAATGTGATAGATGGAATAT
SpinSel consensus   CACTGCGAATTTACTTATTTCACTGTGTATTAGGAATCATTGAATGTGATAGATGGAATAT
TA4 consensus       CACTGCGAATTTACTTATTTCACTGTGTATTAGGAATCATTGAATGTGATAGATGGAATAT

Spin consensus      ATAATGTACCTACTAATATGATAGACTAGAATGAAAATTAGCACCTTTAGAATTGTAGTGA
SpinSel consensus   ATAACGTACCTACTAATATGATAGACTGGAATGAAAATTAGCACCTTTAGAATTTTAGT--
TA4 consensus       ATAACGTACCTACTAATATGATAGACTGGAATGAAAATTAGCACCTTTAGAATTTTAGT--

Spin consensus      TCTATATTAAAAGCTAGAAAACTTTGTAAAACCAAGTGA----TACTTCATCAATTTATTC
SpinSel consensus   ----------AAGCTAAGAAACGTTGTAAAAGCAAATGATAAGTACTTCATCAATTTATTC
TA4 consensus       ----------AAGCTAAGAAACGTTGTAAAAGCAAATGATAAGTACTTCATCAATTTATTC

Spin consensus      GTAAAAAA--------ACAGCCCCAAATCTTGCCTCAACTCGTCAGAAATCCAGGCTGGTA
SpinSel consensus   GTAGAAAAACGAAAGTACAGCCCCAAATCTTGCCTCAACTTGTTAGAAATCCTGGCTGGTA
TA4 consensus       GTAGAAAAACGAAAGTACAGCCCCAAATCTTGCCTCAACTTGTTAGAAATCCTGGCTGGTA

Spin consensus      ACAAAATAGCACAAATTTTAAAGAGTCTTAAACATTTCAGCAAAAGGGTGATGGTGGCGGG
SpinSel consensus   ACAAAATAGCACAAATTTTAAAGAGTCTTAAACATTTCAGCAAAAGGGTGATGGTGGCGGG
TA4 consensus       ACAAAATAGCACAAATTTTAAAGAGTCTTAAACATTTCAGCAAAAGGGTGATGGTGGCGGG

Spin consensus      AGGTAGAGCGCGCCAAGCGGCGGTTGTCGCATGGCCGTCGGTGTTATGTTGTCACTCGCTC
SpinSel consensus   AGGTAGAGCGCGCCAAGCGGCGGTTGTCGCATGGCCGTCGGTGTTATGTTGTCACTCGCTC
TA4 consensus       AGGTAGAGCGCGCCAAGCGGCGGTTGTCGCATGGCCGTCGGTGTTATGTTGTCACTCGCTC

Spin consensus      GGTATAGGTGCCGTGCGGGATACATTGATTGCGCCACTTCGTACAAGCTTTAAGACGCAAC
SpinSel consensus   GGTATAGGTGCCGCGCGGGATACATTGATTGCGCCACTTCGTACAAGCTTTAAGACGCAAC
TA4 consensus       GGTATAGGTGCCGCGCGGGATACATTGATTGCGCCACTTCGTACAAGCTTTAAGACGCAAC

Spin consensus      CACTACTGCTGGTTTAAAGTGTTGCAATTTTATTCAAAAGGCTTTTACTAACTGGCGCTTT
SpinSel consensus   CACTACTGCGGGTTTAAAGTATTGCAATTTTATTCAAAAGGCTTTTACTAACTGGCGCTTT
TA4 consensus       CACTACTGCGGGTTTAAAGTATTGCAATTTTATTCAAAAGGCTTTTACTAACTGGCGCTTT

Spin consensus      TATGAGCCCGCTAGGTTCCGTTAGCTCTGTGTGGCGGCTCGCGGGGACTGCTCGATCGCAT
SpinSel consensus   TATGAGCCCGCAAGGTTCCGTTAGCTCTGTGTGGCGGCTCGCGGGGACTGCTCGATCGCAT
TA4 consensus       TATGAGCCCGCAAGGTTCCGTTAGCTCTGTGTGGCGGCTCGCGGGGACTGCTCGATCGCAT

Spin consensus      GTTGGCAGCTCCTGTGTGCCGCTGGTCCTCGTAATGTGTCATCCAGGTGTCGATCACAGTG
SpinSel consensus   GTTGGCAGCTCCTGTGTGCCGCTGGTCCTCGTAATGTGTCATCCAGGTGTCGATCACAGTG
TA4 consensus       GTTGGCAGCTCCTGTGTGCCGCTGGTCCTCGTAATGTGTCATCCAGGTGTCGATCACAGTG

Spin consensus      TTTAGATCATTATACTGACTGCGCCCTTTCTTTCTGTTTTTGTTGACATCGTCTAACCCAA
SpinSel consensus   TTTAGATCATTATACTGACTGCGCCCTTTCTTTCTGTTTTTGTTGACATCGTCTAACCCAA
TA4 consensus       TTTAGATCATTATACTGACTGCGCCCTTTCTTTCTGTTTTTGTTGACATCGTCTAACCCAA

Spin consensus      TAGGATGAGAAGAATCAACTACTTATAACCAATATATGGCTGTCATTGGTAAGTGTGTATA
SpinSel consensus   TAGGATGAGAAGAATCAACTACTTATAACCAATATATGGCTGTCATTGGTAAGTGTGTATA
TA4 consensus       TAGGATGAGAAGAATCAACTACTTATAACCAATATATGGCTGTCATTGGTAAGTGTGTATA

Spin consensus      AAAGAACGACCTAAATTAATAATTAAAACTTCTAATGTTCCGTTCTTTACTTACTTTCTTT
SpinSel consensus   AAAGAACGACCTAAATTAATAATTAAAACTTCTAATGTTCCGTTCTTTACTTACTTTCTTT
TA4 consensus       AAAGAACGACCTAAATTAATAATTAAAACTTCTAATGTTCCGTTCTTTACTTACTTTCTTT
```

Figure 6.7. Alignment of Spin, SpinSel and TA4 genomic DNA sequences spanning the region containing exon3a (red box) and 3b (blue box) of the nAChR α6 subunit of *T. absoluta*. Nucleotides which differ between populations are highlighted. A longer version of this figure with an additional 1700 bp of intron 2 and an additional 1300 bp of intron 3 is available in appendix 5. Site of a predicted polypyrimidine tract is boxed in purple.

6.3.4 RNA-seq analysis of Spin and SpinSel

To explore the possibility of changes in the expression of trans-acting proteins modifying splicing in the SpinSel strain illumina RNA sequencing was used to look for differences in gene expression between Spin and SpinSel (chapter 5). The raw reads were mapped against the transcriptomes to create a count matrix of the abundance of each transcript for each replicate. Two programmes were used to find differentially expressed genes (FDR< 0.05): EdgeR and DEseq2. DEseq2 found a greater number of differentially expressed contigs than EdgeR. There were 440 genes classed as differentially expressed by both algorithms in Assembly 5, and 366 in Assembly 6 (Figure 6.8; Appendix 6). About 30% of the DE genes in assembly 6 were up-regulated and 60% down-regulated. No cytochromes P450s were found to be differentially expressed in either assembly, consistent with the view that these genes do not contribute to resistance as judged by the P450 inhibitor PBO studies (Chapter 4).

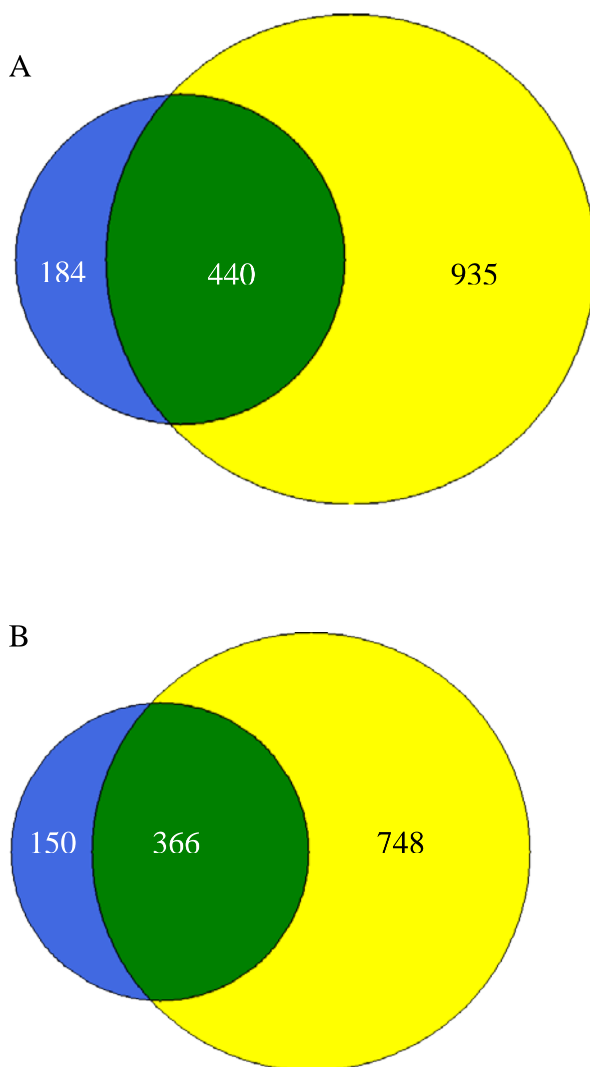


Figure 6.8. Comparison of number of differentially expressed contigs between Spin and SpinSel found using EdgeR and DEseq2 (False discovery rate < 0.05), Reads mapped to Assembly 5 (A), Assembly 6 (B). Blue = EdgeR only, yellow = DEseq2 only, green = both. Created using Venn Diagram Generator, Whitehead Institute for Biomedical Research: http://jura.wi.mit.edu/bioc/tools/venn.php (accessed 14/07/2015)

6.3.4.1 Expression of regulators of splicing

As differences in splicing between Spin and SpinSel larvae had been identified, the DE contig lists described in 6.3.4.1 from assembly 5 and 6 were searched for transcripts encoding known regulators of splicing. Six known *trans*-acting splice regulators were found to be differentially expressed, all with lower expression in SpinSel (Table 6.5). In our data, EdgeR had more conservative FDRs but DEseq2 had lower fold-change estimates for the same genes (Table 6.5). This is likely to be due to an additional fold-change shrinkage step which is incorporated into DEseq2 to stop transcripts with low expression from getting exaggerated fold change estimates (Love et al., 2014).

The DE contigs from *T. absoluta* included two subunits of the integrator complex and a small nuclear ribonucleoprotein. The integrator complex is involved in the formation of small nuclear RNAs and a reduction in integrator proteins has been shown to cause splicing defects (Reviewed in (Chen and Wagner, 2010). However, the greatest fold change was seen for the protein suppressor of white apricot (SWAP), a member of the serine-arginine-rich (SR) protein family which interact with RNA and other splice factors to regulate a wide variety of alternatively spliced mRNAs, through both activation and repression of splicing (Sarkissian et al., 1996). Another member of the SR protein family, RNA-binding Protein 1 (RBP1), was also down-regulated in *T. absoluta*. In *D. melanogaster*, RBP1 activates female-specific splicing of double-sex pre-mRNA (Heinrichs and Baker, 1995). However, the homologue of *D. melanogaster rbp1* in *B. mori* showed equal expression in males and females (Wang et al., 2010b) and so likely has alternative/additional roles.

SR proteins bound to exon sequences have been shown to stabilise pre-mRNA and improve the efficiency of splicing (Ibrahim et al., 2005). Using a pre-mRNA with one 5' splice site and two duplicated 3' splice sites, it was found that increasing the concentration of SR protein increased the use of the first 3' splice site and suppressed the use of the distal 3' splice site. This indicates that SR proteins bound to exonic enhancer elements prevent exon skipping

(Ibrahim et al., 2005), so down-regulation of these proteins may be allowing exon skipping to occur, in the SpinSel larvae.

QPCR analysis was done to check the validity of the interesting RNA-seq results (Figure 6.9).

Table 6.5. Splice factors chosen for qPCR validation. A = assembly number, FC = Fold change, FDR = False discovery rate (adjusted P value).

			DeSeq2		EdgeR	
A	Contig	Blast hit	FC	FDR	FC	FDR
5	comp72316_c0_seq4	protein suppressor of white apricot	-53	<0.001	-624	<0.001
6	comp157733_c1_seq7	integrator complex subunit 4	-29	<0.001	-346	0.009
5	comp62457_c0_seq2	gem-associated protein 5	-26	<0.001	-387	0.007
6	comp147101_c0_seq2	integrator complex subunit 12	-22	<0.001	-80	0.038
6	comp142486_c0_seq8	RNA-binding protein 1	-21	<0.001	-216	0.046
5	comp67725_c1_seq2	u11 u12 small nuclear ribonucleoprotein	-21	<0.001	-63	0.024

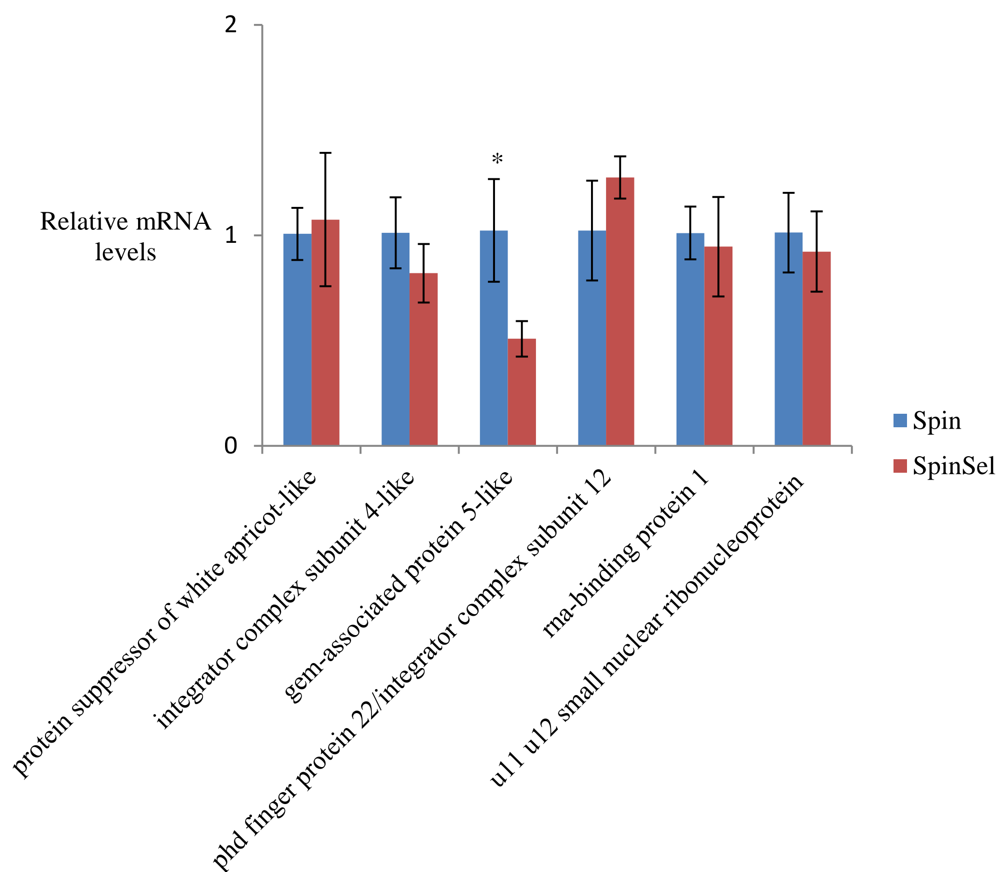


Figure 6.9. QPCR results showing relative expression of splice factors in *T. absoluta*. Asterisk indicates significant difference (P<0.05).

In this analysis the only splice factor found to be expressed differentially was the gem-associated protein 5 which showed approx. 2-fold down regulation. This was less than that predicted by DEseq2 which estimated 26-fold down regulation. Gemin5 is a component of the spliceosomal complex, responsible for small nuclear RNA binding. A study of the role of gemini5 in human cancer cells found that overexpression of Gemini5 caused alternative splicing events in at least 16 genes (Lee et al., 2008). One gene had an intron retained, two genes had alterative splice donor sites, leading to a shortened or lengthened exon, and eight genes had novel exons. Two genes had internal exon sequences recognised as introns and spliced out. Three genes, calcium/calmodulin-dependent protein kinase IV, STK32C and TIE1 had deleted exons, showing that Gemin5 can mediate exon skipping (Lee et al., 2008).

The qPCR analysis did not find significantly different expression for any of the other splice factors. The Trinity *de-novo* assembler assigns distinct isoforms of the same gene as separate contigs. If specific isoforms were differentially expressed, but the overall expression of the combined isoforms for a particular gene was the same, then this would be picked up by RNA-seq but not necessarily by qPCR. This may be particularly relevant for the two SR proteins differentially expressed as both RBP1 and SWAP are known to auto-regulate their expression through alternative splicing of their own transcripts (Zachar et al., 1987, Kumar and Lopez, 2005).

To follow this up the down-regulated isoforms of RBP1 and SWAP were compared to other isoforms of the same contig which were not differentially expressed. No unique differences were found in the down-regulated isoform of RBP1. However, the down-regulated isoform of SWAP had identical coding sequence but contained a 15 bp insertion in the 5' UTR compared to the most closely related isoform which was not differentially expressed (Figure 6.10). Further analysis with allele-specific qPCR could be used to investigate this in more detail.

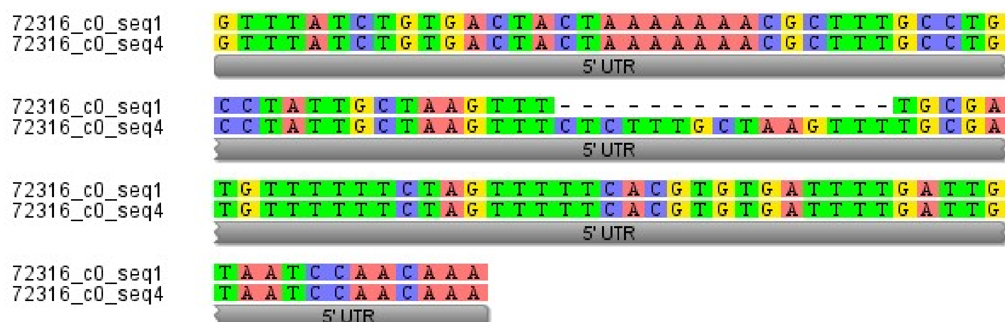


Figure 6.10. Alignment of 5' UTR of transcripts of Protein Suppressor of White Apricot. Top = comp72316_c0_seq1 (not differentially expressed); bottom = comp72361_c0_seq4 (differentially expressed).

6.3.5 Comparison of nAChR α6 subunit of *T. absoluta* in different life stages

To check if the exon skipping observed in the nAChR α6 subunit of *T. absoluta* is life-stage specific, RNA was extracted from second, third and fourth-instar larvae, pupae and adults of Spin and SpinSel, cDNA was synthesised and PCR used to check the sequences. Only the SpinSel larvae were missing the third exon, with either exon 3a or 3b present in pupae and adults of SpinSel (Figure 6.11). No differences were found between larvae and adults of Spin. Therefore, the exon-skipping in SpinSel is specific to the larvae and only these would be expected to be resistant to spinosad. Life-stage specific resistance to insecticides has been reported previously in *B. tabaci*. Adults were resistant to imidacloprid and could metabolise it, whilst nymphs were susceptible and this was found to correspond with expression of the P450 CYP6CM1 which is expressed at higher levels in adults than nymphs (Jones et al., 2011). Both nymph and adult *B. tabaci* feed on the phloem in contrast, only the larvae of *T. absoluta* feed on tomatoes, so the inverse pattern of having insecticide-resistant larva would be advantageous from an evolutionary standpoint.

The life-stage specificity of exon-skipping is slightly surprising as we previously found that both adults and larvae of SpinSel were resistant to spinosad (Chapter 4), although SpinSel adults were only 44-fold resistant, compared to 160-fold resistance for larvae. Since larvae without exon 3 would produce an α6 subunit missing binding loop D, it is highly likely this is a major mechanism of resistance. However, there must be a second mechanism which gives the SpinSel adults more moderate resistance to spinosad.

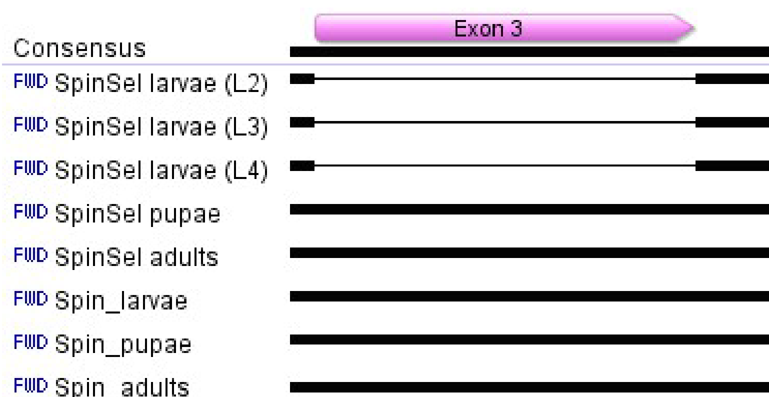


Figure 6.11. Skipping of exon 3 in SpinSel is life-stage specific.

6.3.6 Relative expression of nAChR α6 subunit of *T. absoluta*

Transcriptomes of Spin and SpinSel cDNA were assembled for each population both separately and combined (chapter 5). The separate assemblies (assembly 3 and assembly 4) were annotated to look for the α6 subunit, in order to check if the sequences matched those reported in 6.3.2. Unfortunately, no transcripts with hits to the α6 subunit were present in the SpinSel transcriptome, possibly due to lower expression levels. The α6 subunit was not found to be significantly differently expressed by EdgeR or Deseq2. However, the count matrix showed zero counts of this subunit in SpinSel, compared to multiple counts in the Spin population (Table 6.6). Genes with lower expression may be missed from lists of DE genes in RNA-seq experiments (Love et al., 2014).

Therefore, qPCR was carried out on larvae and adults of Spin and SpinSel with primers designed in different regions of the α6 subunit and in cases the α6 subunit had significantly lower expression in SpinSel compared to Spin (Figure 6.12). The α6 subunit is not essential for *D. melanogaster* viability, suggesting there may be flexibility in the subunits which form nAChRs (Watson et al., 2010). To check if members of SpinSel were substituting other nAChR subunits to compensate for the lack of α6, the RNA-seq data was re-examined

to look at counts of other subunits but the counts did not appear to differ between Spin and SpinSel (Table 6.6).

Down-regulation of the α6 subunit would be expected to give resistance to spinosad. This mechanism was present in both larvae and adults and may therefore explain the resistance observed in SpinSel adults (Chapter 4). Since SpinSel adults with wild-type α6 transcripts still had lower levels of expression, this mechanism is expected to be independent of exon skipping.

Table 6.6. Counts of transcripts with hit against nAChR subunits

Subunit	Spin1	Spin2	Spin3	Spinsel1	Spinsel2	Spinsel3	Average Spin	Average SpinSel
α1	101	128	174	109	167	119	**134**	**132**
α2	28	36	46	45	56	46	**37**	**49**
α3	82	85	114	48	93	73	**94**	**71**
α4	35	43	29	27	57	30	**36**	**38**
α5	0	2	0	3	5	2	**1**	**3**
α6	11	14	8	0	0	0	**11**	**0**
α7	287	399	450	197	332	278	**379**	**269**
α8	14	11	4	6	14	11	**10**	**10**
α9	1337	1212	1278	1374	1395	1052	**1275**	**1274**
β1	141	187	211	159	183	221	**180**	**188**
β3	43	39	53	65	80	11	**45**	**52**

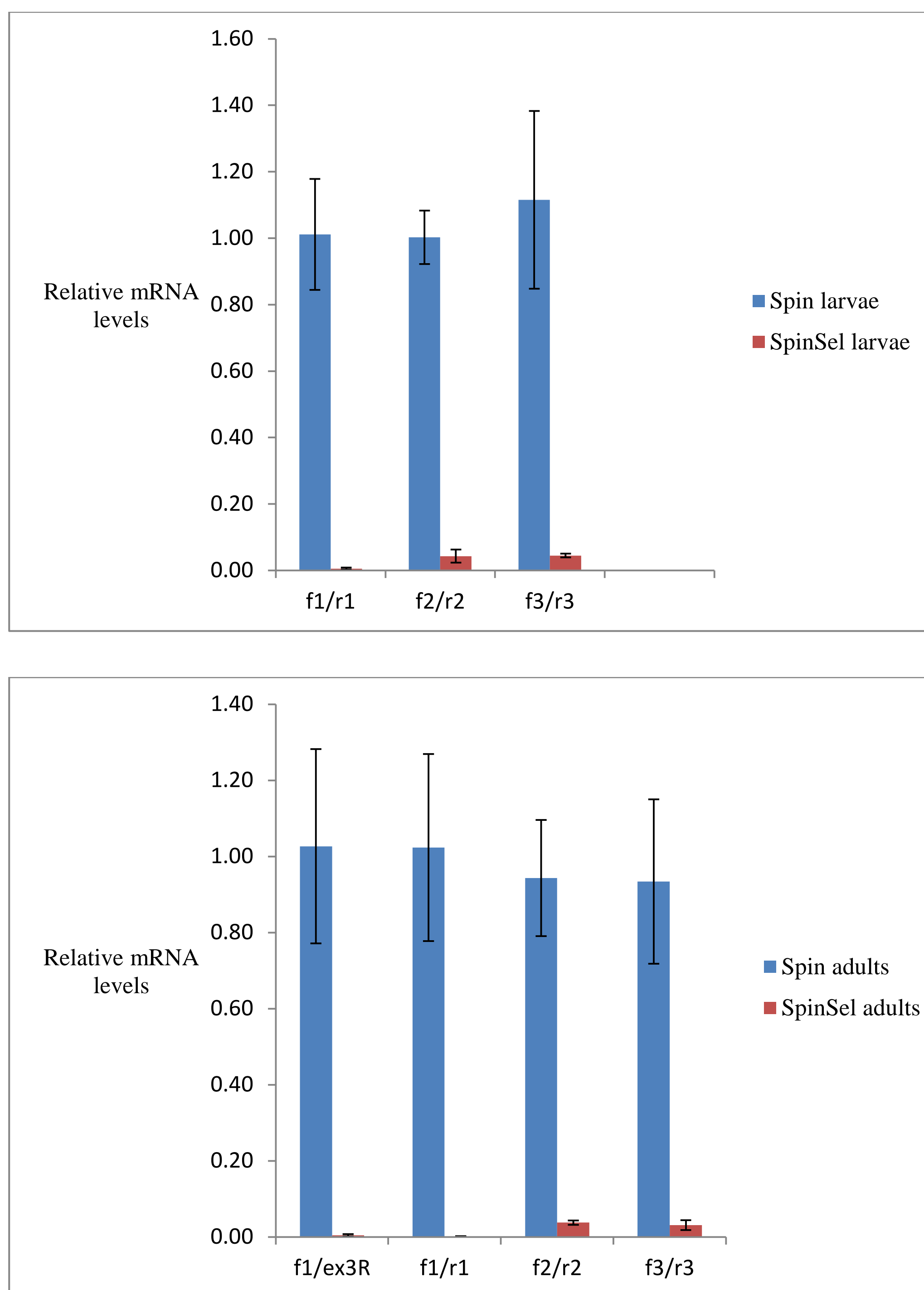


Figure 6.12. Expression of nAChR α6 subunit of *T. absoluta* 6 subunit in Spin and SpinSel larvae and adults. F1/r1 = exons 2-4, f1/ex3R = exons 1-3, f2/r2 = exons 5-6 f3/r3 = exons 7/8.

6.4 Conclusions

In this chapter the nAChR α6 subunit of *T. absoluta* the target site of spinosad was compared between spinosad resistant and susceptible populations. SpinSel resistant larvae were found to have an alternatively spliced α6 subunit which was missing exon 3, which encodes binding loop D of the protein. As far as we are aware, the skipping of exon 3, without any subsequent premature stop codons, has not been reported in spinosad-resistant insects. This new mechanism of resistance should therefore be monitored carefully, as it could have big implications on the efficacy of spinosad to control insect pests *in vivo*. The mechanism was found to be larvae specific, with pupae and adults unaffected.

A second mechanism, the down-regulation of α6 was also present in both larvae and adults. Since SpinSel adults did not have any mutations or altered splicing compared to susceptible populations, the down-regulation of α6 is expected to be the main mechanism responsible for resistance in this life stage. Further studies should be carried out to investigate the variation in expression of *T. absoluta* α6 between different populations and developmental stages and to look for potential *cis*-acting mutations in the promoter region of this gene in resistant strains. Furthermore, RNA-seq of adult RNA would rule out any other possible mechanisms of resistance.

DNA sequencing and expression of splice factors were investigated to find the mechanism responsible for the exon-skipping in SpinSel larvae. No unique differences were found in exon or intron sequence of SpinSel gDNA but several splice factor isoforms were found to be down-regulated in SpinSel. These included SR family proteins, which have been associated with both constitutive and alternative splicing, and regulation of gene expression (Howard and Sanford, 2015). Gemini-5, a member of the spliceosomal complex, was found to be significantly down-regulated by both RNA-seq and qPCR. SWAP was not significantly down-regulated in qPCRs but alternative isoforms of genes encoding this protein were differentially expressed in the transcriptome. These alternative isoforms may have different effects on splicing and therefore could play a role in the exon skipping observed in

SpinSel. In Chapter 7, I will outline further work which could be done to pin-point the precise mechanism responsible for the skipping of *T. absoluta* nAChR α6 exon 3 in SpinSel larvae.

7. General Discussion

In this chapter I summarise the main findings of this thesis and relate this to the wider scientific literature. The implications of these findings with relation to the management of *T. absoluta* are subsequently discussed. Finally, future work which would build on the results found during my PhD is outlined.

7.1 Pyrethroid resistance in *T. absoluta*

In chapter 3, multiple pyrethroid resistance mutations were found to be widespread across the range of *T. absoluta.* The L1014F substitution was found at 100% frequency in all of the invasive populations, and in all South American countries tested except Columbia. In addition to L1014F, all of the populations tested had at least one additional *skdr* substitution (M918T, T929I or L925M). Substitutions in the *para*-type sodium channel at positions 918, 925, 929 and 1014 have arisen in many species of insect (Table 7.1; reviewed in (Rinkevich et al., 2013). This is an example of convergent evolution. It suggests that these mutations at these hot spots are able to confer resistance to pyrethroids without completely impairing the native function of the sodium channel.

Table 7.1. Substitutions at positions 918, 925, 929 and 1014 of the para-type sodium channel in selected insect pests.

	Amino acid position				
Species	918	925	929	1014	Reference(s)
T. absoluta	M918T	L925M	T929I	L1014F	(Haddi, 2012)
P. xylostella	M918I		T929I	L1014F	(Sonoda et al., 2008a)
T. vaporariorum	M918L	L925I	T929I		(Karatolos et al., 2012a)
B. tabaci	M918V	L915I	T929V		(Morin et al., 2002), (Alon et al., 2006)
T. tabaci	M918T		T929I	L1014F	(Toda and Morishita, 2009)
M. persicae	M918T			L1014F	(Eleftherianos et al., 2008)
A. gambiae				L1014F, L1014S	(Martinez-Torres et al., 1998), (Ranson et al., 2000)
H. zea				L1014H	(Hopkins and Pietrantonio, 2010)
Ctenocephalides felis			T929V	L1014F	(Bass et al., 2004)
M. domestica	M918T			L1014F, L1014H	(Williamson et al., 1996) (Liu and Pridgeon, 2002)

An interesting finding of the work described in chapter 3 is that four different target site alterations were found in *T. absoluta* rather than just one predominant mutation. It is possible that the L1014F mutation alone may be insufficient to provide protection against the field rate of pyrethroids used against *T. absoluta*, so there was selective pressure to develop the additional *skdr* mutations. In support of this hypothesis pollen beetles homozygous for the L1014F mutation in Denmark displayed 100% mortality at the field rate of a pyrethroid (Højland, 2015) indicating that L1014F alone was not sufficient to survive this pyrethroid application. *M. periscae* with both L1014F and M918T were significantly more resistant to pyrethroids than individuals with L1014F alone (Eleftherianos et al., 2008). However, it is not clear from our data whether L1014F evolved before or after M918T and T929I, which have been found in the absence of *kdr* in some insects (Araujo et al., 2011, Nyoni et al., 2011). The presence of three alternative skdr mutations: M918T, L925M and T929I in some populations of *T. absoluta* is intriguing. In *B. tabaci* M918V was found initially but was later replaced by L925I, suggesting that L925I gave some advantage over M918V (Morin et al., 2002). Therefore it is possible that in *T. absoluta* the different mutations provide different levels of selective advantage to different pyrethroid insecticides and if so L925M whilst currently rare may increase in frequency over time.

Work on resistance can give insight into the population genetics of invasive species. Phylogenetic analyses suggest a single point of origin for *T. absoluta* in the Mediterranean basin (Cifuentes et al., 2011, Guillemaud et al., 2015). Our finding of L1014F, M918T and T929I in all of the invasive countries supports this and suggests that this population of *T. absoluta* arrived in Europe already carrying resistance to pyrethroids as a result of past insecticide use in South America. It is interesting L1014F was fixed in all countries except Columbia, where some insects were heterozygous. This could point to greater genetic diversity of *T. absoluta* in Columbia. *T. absoluta* is thought to be originally from Peru but has been present in Columbia since the 1960s (Guedes and Picanço, 2012). This predates the discovery of photo-stable synthetic pyrethroids in the 1970s (Elliott et al., 1973) but not that of DDT which share the same target-site.

Fitness costs to *kdr* mutations have been reported in other species, for example *M. persicae* homozygous for L1014F were slower to respond to alarm pheromone (Foster et al., 1999) and were more likely to be parasitised by *Diaeretiella rapae* (Foster et al., 2011). Since all of the populations of *T. absoluta* in the Rothamsted insectary were homozygous for L1014F fitness cost studies would not be possible to carry out, as there would be no susceptible population for comparison.

Although all of the populations in the Rothamsted insectary carried *kdr* and *skdr* mutations, there was still substantial variation in susceptibility to pyrethroids. The Brazilian populations of *T. absoluta* which were found to have M918T, T929I and L1014F in chapter 3, were tested for metabolic resistance (Silva et al., 2015). A positive correlation between glutathione-*s*-transferase and cytochrome P450 mediated N-demethylation activity and resistance to permethrin and deltamethrin was found, but not for α-cypermethrin (Silva et al., 2015). It would be interesting to test enzyme activity in the populations in the Rothamsted Insectary to explore if metabolism may be a secondary mechanism of resistance in some populations.

7.2 Spinosad resistance in *T. absoluta*

In this thesis, exon skipping in the nAChR α6 subunit was found in a population of *T. absoluta* with high resistance to spinosad. In *P. xylostella* two field populations in Hawaii (Pearl and Ewa) developed LC_{50}s above the field rate just two and a half years after spinosad first became commercially available (Zhao et al., 2002). The Pearl-parent strain had an initial LC_{50} of 151 mg L^{-1}; after selection in the laboratory the LC50 of this strain (Pearl-Sel) rose to 837 mg L^{-1}. Synergist tests suggested that P450s and esterase were not involved in the resistance observed in Pearl-Sel (Zhao et al., 2002). The resistance in Pearl-Sel was found to be caused by premature stop codons in the nAChR α6 subunit leading to truncated proteins (Baxter et al., 2010, Rinkevich et al., 2010). One mechanism was a point mutation (GT to AT) in the intron 9 donor splice site of the nAChR α6 subunit, which resulted in an extra 40 bp of sequence added to the mRNA (Baxter et al., 2010). An alternative target site alteration, a splice variant with an extra 7 bp of intron sequence added to exon 3 was also found (Rinkevich et al., 2010). Both of these mechanisms involved an insertion of intron sequence which was not divisible by 3, thus disrupting the reading frame and ultimately generating premature stop codons. This is different from the skipping of exon 3 in *T. absoluta* which is 45 bp so does not alter the reading frame, allowing the protein to be transcribed until the normal stop codon is reached.

In *B. dorsalis* all transcripts from spinosad resistant strains had premature stop codons in exon 7, and additionally were missing either exons 3-6 or 4-6 (Hsu et al., 2012). Analysis of gDNA found that exon 5 could not be amplified, and an A to T mutation was found in intron 2 of resistant *B. dorsalis* (Hsu et al., 2012).

Subsequent studies have found a point mutation in a transmembrane region III which gave high resistance to spinosad in *F. occidentalis* and *T. palmi* (Puinean et al., 2012, Bao et al., 2014). All of the target site alterations in other spinosad resistant pest species to date affect the transmembrane domains, whereas the exon skipping in *T. absoluta* only disrupts ligand binding loop D (Figure 7.1)

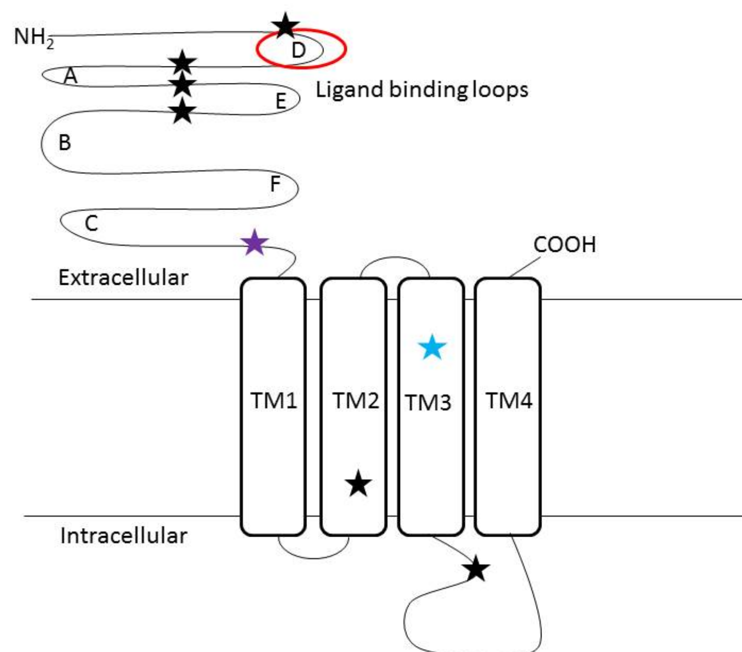


Figure 7.1. Spinosad target-site resistance mechanisms. Adapted from (Rinkevich et al., 2010). Black stars = premature stop codons found in *P. xylostella* (Baxter et al., 2010, Rinkevich et al., 2010). Light blue star = G275E mutation found in *F. occidentalis* and *T. palmi* (Puinean et al., 2012, Bao et al., 2014). Purple star = approximate location of premature stop codons in *B. dorsarlis* (Hsu et al., 2012). Red circle = site of exon skipping in *T. absoluta*.

Whilst the structure of the vertebrate nAChRs has been studied in detail, less is known about the composition of insect nAChRs. Functional expression of *D. melanogaster* subunits in Xenopus oocytes *showed* that α6 could not be expressed as a homomer, but could be expressed as a heteromer with α5 (Watson et al., 2010). This created a receptor with sensitivity to acetylcholine, nicotine and spinosyns, but not the neonicotinoid imidacloprid. Therefore, it is thought that insects must have nAChRs with alternative subunits which are targeted by neonicotinoids. Strains of *D. melanogaster* with mutations in α1 and β1 have been shown to be resistant to neonicotinoids (Perry et al., 2012).

Whilst the nAChR gene family has been cloned and sequenced in many insects (Jones et al., 2006, Jones et al., 2005, Sattelle et al., 2005, Shao et al., 2007), more work needs to be done to see what subunit combinations are *expressed in vivo*, and whether expression is tissue-specific like in vertebrates.

It is interesting that even in susceptible insects, the nAChR and specifically the α6 subunit displays a large diversity of different transcripts. Most insects have mutually exclusive exons 3a and 3b and it is unknown how these are regulated, or why the alternative exons exist. Remarkably, skipping (and duplication) of exon 3 has been found at low levels in wild-type insects suggesting the expression of a wide diversity of α6 subunits is the norm in insects (Shao et al., 2009). Further diversity of α6 is generated by alternative isoforms of exon 8 and by RNA-editing in exon 5 (Sattelle et al., 2005).

Without knowing how the splicing of alternative exons in nAChRs are regulated in wild-type insects, it will difficult to definitively determine the mechanism responsible for the exon skipping that we found in SpinSel. We did not find any mutations in intron sequence, in contrast with spinosad-resistant *B. doralis* where a point mutations in intron 2 was discovered (Hsu et al., 2012). Interestingly, in *T. absoluta* intron 2 was over 10 kb long, whilst in *B. dorsalis* and *P. xylostella* intron 2 was only 304 and 2864 nucleotides respectively (Hsu et al., 2012, Baxter et al., 2010). We did find differences in the expression of specific transcripts of splice-regulating proteins, but further work will be needed to prove that these are involved. Furthermore, we have not yet investigated methylation which can also affect splicing (Lyko et al., 2010).

Splicing as a mechanism of insecticide resistance is unusual, but has been reported for insecticides other than spinosad. In pyrethroid-resistant field strains of *P. xylostella*, alternatively spliced sodium channel transcripts were identified (Sonoda et al., 2008a, Sonoda et al., 2006). A 36 bp deletion in cDNA encoding the glutamate-gated chloride channel has been reported in abamectin resistant *P. xylostella* (Liu et al., 2014). The gDNA was not sequenced so it is ambiguous whether this was caused by alternative splicing. One important conclusion from our work is that scientists working on insecticide resistance must look at cDNA as well as gDNA, as we would not

have detected the difference in SpinSel if only gDNA had been studied. This is an especially important point for candidate gene association studies which almost exclusively look for SNPs and indels associated with resistance in genomic DNA.

We did not find exon skipping in adults of SpinSel, even though they displayed resistance to spinosad. Most studies only look at one life stage of an insect, so life-stage specific mechanisms could be missed. We found a possible second mechanism of resistance, the down-regulation of the α6 subunit in both adults and larvae of SpinSel. Reduced expression of α6 has not been reported as a spinosad-resistance mechanism to date. Surprisingly, RNAi which reduced expression of α6 by up to 75% did not reduce the spinosad sensitivity of *D. melanogaster* or *T. castaneum* (Rinkevich and Scott, 2013). However, the down-regulation we found in SpinSel was much higher, with α6 at least 23-fold under expressed. More work is needed to elucidate how much natural variation there is in expression of α6 between different populations, and how this changes sensitivity to spinosad.

Fitness costs were not explored in SpinSel, although the fact that the original Spin-parent strain became less resistant to spinosad over time in the absence of selection suggests that there may be some fitness penalty associated with spinosad resistance in *T. absoluta*. In *H. armigera* populations with 24-fold spinosad resistance had a longer development time, a lower proportion of eggs hatching, reduced adult emergence and reduced pupal weight (Wang et al., 2010a). This strain had significantly reduced resistance in the presence of PBO, suggesting that the resistance could be mediated by P450 metabolism (Wang et al., 2009). In contrast, no evidence of reduced fitness was found in a *D. melanogaster* α6 knockout strain with high spinosad resistance, with no reduction in survival observed (Perry et al., 2007). This suggests that target site resistance to spinosad may not carry a fitness cost, although further work is needed to explore sub-lethal effects.

7.3 Implications for resistance management of *T. absoluta*

Clearly the findings in this thesis have implications for the management of *T. absoluta*. In terms of pyrethroid resistance, the L1014F mutation was fixed in all but one population. Since the mating of two homozygous resistant individuals produces 100% homozygous resistant offspring, insects cannot return to susceptibility without reversing the mutation. A population of *T. absoluta* in Argentina which had received only 1 spray of pyrethroids in the previous year was still heavily resistant to pyrethroids (Lietti et al., 2005). The authors suggested that the resistance in this strain could be due to migration of resistant insects from nearby glasshouses (Lietti et al., 2005), but based on our results I would suggest that the resistance may have been fixed in this population many years previously.

In this thesis a population from Portugal with reported control failure using spinosad displayed 8-fold resistance to spinosad compared to the most susceptible population tested. However, the LC_{50} of 15 mg L^{-1} was still below the field rate of 80 mg L^{-1}. Selection in the laboratory increased this to 277-fold resistance, with an LC_{50} of 498 mg L^{-1}, greater than the field rate. A recent study of the effectiveness of spinosad in Brazil found resistance ratios of up to 93, but both the reported LC_{50} and LC_{99} values were well below the field rate (Campos et al., 2014a). Seven generations of laboratory selection increased this resistance to 180,000-fold with an LC_{50} of 1700 mg L^{-1}(Campos et al., 2014b).This shows that *T. absoluta* is capable of rapidly developing resistance to the label rate of spinosad under laboratory conditions. The question is whether these levels of resistance will develop in field and greenhouse populations.

In the UK, the current recommendation for the control of *T. absoluta* is an Integrated Pest management (IPM) regime using spinosad, the diamide chlorantraniliprole and the predatory bug *M. pygmaeus* (Howlett, 2013). However, recent control failures have been reported by three UK growers (Rob Jacobson, Personal communication 2015). These populations have been tested at Rothamsted Research and high spinosad resistance at the field rate was found in two of the populations (John Risley, Personal communication).

Furthermore, resistance to diamides has recently been reported in populations of *T. absoluta* in Sicily, Italy and Crete, Greece. In Greece, 14-fold resistance to chlorantraniliprole was reported whilst in Italy a population was found with over 2000-fold resistance. The LC_{50} was below the field rate for all of the Greek strains, but three out of four Italian populations tested had LC_{50}s higher than the field rate (Roditakis et al., 2015).

It is particularly difficult to substitute insecticides within an integrated pest management programme, as the insecticides must have low toxicity to the biological control species (Bielza, 2015). However, this could be potentially overcome by selecting the biological control species with insecticide so that it becomes resistant to the insecticide or exploiting natural variation in the sensitivity of biological control agents to insecticides (Bielza, 2015). Ideally to manage resistance three different active ingredients should be available to rotate between (IRAC, 2011). Unfortunately, *T. absoluta* has developed resistance to almost all chemical classes currently registered for use (Table 7.2). Therefore there is a need for more active ingredients to come on to the market. In the meantime, I would advise careful monitoring of resistance to spinosad and diamides. Rotation should be used, leaving a sixty day window without a particular mode of action, to prevent or slow down the development of resistance to insecticides (IRAC, 2011). If resistance to either spinosad or diamides is found then these should not be used and insecticides with alternative modes of action should be used instead. Indoxacarb or *B. thuringiensis* are other insecticides which can be used as part of the control of *T. absoluta* in the UK (Howlett, 2013). I would not recommend the use of pyrethroids at all, as the resistance to these insecticides appears to be fixed.

Table 7.2. Insecticide classes registered for use against *T. absoluta* (IRAC, 2011) and first reports of resistance, adapted from (www.pesticideresistance.org, 2015).

				First report of resistance in *T. absoluta*		
MOA Group	Chemical class	Target	Example	Date	Location	Reference
3	Pyrethroids	Sodium channel	Deltamethrin	1994	Argentina	(Lietti et al., 2005)
6	Avermectins	Chloride channel	Abamectin	1998	Brazil	(Siqueira et al., 2001)
14	Nereistoxin analogues	NAChR	Cartap	1999	Brazil	(Siqueira et al., 2000a)
1	Organophosphates	Acetylcholinesterase	Methamidophos	2000	Brazil	(Siqueira et al., 2000b)
15	Benozylureas	Chitin biosynthesis	Diflubenzuron	2005	Brazil	(Silva et al., 2011)
22	Oxadiozines	Sodium channel	Indoxacarb	2005	Brazil	(Silva et al., 2011)
5	Spinosyns	NAChR	Spinosad, spinetoram	2011	Chile	(Reyes et al., 2012)
11	Microbes	Insect midgut	*Bacillus thuringiensis*	2011	Brazil	(Silva et al., 2011)
28	Diamides	Ryanodine receptor	Chlorantraniliprole	2014	Italy	(Roditakis et al., 2015)
18	Diacylhydrazines	Ecdysone receptor	Tebufenozide	Not Reported		
13	Pyrroles	Oxidative phosphorylation	Chlorfenapyr	Not Reported		
Un	Tertranortiterepenoid	Unknown	Azadirachtin	Not Reported		

7.4 Future Work

The results of this thesis could be explored further as follows:

1) There was a high level of variation in pyrethroid resistance between populations of *T. absoluta* in the Rothamsted insectary which could not be fully explained by the target site mutations found (Haddi et al., 2012). This suggests that additional mechanisms could also be present in *T. absoluta*, for example differences in metabolism or reduced penetration. This could be explored further, making use of the reference transcriptome (chapter 5).
2) Evidence of down-regulation of specific isoforms splice factor proteins in SpinSel was shown in chapter 6. Allele-specific qPCR could be used to explore this further. RNAi could then be used to knockdown expression of specific splice factors in susceptible strains of *T. absoluta* to elucidate whether they affect exon-skipping *in vivo*.
3) In addition to mutations in intron or exon sequence of a gene or differences in expression of RNA-binding proteins, alternative splicing can also be caused by differences in methylation (Lyko et al., 2010). Bisulphite PCR and sequencing could be used to explore this.
4) Down-regulation of nAChR α6 was also found in SpinSel. Genome-walking could be used to look for differences in the promoter region of this gene.
5) At the moment both spinosad resistance mechanisms, exon-skipping and down-regulation of α6, can only be diagnosed by amplifying RNA. To monitor the frequency of these mechanisms in field populations of *T. absoluta,* ideally a DNA-based diagnostic would be required. This would be dependent on finding the DNA change underpinning these resistance mechanisms.

References

ADAMS, M. D., CELNIKER, S. E., HOLT, R. A., EVANS, C. A., GOCAYNE, J. D., AMANATIDES, P. G., SCHERER, S. E., LI, P. W., HOSKINS, R. A., GALLE, R. F., GEORGE, R. A., LEWIS, S. E., RICHARDS, S., ASHBURNER, M., HENDERSON, S. N., SUTTON, G. G., WORTMAN, J. R., YANDELL, M. D., ZHANG, Q., CHEN, L. X., BRANDON, R. C., ROGERS, Y.-H. C., BLAZEJ, R. G., CHAMPE, M., PFEIFFER, B. D., WAN, K. H., DOYLE, C., BAXTER, E. G., HELT, G., NELSON, C. R., GABOR, G. L., MIKLOS, ABRIL, J. F., AGBAYANI, A., AN, H.-J., ANDREWS-PFANNKOCH, C., BALDWIN, D., BALLEW, R. M., BASU, A., BAXENDALE, J., BAYRAKTAROGLU, L., BEASLEY, E. M., BEESON, K. Y., BENOS, P. V., BERMAN, B. P., BHANDARI, D., BOLSHAKOV, S., BORKOVA, D., BOTCHAN, M. R., BOUCK, J., BROKSTEIN, P., BROTTIER, P., BURTIS, K. C., BUSAM, D. A., BUTLER, H., CADIEU, E., CENTER, A., CHANDRA, I., CHERRY, J. M., CAWLEY, S., DAHLKE, C., DAVENPORT, L. B., DAVIES, P., PABLOS, B. D., DELCHER, A., DENG, Z., MAYS, A. D., DEW, I., DIETZ, S. M., DODSON, K., DOUP, L. E., DOWNES, M., DUGAN-ROCHA, S., DUNKOV, B. C., DUNN, P., DURBIN, K. J., EVANGELISTA, C. C., FERRAZ, C., FERRIERA, S., FLEISCHMANN, W., FOSLER, C., GABRIELIAN, A. E., GARG, N. S., GELBART, W. M., GLASSER, K., GLODEK, A., GONG, F., GORRELL, J. H., GU, Z., GUAN, P., HARRIS, M., HARRIS, N. L., HARVEY, D., HEIMAN, T. J., HERNANDEZ, J. R., HOUCK, J., HOSTIN, D., HOUSTON, K. A., HOWLAND, T. J., WEI, M.-H., et al. 2000. The genome sequence of *Drosophila melanogaster*. *Science,* 287**,** 2185-2195.

AHMAD, M., DENHOLM, I. & BROMILOW, R. H. 2006. Delayed cuticular penetration and enhanced metabolism of deltamethrin in pyrethroid-resistant strains of *Helicoverpa armigera* from China and Pakistan. *Pest Management Science,* 62**,** 805-810.

AI, J., ZHU, Y., DUAN, J., YU, Q., ZHANG, G., WAN, F. & XIANG, Z.-H. 2011. Genome-wide analysis of cytochrome P450 monooxygenase genes in the silkworm, *Bombyx mori*. *Gene,* 480**,** 42-50.

ALON, M., BENTING, J., LUEKE, B., PONGE, T., ALON, F. & MORIN, S. 2006. Multiple origins of pyrethroid resistance in sympatric biotypes of *Bemisia tabaci* (Hemiptera: Aleyrodidae). *Insect Biochemistry and Molecular Biology,* 36**,** 71-79.

ARAUJO, R. A., WILLIAMSON, M. S., BASS, C., FIELD, L. M. & DUCE, I. R. 2011. Pyrethroid resistance in *Sitophilus zeamais* is associated with a mutation (T929I) in the voltage-gated sodium channel. *Insect molecular biology,* 20**,** 437-45.

ARENSBURGER, P., MEGY, K., WATERHOUSE, R. M., ABRUDAN, J., AMEDEO, P., ANTELO, B., BARTHOLOMAY, L., BIDWELL, S., CALER, E., CAMARA, F., CAMPBELL, C. L., CAMPBELL, K. S., CASOLA, C., CASTRO, M. T., CHANDRAMOULISWARAN, I., CHAPMAN, S. B., CHRISTLEY, S., COSTAS, J., EISENSTADT, E., FESCHOTTE, C., FRASER-LIGGETT, C., GUIGO, R., HAAS, B., HAMMOND, M., HANSSON, B. S., HEMINGWAY, J., HILL, S. R., HOWARTH, C., IGNELL, R., KENNEDY, R. C., KODIRA, C. D., LOBO, N. F., MAO, C., MAYHEW, G., MICHEL, K., MORI, A., LIU, N., NAVEIRA, H., NENE, V., NGUYEN, N., PEARSON, M. D., PRITHAM, E. J., PUIU, D., QI, Y., RANSON, H., RIBEIRO, J. M. C., ROBERSTON, H. M., SEVERSON, D. W., SHUMWAY, M., STANKE, M., STRAUSBERG, R. L., SUN, C., SUTTON, G., TU, Z., TUBIO, J. M. C., UNGER, M. F., VANLANDINGHAM, D. L., VILELLA, A. J., WHITE, O., WHITE, J. R., WONDJI, C. S., WORTMAN, J., ZDOBNOV, E. M., BIRREN, B., CHRISTENSEN, B. M., COLLINS, F. H., CORNEL, A., DIMOPOULOS, G., HANNICK, L. I., HIGGS, S., LANZARO, G. C., LAWSON, D., LEE, N. H., MUSKAVITCH, M. A. T., RAIKHEL, A. S. & ATKINSON, P. W. 2010. Sequencing of *Culex quinquefasciatus* establishes a platform for mosquito comparative genomics. *Science,* 330**,** 86-88.

BALE, J. S., VAN LENTEREN, J. C. & BIGLER, F. 2008. Biological control and sustainable food production. *Philosophical Transactions of the Royal Society B-Biological Sciences,* 363**,** 761-776.

BAO, W. X., NARAI, Y., NAKANO, A., KANEDA, T., MURAI, T. & SONODA, S. 2014. Spinosad resistance of melon thrips, *Thrips palmi*, is conferred by G275E mutation in

α6 subunit of nicotinic acetylcholine receptor and cytochrome P450 detoxification. *Pesticide Biochemistry and Physiology,* 112**,** 51-55.

BARRIENTOS, Z., APABLAZA, H., NORERO, S. & ESTAY, P. 1998. Temperatura base y constate termica de desarrollo de la polilla del tomate *Tuta absoluta* (Lepidoptera: Gelechiidae). *Ciencia e Ivestigacion Agraria,* 25**,** 133-137.

BASS, C., NIKOU, D., DONNELLY, M., WILLIAMSON, M., RANSON, H., BALL, A., VONTAS, J. & FIELD, L. 2007. Detection of knockdown resistance (kdr) mutations in *Anopheles gambiae*: a comparison of two new high-throughput assays with existing methods. *Malaria Journal,* 6**,** 111.

BASS, C., PUINEAN, A. M., ANDREWS, M. C., CULTER, P., DANIELS, M., ELIAS, J., LAURA PAUL, V., CROSSTHWAITE, A. J., DENHOLM, I., FIELD, L. M., FOSTER, S. P., LIND, R., WILLIAMSON, M. S. & SLATER, R. 2011. Mutation of a nicotinic acetylcholine receptor β subunit is associated with resistance to neonicotinoid insecticides in the aphid *Myzus persicae*. *BMC Neuroscience* 12**,** 51.

BASS, C., SCHROEDER, I., TURBERG, A., M FIELD, L. & S WILLIAMSON, M. 2004. Identification of mutations associated with pyrethroid resistance in the para-type sodium channel of the cat flea, *Ctenocephalides felis*. *Insect Biochemistry and Molecular Biology,* 34**,** 1305-1313.

BAXTER, S. W., CHEN, M., DAWSON, A., ZHAO, J.-Z., VOGEL, H., SHELTON, A. M., HECKEL, D. G. & JIGGINS, C. D. 2010. Mis-Spliced transcripts of nicotinic acetylcholine receptor α6 are associated with field evolved spinosad resistance in *Plutella xylostella* (L.). *PLoS Genet,* 6**,** e1000802.

BEZANILLA, F. & ARMSTRONG, C. M. 1977. Inactivation of the sodium channel. I. Sodium current experiments. *The Journal of General Physiology,* 70**,** 549-566.

BIELZA, P. The silver lining of insectide resistance: resistant natural enemies. Resistance 2015 Rothamsted Research.

BONGAARTS, J. 2015. Global fertility and population trends. *Seminars in Reproductive Medicine,* 33**,** 5-10.

BUENO, V. H. P., VAN LENTEREN, J. C., LINS, J. C., CALIXTO, A. M., MONTES, F. C., SILVA, D. B., SANTIAGO, L. D. & PÉREZ, L. M. 2013. New records of *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) predation by Brazilian Hemipteran predatory bugs. *Journal of Applied Entomology,* 137**,** 29-34.

BURSET, M., SELEDTSOV, I. A. & SOLOVYEV, V. V. 2000. Analysis of canonical and non-canonical splice sites in mammalian genomes. *Nucleic Acids Research,* 28**,** 4364-4375.

BURTON, M. J., MELLOR, I. R., DUCE, I. R., DAVIES, T. G. E., FIELD, L. M. & WILLIAMSON, M. S. 2011. Differential resistance of insect sodium channels with kdr mutations to deltamethrin, permethrin and DDT. *Insect Biochemistry and Molecular Biology,* 41**,** 723-732.

CALTAGIRONE, L. E. 1981. Landmark examples in classical biological control. *Annual Review of Entomology,* 26**,** 213-232.

CAMPBELL, P. M., NEWCOMB, R. D., RUSSELL, R. J. & OAKESHOTT, J. G. 1998. Two different amino acid substitutions in the ali-esterase, E3, confer alternative types of organophosphorus insecticide resistance in the sheep blowfly, *Lucilia cuprina*. *Insect Biochemistry and Molecular Biology,* 28**,** 139-150.

CAMPOS, M., SILVA, T. M., SILVA, W., SILVA, J. & SIQUEIRA, H. A. 2014a. Spinosyn resistance in the tomato borer *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae). *Journal of Pest Science***,** 1-8.

CAMPOS, M. R., RODRIGUES, A. R. S., SILVA, W. M., SILVA, T. B. M., SILVA, V. R. F., GUEDES, R. N. C. & SIQUEIRA, H. A. A. 2014b. Spinosad and the tomato borer *Tuta absoluta* a bioinsecticide, an invasive pest threat, and high insecticide resistance. *Plos One,* 9**,** e103235.

CASIDA, J. E. & DURKIN, K. A. 2013. Neuroactive insecticides: targets, selectivity, resistance, and secondary effects. *Annual Review of Entomology,* 58**,** 99-117.

CATTERALL, W. 1988. Structure and function of voltage-sensitive ion channels. *Science,* 242**,** 50-61.

CHAILLEUX, A., DESNEUX, N., SEGURET, J., DO THI KHANH, H., MAIGNET, P. & TABONE, E. 2012. Assessing european egg parasitoids as a mean of controlling the invasive South American tomato pinworm *Tuta absoluta*. *Plos One,* 7**,** e48068.

CHEN, J. & WAGNER, E. J. 2010. snRNA 3′ end formation: the dawn of the Integrator complex. *Biochemical Society transactions,* 38**,** 1082-1087.

CIFUENTES, D., CHYNOWETH, R. & BIELZA, P. 2011. Genetic study of Mediterranean and South American populations of tomato leafminer *Tuta absoluta* (Povolny, 1994) (Lepidoptera: Gelechiidae) using ribosomal and mitochondrial markers. *Pest management science*.

COCCO, A., DELIPERI, S. & DELRIO, G. 2013. Control of *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) in greenhouse tomato crops using the mating disruption technique. *Journal of Applied Entomology,* 137**,** 16-28.

CONESA, A., GÖTZ, S., GARCÍA-GÓMEZ, J. M., TEROL, J., TALÓN, M. & ROBLES, M. 2005. Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics,* 21**,** 3674-3676.

CORBETT, A. & ROSENHEIM, J. A. 1996. Impact of a natural enemy overwintering refuge and its interaction with the surrounding landscape. *Ecological Entomology,* 21**,** 155-164.

CUTHBERTSON, A., MATHERS, J., BLACKBURN, L., KORYCINSKA, A., LUO, W., JACOBSON, R. & NORTHING, P. 2013. Population development of *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) under simulated UK glasshouse conditions. *Insects,* 4**,** 185-197.

DABORN, P., BOUNDY, S., YEN, J., PITTENDRIGH, B. & FFRENCH-CONSTANT, R. 2001. DDT resistance in *Drosophila* correlates with Cyp6g1 over-expression and confers cross-resistance to the neonicotinoid imidacloprid. *Molecular Genetics and Genomics,* 266**,** 556-563.

DABORN, P. J., YEN, J. L., BOGWITZ, M. R., LE GOFF, G., FEIL, E., JEFFERS, S., TIJET, N., PERRY, T., HECKEL, D., BATTERHAM, P., FEYEREISEN, R., WILSON, T. G. & FFRENCH-CONSTANT, R. H. 2002. A Single P450 Allele Associated with Insecticide Resistance in *Drosophila*. *Science,* 297**,** 2253-2256.

DAVIES, T. G., FIELD, L. M., USHERWOOD, P. N. & WILLIAMSON, M. S. 2007. DDT, pyrethrins, pyrethroids and insect sodium channels. *IUBMB life,* 59**,** 151-62.

DEFRA. 2015. Available: https://secure.fera.defra.gov.uk/phiw/riskRegister/index.cfm [Accessed 08/09/2015.]

DEL GATO-KONCZAK, F., BOURGEOIS, C. F., LE GUINER, C., KISTER, L., GESNEL, M. C., STEVENIN, J. & BREATHNACH, R. 2000. The RNA-binding protein TIA-1 is a novel mammalian splicing regulator acting through intron sequences adjacent to a 5 ' splice site. *Molecular and Cellular Biology,* 20**,** 6287-6299.

DESNEUX, N., LUNA, M., GUILLEMAUD, T. & URBANEJA, A. 2011. The invasive South American tomato pinworm, *Tuta absoluta*, continues to spread in Afro-Eurasia and beyond: the new threat to tomato world production. *Journal of Pest Science,* 84**,** 403-408.

DESNEUX, N., WAJNBERG, E., WYCKHUYS, K., BURGIO, G., ARPAIA, S., NARVÁEZ-VASQUEZ, C., GONZÁLEZ-CABRERA, J., CATALÁN RUESCAS, D., TABONE, E., FRANDON, J., PIZZOL, J., PONCET, C., CABELLO, T. & URBANEJA, A. 2010. Biological invasion of European tomato crops by *Tuta absoluta* ecology, geographic expansion and prospects for biological control. *Journal of Pest Science,* 83**,** 197-215.

DEVONSHIRE, A. L. & MOORES, G. D. 1982. A carboxylesterase with broad substrate specificity causes organophosphorus, carbamate and pyrethroid resistance in peach-potato aphids (*Myzus persicae*). *Pesticide Biochemistry and Physiology,* 18**,** 235-246.

DIGGLE, A. J., NEVE, P. B. & SMITH, F. P. 2003. Herbicides used in combination can reduce the probability of herbicide resistance in finite weed populations. *Weed Research,* 43**,** 371-382.

DRIPPS, J., OLSON, B., SPARKS, T. & CROUSE, G. 2008. Spinetoram: how artificial intelligence combined natural fermentation with synthetic chemistry to produce a new spinosyn insecticide. *Plant Health Progress***,** PHP-2008-0822-01-PS.

EDGAR, R. C. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research,* 32**,** 1792-1797.

EDI, C. V., DJOGBÉNOU, L., JENKINS, A. M., REGNA, K., MUSKAVITCH, M. A. T., POUPARDIN, R., JONES, C. M., ESSANDOH, J., KÉTOH, G. K., PAINE, M. J. I., KOUDOU, B. G., DONNELLY, M. J., RANSON, H. & WEETMAN, D. 2014. CYP6

P450 enzymes and *ACE-1* duplication produce extreme and multiple insecticide resistance in the malaria mosquito *Anopheles gambiae*. *PLoS Genet,* 10, e1004236.

ELEFTHERIANOS, I., FOSTER, S. P., WILLIAMSON, M. S. & DENHOLM, I. 2008. Characterization of the M918T sodium channel gene mutation associated with strong resistance to pyrethroid insecticides in the peach-potato aphid, *Myzus persicae* (Sulzer). *Bulletin of Entomological Research,* 98, 183-191.

ELLIOTT, M., FARNHAM, A. W., JANES, N. F., NEEDHAM, P. H., PULMAN, D. A. & STEVENSON, J. H. 1973. A Photostable Pyrethroid. *Nature,* 246, 169-170.

EPERON, I. C., MAKAROVA, O. V., MAYEDA, A., MUNROE, S. H., CACERES, J. F., HAYWARD, D. G. & KRAINER, A. R. 2000. Selection of alternative 5' splice sites: role of U1 snRNP and models for the antagonistic effects of SF2/ASF and hnRNP A1. *Mol Cell Biol,* 20, 8303-18.

FAOSTAT. 2012. Available: http://faostat3.fao.org/download/Q/QC/E [Accessed 24/09/15].

FAOSTAT. 2013. Available: http://faostat3.fao.org/download/Q/QC/E [Accessed 06/09/2015].

FAOSTAT. 2015. Available: http://faostat3.fao.org/browse/D/*/E [Accessed 08/05/2015] .

FEYEREISEN, R. 1995. Molecular biology of insecticide resistance. *Toxicology Letters,* 82-3, 83-90.

FEYEREISEN, R. 2011. Arthropod CYPomes illustrate the tempo and mode in P450 evolution. *Biochimica et Biophysica Acta (BBA) - Proteins & Proteomics,* 1814, 19-28.

FFRENCH-CONSTANT, R. H., ROCHELEAU, T. A., STEICHEN, J. C. & CHALMERS, A. E. 1993. A point mutation in a *Drosophila* GABA receptor confers insecticide resistance. *Nature,* 363, 449-451.

FIELD, L. M., DEVONSHIRE, A. L. & FORDE, B. G. 1988. Molecular evidence that insecticide resistance in peach-potato aphids (*Myzus persicae* Sulz.) results from amplification of an esterase gene. *Biochemical Journal,* 251, 309-312.

FIGUEIREDO, M. D. C., CRUZ, I., DA SILVA, R. B. & FOSTER, J. E. 2015. Biological control with *Trichogramma pretiosum* increases organic maize productivity by 19.4%. *Agronomy for Sustainable Development,* 35, 1175-1183.

FINNEY, D. J. 1947. *Probit analysis; a statistical treatment of the sigmoid response curve.,* Oxford, England, Macmillan.

FOSTER, S. P., DENHOLM, I. & DEVONSHIRE, A. L. 2000. The ups and downs of insecticide resistance in peach-potato aphids (*Myzus persicae*) in the UK. *Crop Protection,* 19, 873-879.

FOSTER, S. P., DENHOLM, I., POPPY, G. M., THOMPSON, R. & POWELL, W. 2011. Fitness trade-off in peach-potato aphids (*Myzus persicae*) between insecticide resistance and vulnerability to parasitoid attack at several spatial scales. *Bulletin of Entomological Research,* 101, 659-666.

FOSTER, S. P., WOODCOCK, C. M., WILLIAMSON, M. S., DEVONSHIRE, A. L., DENHOLM, I. & THOMPSON, R. 1999. Reduced alarm response by peach–potato aphids, *Myzus persicae* (Hemiptera: Aphididae), with knock-down resistance to insecticides (kdr)may impose a fitness cost through increased vulnerability to natural enemies. *Bulletin of Entomological Research,* 89, 133-138.

GARZIA, G. S., GAETONO, S., BIONDI, A. & ZAPPALA, L. Biology, distribution and damage of *Tuta absoluta* an exotic invasive pest from South America. EPPO/IOBC/FAO/NEPPO Joint International Symposium on management of *Tuta absoluta*, 16-18th November 2011 Agadir, Morocco.

GODFRAY, H. C. J., BEDDINGTON, J. R., CRUTE, I. R., HADDAD, L., LAWRENCE, D., MUIR, J. F., PRETTY, J., ROBINSON, S., THOMAS, S. M. & TOULMIN, C. 2010. Food Security: The Challenge of Feeding 9 Billion People. *Science,* 327, 812-818.

GOECKS, J., NEKRUTENKO, A., TAYLOR, J. & GALAXY, T. 2010. Galaxy: a comprehensive approach for supporting accessible, reproducible, and transparent computational research in the life sciences. *Genome Biology,* 11.

GONTIJO, P. C., PICANÇO, M. C., PEREIRA, E. J. G., MARTINS, J. C., CHEDIAK, M. & GUEDES, R. N. C. 2012. Spatial and temporal variation in the control failure likelihood of the tomato leaf miner, *Tuta absoluta*. *Annals of Applied Biology*, n/a-n/a.

GONZÁLEZ-CABRERA, J., MOLLÁ, O., MONTÓN, H. & URBANEJA, A. 2011. Efficacy of *Bacillus thuringiensis* (Berliner) in controlling the tomato borer, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae). *BioControl,* 56, 71-80.

GOODACRE, S. L. & MARTIN, O. Y. 2012. Modification of insect and arachnid behaviours by vertically transmitted endosymbionts: infections as drivers of behavioural change and evolutionary novelty. *Insects,* 3**,** 246-261.

GRABHERR, M. G., HAAS, B. J., YASSOUR, M., LEVIN, J. Z., THOMPSON, D. A., AMIT, I., ADICONIS, X., FAN, L., RAYCHOWDHURY, R., ZENG, Q., CHEN, Z., MAUCELI, E., HACOHEN, N., GNIRKE, A., RHIND, N., DI PALMA, F., BIRREN, B. W., NUSBAUM, C., LINDBLAD-TOH, K., FRIEDMAN, N. & REGEV, A. 2011. Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nat Biotech,* 29**,** 644-652.

GRAUSO, M., REENAN, R. A., CULETTO, E. & SATTELLE, D. B. 2002. Novel putative nicotinic acetylcholine receptor subunit genes, Dα5, Dα6 and Dα7, in *Drosophila melanogaster* identify a new and highly conserved target of adenosine deaminase acting on RNA-mediated A-to-I Pre-mRNA editing. *Genetics,* 160**,** 1519-1533.

GUEDES, R. N. C. & PICANÇO, M. C. 2012. The tomato borer *Tuta absoluta* in South America: pest status, management and insecticide resistance. *EPPO Bulletin,* 42**,** 211-216.

GUILLEMAUD, T., BLIN, A., LE GOFF, I., DESNEUX, N., REYES, M., TABONE, E., TSAGKARAKOU, A., NIÑO, L. & LOMBAERT, E. 2015. The tomato borer, *Tuta absoluta*, invading the Mediterranean Basin, originates from a single introduction from Central Chile. *Sci. Rep.,* 5.

GUO, Z., KANG, S., ZHU, X., XIA, J., WU, Q., WANG, S., XIE, W. & ZHANG, Y. 2015. Down-regulation of a novel ABC transporter gene (Pxwhite) is associated with Cry1Ac resistance in the diamondback moth, *Plutella xylostella* (L.). *Insect Biochemistry and Molecular Biology,* 59**,** 30-40.

HAAS, B. J., PAPANICOLAOU, A., YASSOUR, M., GRABHERR, M., BLOOD, P. D., BOWDEN, J., COUGER, M. B., ECCLES, D., LI, B., LIEBER, M., MACMANES, M. D., OTT, M., ORVIS, J., POCHET, N., STROZZI, F., WEEKS, N., WESTERMAN, R., WILLIAM, T., DEWEY, C. N., HENSCHEL, R., LEDUC, R. D., FRIEDMAN, N. & REGEV, A. 2013. De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. *Nat Protoc,* 8**,** 1494-512.

HADDI, K. 2012. Studies on insecticide resistance in *Tuta absoluta* (Meyrick), with special emphasis on characterisation of two target site mechanisms., University of Catania.

HADDI, K., BERGER, M., BIELZA, P., CIFUENTES, D., FIELD, L. M., GORMAN, K., RAPISARDA, C., WILLIAMSON, M. S. & BASS, C. 2012. Identification of mutations associated with pyrethroid resistance in the voltage-gated sodium channel of the tomato leaf miner (*Tuta absoluta*). *Insect Biochemistry and Molecular Biology,* 42**,** 506-513.

HARDSTONE, M., LAZZARO, B. & SCOTT, J. 2009. The effect of three environmental conditions on the fitness of cytochrome P450 monooxygenase-mediated permethrin resistance in *Culex pipiens quinquefasciatus*. *Bmc Evolutionary Biology,* 9**,** 42.

HARDSTONE, M. C., LEICHTER, C., HARRINGTON, L. C., KASAI, S., TOMITA, T. & SCOTT, J. G. 2007. Cytochrome P450 monooxygenase-mediated permethrin resistance confers limited and larval specific cross-resistance in the southern house mosquito, *Culex pipiens quinquefasciatus*. *Pesticide Biochemistry and Physiology,* 89**,** 175-184.

HE, G., SUN, Y. & LI, F. 2012. RNA interference of two acetylcholinesterase genes in *Plutella Xylostella* reveals their different functions. *Archives of Insect Biochemistry and Physiology,* 79**,** 75-86.

HEDGES, L. M., BROWNLIE, J. C., O'NEILL, S. L. & JOHNSON, K. N. 2008. Wolbachia and virus protection in insects. *Science,* 322**,** 702.

HEGRENESS, M., SHORESH, N., DAMIAN, D., HARTL, D. & KISHONY, R. 2008. Accelerated evolution of resistance in multidrug environments. *Proceedings of the National Academy of Sciences,* 105**,** 13977-13981.

HEINRICHS, V. & BAKER, B. S. 1995. The *Drosophila* Sr Protein Rbp1 contributes to the regulation of doublesex alternative splicing by recognizing rbp1 RNA target sequences. *Embo Journal,* 14**,** 3987-4000.

HODGKIN, A. L. & HUXLEY, A. F. 1952. A quantitative description of membrane current and its application to conduction and excitation in nerve. *The Journal of Physiology,* 117**,** 500-544.

HOGENHOUT, S. A., AMMAR, E. D., WHITFIELD, A. E. & REDINBAUGH, M. G. 2008. Insect vector interactions with persistently transmitted viruses. *Annual Review of Phytopathology,* 46**,** 327-359.

HØJLAND, D. Pyrethroid monitoring of cabbage stem flea beetles in Europe. Resistance 2015 Rothamsted Research.

HOLT, R. A., SUBRAMANIAN, G. M., HALPERN, A., SUTTON, G. G., CHARLAB, R., NUSSKERN, D. R., WINCKER, P., CLARK, A. G., RIBEIRO, J. M. C., WIDES, R., SALZBERG, S. L., LOFTUS, B., YANDELL, M., MAJOROS, W. H., RUSCH, D. B., LAI, Z. W., KRAFT, C. L., ABRIL, J. F., ANTHOUARD, V., ARENSBURGER, P., ATKINSON, P. W., BADEN, H., DE BERARDINIS, V., BALDWIN, D., BENES, V., BIEDLER, J., BLASS, C., BOLANOS, R., BOSCUS, D., BARNSTEAD, M., CAI, S., CENTER, A., CHATUVERDI, K., CHRISTOPHIDES, G. K., CHRYSTAL, M. A., CLAMP, M., CRAVCHIK, A., CURWEN, V., DANA, A., DELCHER, A., DEW, I., EVANS, C. A., FLANIGAN, M., GRUNDSCHOBER-FREIMOSER, A., FRIEDLI, L., GU, Z. P., GUAN, P., GUIGO, R., HILLENMEYER, M. E., HLADUN, S. L., HOGAN, J. R., HONG, Y. S., HOOVER, J., JAILLON, O., KE, Z. X., KODIRA, C., KOKOZA, E., KOUTSOS, A., LETUNIC, I., LEVITSKY, A., LIANG, Y., LIN, J. J., LOBO, N. F., LOPEZ, J. R., MALEK, J. A., MCINTOSH, T. C., MEISTER, S., MILLER, J., MOBARRY, C., MONGIN, E., MURPHY, S. D., O'BROCHTA, D. A., PFANNKOCH, C., QI, R., REGIER, M. A., REMINGTON, K., SHAO, H. G., SHARAKHOVA, M. V., SITTER, C. D., SHETTY, J., SMITH, T. J., STRONG, R., SUN, J. T., THOMASOVA, D., TON, L. Q., TOPALIS, P., TU, Z. J., UNGER, M. F., WALENZ, B., WANG, A. H., WANG, J., WANG, M., WANG, X. L., WOODFORD, K. J., WORTMAN, J. R., WU, M., YAO, A., ZDOBNOV, E. M., ZHANG, H. Y., ZHAO, Q., et al. 2002. The genome sequence of the malaria mosquito *Anopheles gambiae*. *Science,* 298**,** 129-149.

HOPKINS, B. W. & PIETRANTONIO, P. V. 2010. The *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) voltage-gated sodium channel and mutations associated with pyrethroid resistance in field-collected adult males. *Insect Biochemistry and Molecular Biology,* 40**,** 385-393.

HOWARD, J. M. & SANFORD, J. R. 2015. The RNAissance family: SR proteins as multifaceted regulators of gene expression. *Wiley Interdisciplinary Reviews: RNA,* 6**,** 93-110.

HOWLETT, P. 2013. *Tomato: Phase 4 of the development of a robust IPM programme for Tuta absoluta* [Online]. Available: http://horticulture.ahdb.org.uk/project/tomato-phase-4-development-robust-ipm-programme-tuta-absoluta.

HSU, J.-C., FENG, H.-T., WU, W.-J., GEIB, S. M., MAO, C.-H. & VONTAS, J. 2012. Truncated transcripts of nicotinic acetylcholine subunit gene Bdα6 are associated with spinosad resistance in *Bactrocera dorsalis*. *Insect Biochemistry and Molecular Biology,* 42**,** 806-815.

HUANG, H.-S., HU, N.-T., YAO, Y.-E., WU, C.-Y., CHIANG, S.-W. & SUN, C.-N. 1998. Molecular cloning and heterologous expression of a glutathione S-transferase involved in insecticide resistance from the diamondback moth, *Plutella xylostella*. *Insect Biochemistry and Molecular Biology,* 28**,** 651-658.

IAGC, International Aphid Genome Consortium 2010. Genome sequence of the pea aphid *Acyrthosiphon pisum*. *PLoS biology,* 8**,** e1000313.

IBRAHIM, E. C., SCHAAL, T. D., HERTEL, K. J., REED, R. & MANIATIS, T. 2005. Serine/arginine-rich protein-dependent suppression of exon skipping by exonic splicing enhancers. *Proceedings of the National Academy of Sciences of the United States of America,* 102**,** 5002-5007.

IRAC 2011. *Tuta absoluta*, the tomato leafminer or tomato borer, recommendations for sustainable and effective resistance management.

ISGC, International Silkworm Genome Consortium 2008. The genome of a lepidopteran model insect, the silkworm *Bombyx mori*. *Insect Biochemistry and Molecular Biology,* 38**,** 1036-1045.

JIN, Y., TIAN, N., CAO, J., LIANG, J., YANG, Z. & LV, J. 2007. RNA editing and alternative splicing of the insect nAChR subunit alpha6 transcript: evolutionary conservation, divergence and regulation. *Bmc Evolutionary Biology,* 7**,** 98.

JONES, A. K., GRAUSO, M. & SATTELLE, D. B. 2005. The nicotinic acetylcholine receptor gene family of the malaria mosquito, *Anopheles gambiae*. *Genomics,* 85**,** 176-187.

JONES, A. K., RAYMOND-DELPECH, V., THANY, S. H., GAUTHIER, M. & SATTELLE, D. B. 2006. The nicotinic acetylcholine receptor gene family of the honey bee, *Apis mellifera*. *Genome Research,* 16**,** 1422-1430.

JONES, C. M., DANIELS, M., ANDREWS, M., SLATER, R., LIND, R. J., GORMAN, K., WILLIAMSON, M. S. & DENHOLM, I. 2011. Age-specific expression of a P450 monooxygenase (CYP6CM1) correlates with neonicotinoid resistance in *Bemisia tabaci*. *Pesticide Biochemistry and Physiology,* 101**,** 53-58.

JONES, D. G. 1998. *Piperonyl butoxide*, Academic Press.

JONES, F. G. W. & JONES, M. G. 1984. *Pests of Field Crops*, Edward Arnold Ltd. .

JOUSSEN, N., HECKEL, D. G., HAAS, M., SCHUPHAN, I. & SCHMIDT, B. 2008. Metabolism of imidacloprid and DDT by P450 GYP6G1 expressed in cell cultures of *Nicotiana tabacum* suggests detoxification of these insecticides in Cyp6g1-overexpressing strains of *Drosophila melanogaster,* leading to resistance. *Pest Management Science,* 64**,** 65-73.

KAKANI, E. G., ZYGOURIDIS, N. E., TSOUMANI, K. T., SERAPHIDES, N., ZALOM, F. G. & MATHIOPOULOS, K. D. 2010. Spinosad resistance development in wild olive fruit fly *Bactrocera oleae* (Diptera: Tephritidae) populations in California. *Pest Management Science,* 66**,** 447-453.

KARATOLOS, N., GORMAN, K., WILLIAMSON, M. S. & DENHOLM, I. 2012a. Mutations in the sodium channel associated with pyrethroid resistance in the greenhouse whitefly, *Trialeurodes vaporariorum*. *Pest Management Science,* 68**,** 834-838.

KARATOLOS, N., PAUCHET, Y., WILKINSON, P., CHAUHAN, R., DENHOLM, I., GORMAN, K., NELSON, D., BASS, C., FFRENCH-CONSTANT, R. & WILLIAMSON, M. 2011. Pyrosequencing the transcriptome of the greenhouse whitefly, *Trialeurodes vaporariorum* reveals multiple transcripts encoding insecticide targets and detoxifying enzymes. *Bmc Genomics,* 12**,** 56.

KARATOLOS, N., WILLIAMSON, M. S., DENHOLM, I., GORMAN, K., FFRENCH-CONSTANT, R. H. & BASS, C. 2012b. Over-expression of a Cytochrome P450 Is associated with resistance to pyriproxyfen in the greenhouse whitefly *Trialeurodes vaporariorum*. *Plos One,* 7**,** e31077.

KARUNKER, I., BENTING, J., LUEKE, B., PONGE, T., NAUEN, R., RODITAKIS, E., VONTAS, J., GORMAN, K., DENHOLM, I. & MORIN, S. 2008. Over-expression of cytochrome P450 *CYP6CM1* is associated with high resistance to imidacloprid in the B and Q biotypes of *Bemisia tabaci* (Hemiptera : Aleyrodidae). *Insect Biochemistry and Molecular Biology,* 38**,** 634-644.

KEELING, C. I., HENDERSON, H., LI, M., YUEN, M., CLARK, E. L., FRASER, J. D., HUBER, D. P. W., LIAO, N. Y., RODERICK DOCKING, T., BIROL, I., CHAN, S. K., TAYLOR, G. A., PALMQUIST, D., JONES, S. J. M. & BOHLMANN, J. 2012. Transcriptome and full-length cDNA resources for the mountain pine beetle, *Dendroctonus ponderosae* Hopkins, a major insect pest of pine forests. *Insect Biochemistry and Molecular Biology,* 42**,** 525-536.

KEREN, H., LEV-MAOR, G. & AST, G. 2010. Alternative splicing and evolution: diversification, exon definition and function. *Nat Rev Genet,* 11**,** 345-355.

KIBBE, W. A. 2007. OligoCalc: an online oligonucleotide properties calculator. *Nucleic Acids Research,* 35**,** W43-W46.

KOL, G., LEV-MAOR, G. & AST, G. 2005. Human-mouse comparative analysis reveals that branch-site plasticity contributes to splicing regulation. *Human Molecular Genetics,* 14**,** 1559-1568.

KONO, Y. & TOMITA, T. 2006. Amino acid substitutions conferring insecticide insensitivity in Ace-paralogous acetylcholinesterase. *Pesticide Biochemistry and Physiology,* 85**,** 123-132.

KUMAR, S. & LOPEZ, A. J. 2005. Negative feedback regulation among SR splicing factors encoded by Rbp1 and Rbp1-like in *Drosophila*. *The EMBO Journal,* 24**,** 2646-2655.

LABBÉ, P., BERTICAT, C., BERTHOMIEU, A., UNAL, S., BERNARD, C., WEILL, M. & LENORMAND, T. 2007. Forty years of erratic insecticide resistance evolution in the mosquito *Culex pipiens*. *Plos Genetics,* 3**,** e205.

LANGMEAD, B., TRAPNELL, C., POP, M. & SALZBERG, S. L. 2009. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biology,* 10**,** 1-10.

LANSDELL, S. J. & MILLAR, N. S. 2004. Molecular characterization of Dα6 and Dα7 nicotinic acetylcholine receptor subunits from Drosophila: formation of a high-affinity α-bungarotoxin binding site revealed by expression of subunit chimeras. *Journal of Neurochemistry,* 90**,** 479-489.

LEE, D.-W., CHOI, J. Y., KIM, W. T., JE, Y. H., SONG, J. T., CHUNG, B. K., BOO, K. S. & KOH, Y. H. 2007. Mutations of acetylcholinesterase1 contribute to prothiofos-resistance in *Plutella xylostella* (L.). *Biochemical and Biophysical Research Communications,* 353**,** 591-597.

LEE, J. H., HORAK, C. E., KHANNA, C., MENG, Z., YU, L. R., VEENSTRA, T. D. & STEEG, P. S. 2008. Alterations in Gemin5 Expression Contribute to Alternative mRNA Splicing Patterns and Tumor Cell Motility. *Cancer Research,* 68**,** 639-644.

LEE, S. H., KANG, J. S., MIN, J. S., YOON, K. S., STRYCHARZ, J. P., JOHNSON, R., MITTAPALLI, O., MARGAM, V. M., SUN, W., LI, H. M., XIE, J., WU, J., KIRKNESS, E. F., BERENBAUM, M. R., PITTENDRIGH, B. R. & CLARK, J. M. 2010. Decreased detoxification genes and genome size make the human body louse an efficient model to study xenobiotic metabolism. *Insect Molecular Biology,* 19**,** 599-615.

LEE, Y., GAMAZON, E. R., REBMAN, E., LEE, Y., LEE, S., DOLAN, M. E., COX, N. J. & LUSSIER, Y. A. 2012. Variants Affecting Exon Skipping Contribute to Complex Traits. *PLoS Genet,* 8**,** e1002998.

LI, B. & DEWEY, C. N. 2011. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics,* 12.

LI, X., SCHULER, M. A. & BERENBAUM, M. R. 2007. Molecular mechanisms of metabolic resistance to synthetic and natural xenobiotics. *Annual Review of Entomology,* 52**,** 231-253.

LIETTI, M. M. M., BOTTO, E. & ALZOGARAY, R. A. 2005. Insecticide resistance in Argentine populations of *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae). *Neotropical Entomology,* 34**,** 113-119.

LIU, F., SHI, X., LIANG, Y., WU, Q., XU, B., XIE, W., WANG, S., ZHANG, Y. & LIU, N. 2014. A 36-bp deletion in the alpha subunit of glutamate-gated chloride channel contributes to abamectin resistance in *Plutella xylostella*. *Entomologia Experimentalis et Applicata,* 153**,** 85-92.

LIU, N. & PRIDGEON, J. W. 2002. Metabolic detoxication and the kdr mutation in pyrethroid resistant house flies, *Musca domestica* (L.). *Pesticide Biochemistry and Physiology,* 73**,** 157-163.

LIU, Z. W. & HAN, Z. J. 2006. Fitness costs of laboratory-selected imidacloprid resistance in the brown planthopper, *Nilaparvata lugens* Stal. *Pest Management Science,* 62**,** 279-282.

LIU, Z. W., WILLIAMSON, M. S., LANSDELL, S. J., DENHOLM, I., HAN, Z. J. & MILLAR, N. S. 2005. A nicotinic acetylcholine receptor mutation conferring target-site resistance to imidacloprid in *Nilaparvata lugens* (brown planthopper). *Proceedings of the National Academy of Sciences of the United States of America,* 102**,** 8420-8425.

LIVAK, K. J. & SCHMITTGEN, T. D. 2001. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. *Methods,* 25**,** 402-408.

LOBELL, D. B., BURKE, M. B., TEBALDI, C., MASTRANDREA, M. D., FALCON, W. P. & NAYLOR, R. L. 2008. Prioritizing climate change adaptation needs for food security in 2030. *Science,* 319**,** 607-610.

LOUGHNEY, K., KREBER, R. & GANETZKY, B. 1989. Molecular analysis of the *para* locus, a sodium channel gene in *Drosophila*. *Cell,* 58**,** 1143-1154.

LOVE, M. I., HUBER, W. & ANDERS, S. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology,* 15**,** 1-21.

LUMJUAN, N., MCCARROLL, L., PRAPANTHADARA, L.-A., HEMINGWAY, J. & RANSON, H. 2005. Elevated activity of an Epsilon class glutathione transferase confers DDT resistance in the dengue vector, *Aedes aegypti*. *Insect Biochemistry and Molecular Biology,* 35**,** 861-871.

LYKO, F., FORET, S., KUCHARSKI, R., WOLF, S., FALCKENHAYN, C. & MALESZKA, R. 2010. The honey bee epigenomes: differential methylation of brain dna in queens and workers. *PLoS Biol,* 8**,** e1000506.

MAMIDALA, P., WIJERATNE, A. J., WIJERATNE, S., KORNACKER, K., SUDHAMALLA, B., RIVERA-VEGA, L. J., HOELMER, A., MEULIA, T., JONES, S. C. & MITTAPALLI, O. 2012. RNA-Seq and molecular docking reveal multi-level pesticide resistance in the bed bug. *Bmc Genomics,* 13.

MARGULIES, M., EGHOLM, M., ALTMAN, W. E., ATTIYA, S., BADER, J. S., BEMBEN, L. A., BERKA, J., BRAVERMAN, M. S., CHEN, Y. J., CHEN, Z., DEWELL, S. B., DU, L., FIERRO, J. M., GOMES, X. V., GODWIN, B. C., HE, W., HELGESEN, S., HO, C. H., IRZYK, G. P., JANDO, S. C., ALENQUER, M. L., JARVIE, T. P., JIRAGE, K. B., KIM, J. B., KNIGHT, J. R., LANZA, J. R., LEAMON, J. H., LEFKOWITZ, S. M., LEI, M., LI, J., LOHMAN, K. L., LU, H., MAKHIJANI, V. B., MCDADE, K. E., MCKENNA, M. P., MYERS, E. W., NICKERSON, E., NOBILE, J. R., PLANT, R., PUC, B. P., RONAN, M. T., ROTH, G. T., SARKIS, G. J., SIMONS, J. F., SIMPSON, J. W., SRINIVASAN, M., TARTARO, K. R., TOMASZ, A., VOGT, K. A., VOLKMER, G. A., WANG, S. H., WANG, Y., WEINER, M. P., YU, P., BEGLEY, R. F. & ROTHBERG, J. M. 2005. Genome sequencing in microfabricated high-density picolitre reactors. *Nature,* 437**,** 376-80.

MARTINEZ-TORRES, D., CHANDRE, F., WILLIAMSON, M. S., DARRIET, F., BERGE, J. B., DEVONSHIRE, A. L., GUILLET, P., PASTEUR, N. & PAURON, D. 1998. Molecular characterization of pyrethroid knockdown resistance (*kdr*) in the major malaria vector *Anopheles gambiae* s.s. *Insect Molecular Biology,* 7**,** 179-184.

MARTINEZ-TORRES, D., DEVONSHIRE, A. L. & WILLIAMSON, M. S. 1997. Molecular studies of knockdown resistance to pyrethroids: cloning of domain II sodium channel gene sequences from insects. *Pesticide Science,* 51**,** 265-270.

MENSAH, R. K. 1997. Local density responses of predatory insects of Helicoverpa spp. to a newly developed food supplement 'Envirofeast' in commercial cotton in Australia. *International Journal of Pest Management,* 43**,** 221-225.

MILLAR, N. S. 2003. Assembly and subunit diversity of nicotinic acetylcholine receptors. *Biochemical Society Transactions,* 31**,** 869-874.

MILLER, J. R., KOREN, S. & SUTTON, G. 2010. Assembly Algorithms for Next-Generation Sequencing Data. *Genomics,* 95**,** 315-327.

MILLIGAN, A. S., DALY, A., PARRY, M. A. J., LAZZERI, P. A. & JEPSON, I. 2001. The expression of a maize glutathione S-transferase gene in transgenic wheat confers herbicide tolerance, both in planta and in vitro. *Molecular Breeding,* 7**,** 301-315.

MITA, K., KASAHARA, M., SASAKI, S., NAGAYASU, Y., YAMADA, T., KANAMORI, H., NAMIKI, N., KITAGAWA, M., YAMASHITA, H., YASUKOCHI, Y., KADONO-OKUDA, K., YAMAMOTO, K., AJIMURA, M., RAVIKUMAR, G., SHIMOMURA, M., NAGAMURA, Y., SHIN-I, T., ABE, H., SHIMADA, T., MORISHITA, S. & SASAKI, T. 2004. The genome sequence of silkworm, *Bombyx mori*. *DNA Research,* 11**,** 27-35.

MIYAZAKI, M., OHYAMA, K., DUNLAP, D. Y. & MATSUMURA, F. 1996. Cloning and sequencing of the *para*-type sodium channel gene from susceptible and *kdr*-resistant German cockroaches (*Blattella germanica*) and house fly (*Musca domestica*). *Molecular and General Genetics,* 252**,** 61-68.

MIYAZAWA, A., FUJIYOSHI, Y. & UNWIN, N. 2003. Structure and gating mechanism of the acetylcholine receptor pore. *Nature,* 423**,** 949-955.

MOLLÁ, O., GONZÁLEZ-CABRERA, J. & URBANEJA, A. 2011. The combined use of *Bacillus thuringiensis* and *Nesidiocoris tenuis* against the tomato borer *Tuta absoluta*. *BioControl,* 56**,** 883-891.

MORGAN, J. A., CORLEY, S. W., JACKSON, L. A., LEW-TABOR, A. E., MOOLHUIJZEN, P. M. & JONSSON, N. N. 2009. Identification of a mutation in the para sodium channel gene of the cattle tick *Rhipicephalus* (Boophilus) microplus associated with resistance to synthetic pyrethroid acaricides. *International Journal for Parasitology*.

MORIN, S., WILLIAMSON, M. S., GOODSON, S. J., BROWN, J. K., TABASHNIK, B. E. & DENNEHY, T. J. 2002. Mutations in the *Bemisia tabaci para* sodium channel gene associated with resistance to a pyrethroid plus organophosphate mixture. *Insect Biochemistry and Molecular Biology,* 32**,** 1781-1791.

NAUEN, R., ELBERT, A., MCCAFFERY, A., SLATER, R. & SPARKS, T. C. 2012. IRAC: Insecticide Resistance, and Mode of Action Classification of Insecticides. *Modern Crop Protection Compounds.* Wiley-VCH Verlag GmbH & Co. KGaA.

NAVOT, N., PICHERSKY, E., ZEIDAN, M., ZAMIR, D. & CZOSNEK, H. 1991. Tomato yellow leaf curl virus: A whitefly-transmitted geminivirus with a single genomic component. *Virology,* 185**,** 151-161.

NYONI, B. N., GORMAN, K., MZILAHOWA, T., WILLIAMSON, M. S., NAVAJAS, M., FIELD, L. M. & BASS, C. 2011. Pyrethroid resistance in the tomato red spider mite, *Tetranychus evansi*, is associated with mutation of the para-type sodium channel. *Pest Management Science,* 67**,** 891-897.

O'REILLY, A. O., KHAMBAY, B. P. S., WILLIAMSON, M. S., FIELD, L. M., WALLACE, B. A. & DAVIES, T. G. E. 2006. Modelling insecticide-binding sites in the voltage-gated sodium channel. *Biochem J,* 396**,** 255-263.

OERKE, E.-C. 2006. Crop losses to pests. *The Journal of Agricultural Science,* 144**,** 31-43.

PAN, G., XU, J., LI, T., XIA, Q., LIU, S.-L., ZHANG, G., LI, S., LI, C., LIU, H., YANG, L., LIU, T., ZHANG, X., WU, Z., FAN, W., DANG, X., XIANG, H., TAO, M., LI, Y., HU, J., LI, Z., LIN, L., LUO, J., GENG, L., WANG, L., LONG, M., WAN, Y., HE, N., ZHANG, Z., LU, C., KEELING, P. J., WANG, J., XIANG, Z. & ZHOU, Z. 2013. Comparative genomics of parasitic silkworm microsporidia reveal an association between genome expansion and host adaptation. *BMC Genomics,* 14**,** 186-186.

PASCUAL, L., JAKUBOWSKA, A. K., BLANCA, J. M., CAÑIZARES, J., FERRÉ, J., GLOECKNER, G., VOGEL, H. & HERRERO, S. 2012. The transcriptome of *Spodoptera exigua* larvae exposed to different types of microbes. *Insect Biochemistry and Molecular Biology,* 42**,** 557-570.

PAYANDEH, J., SCHEUER, T., ZHENG, N. & CATTERALL, W. A. 2011. The crystal structure of a voltage-gated sodium channel. *Nature,* 475**,** 353-358.

PAYNE, R. W., HARDING, S. A., MURRAY, D. A., SOUTAT, D. M., BAIRD, D. B., GLASER, A. I., WELHAM, S. J., GILMOUR, A. R., THOMPSON, R. & WEBSTER, R. 2011. *The Guide to GenStat Release 14, Part 2: statistics.,* Hemel Hempstead UK, VSN International Ltd.

PEREYRA, P. C. & SÁNCHEZ, N. E. 2006. Effect of two solanaceous plants on developmental and population parameters of the tomato leaf miner, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae). *Neotropical Entomology,* 35**,** 671-676.

PERRY, T., CHAN, J. Q., BATTERHAM, P., WATSON, G. B., GENG, C. & SPARKS, T. C. 2012. Effects of mutations in *Drosophila* nicotinic acetylcholine receptor subunits on sensitivity to insecticides targeting nicotinic acetylcholine receptors. *Pesticide Biochemistry and Physiology,* 102**,** 56-60.

PERRY, T., MCKENZIE, J. A. & BATTERHAM, P. 2007. A knockout strain of *Drosophila melanogaster* confers a high level of resistance to spinosad. *Insect Biochemistry and Molecular Biology,* 37**,** 184-188.

PUINEAN, A. M., FOSTER, S. P., OLIPHANT, L., DENHOLM, I., FIELD, L. M., MILLAR, N. S., WILLIAMSON, M. S. & BASS, C. 2010. Amplification of a cytochrome P450 gene is associated with resistance to neonicotinoid insecticides in the aphid *Myzus persicae*. *PLoS genetics,* 6**,** e1000999.

PUINEAN, A. M., LANSDELL, S. J., COLLINS, T., BIELZA, P. & MILLAR, N. S. 2012. A nicotinic acetylcholine receptor transmembrane point mutation (G275E) associated with resistance to spinosad in *Frankliniella occidentalis*. *Journal of Neurochemistry***,** n/a-n/a.

RANSON, H., JENSEN, B., VULULE, J. M., WANG, X., HEMINGWAY, J. & COLLINS, F. H. 2000. Identification of a point mutation in the voltage-gated sodium channel gene of Kenyan *Anopheles gambiae* associated with resistance to DDT and pyrethroids. *Insect Molecular Biology,* 9**,** 491-497.

REYES, M., ROCHA, K., ALARCÓN, L., SIEGWART, M. & SAUPHANOR, B. 2012. Metabolic mechanisms involved in the resistance of field populations of *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) to spinosad. *Pesticide Biochemistry and Physiology,* 102**,** 45-50.

RINKEVICH, F., CHEN, M., SHELTON, A. & SCOTT, J. 2010. Transcripts of the nicotinic acetylcholine receptor subunit gene *Pxylα6* with premature stop codons are associated with spinosad resistance in diamondback moth, *Plutella xylostella*. *Invertebrate Neuroscience,* 10**,** 25-33.

RINKEVICH, F. D., DU, Y. & DONG, K. 2013. Diversity and convergence of sodium channel mutations involved in resistance to pyrethroids. *Pesticide Biochemistry and Physiology,* 106**,** 93-100.

RINKEVICH, F. D. & SCOTT, J. G. 2009. Transcriptional diversity and allelic variation in nicotinic acetylcholine receptor subunits of the red flour beetle, *Tribolium castaneum*. *Insect Molecular Biology,* 18**,** 233-242.

RINKEVICH, F. D. & SCOTT, J. G. 2013. Limitations of RNAi of α6 nicotinic acetylcholine receptor subunits for assessing the in vivo sensitivity to spinosad. *Insect Science,* 20**,** 101-108.

RIVERON, J. M., IRVING, H., NDULA, M., BARNES, K. G., IBRAHIM, S. S., PAINE, M. J. I. & WONDJI, C. S. 2013. Directionally selected cytochrome P450 alleles are driving the spread of pyrethroid resistance in the major malaria vector *Anopheles funestus*. *Proceedings of the National Academy of Sciences,* 110**,** 252-257.

ROBINSON, M. D., MCCARTHY, D. J. & SMYTH, G. K. 2010. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics,* 26**,** 139-140.

RODITAKIS, E. Determination of baseline toxicity of insecticides to *Tuta absoluta*. EPPO/IOBC/FAO/NEPPO Joint International Symposium on management of Tuta absoluta, 16-18th November 2011 Agadir, Morocco.

RODITAKIS, E., SKARMOUTSOU, C. & STAURAKAKI, M. 2013a. Toxicity of insecticides to populations of tomato borer *Tuta absoluta* (Meyrick) from Greece. *Pest Management Science,* 69**,** 834-840.

RODITAKIS, E., SKARMOUTSOU, C., STAURAKAKI, M., DEL ROSARIO MARTÍNEZ-AGUIRRE, M., GARCÍA-VIDAL, L., BIELZA, P., HADDI, K., RAPISARDA, C., RISON, J.-L., BASSI, A. & TEIXEIRA, L. A. 2013b. Determination of baseline susceptibility of European populations of *Tuta absoluta* (Meyrick) to indoxacarb and chlorantraniliprole using a novel dip bioassay method. *Pest Management Science,* 69**,** 217-227.

RODITAKIS, E., VASAKIS, E., GRISPOU, M., STAVRAKAKI, M., NAUEN, R., GRAVOUIL, M. & BASSI, A. 2015. First report of *Tuta absoluta* resistance to diamide insecticides. *Journal of Pest Science,* 88**,** 9-16.

SARKISSIAN, M., WINNE, A. & LAFYATIS, R. 1996. The mammalian homolog of suppressor-of-white-apricot regulates alternative mRNA Splicing of CD45 Exon 4 and fibronectin IIICS. *Journal of Biological Chemistry,* 271**,** 31106-31114.

SATTELLE, D. B., JONES, A. K., SATTELLE, B. M., MATSUDA, K., REENAN, R. & BIGGIN, P. C. 2005. Edit, cut and paste in the nicotinic acetylcholine receptor gene family of *Drosophila melanogaster*. *BioEssays,* 27**,** 366-376.

SAWICKI, R. M. 1962. Insecticidal activity of pyrethrum extract and its four insecticidal constituents against house flies. V.—knock-down activity of the four constituents with piperonyl butoxide. *Journal of the Science of Food and Agriculture,* 13**,** 591-598.

SCHULER, T. H., MARTINEZ-TORRES, D., THOMPSON, A. J., DENHOLM, I., DEVONSHIRE, A. L., DUCE, I. R. & WILLIAMSON, M. S. 1998. Toxicological, electrophysiological, and molecular characterisation of knockdown resistance to pyrethroid insecticides in the diamondback moth, *Plutella xylostella*(L.). *Pesticide Biochemistry and Physiology,* 59**,** 169-182.

SCHWARTZ, S. H., SILVA, J., BURSTEIN, D., PUPKO, T., EYRAS, E. & AST, G. 2008. Large-scale comparative analysis of splicing signals and their corresponding splicing factors in eukaryotes. *Genome Research,* 18**,** 88-103.

SHAO, Y.-M., DONG, K., TANG, Z.-H. & ZHANG, C.-X. 2009. Molecular characterization of a sodium channel gene from the Silkworm *Bombyx mori*. *Insect Biochemistry and Molecular Biology,* 39**,** 145-151.

SHAO, Y.-M., DONG, K. & ZHANG, C.-X. 2007. The nicotinic acetylcholine receptor gene family of the silkworm, *Bombyx mori*. *Bmc Genomics,* 8**,** 324.

SILVA, A. X., JANDER, G., SAMANIEGO, H., RAMSEY, J. S. & FIGUEROA, C. C. 2012. insecticide resistance mechanisms in the green peach aphid *Myzus persicae* (Hemiptera: Aphididae) I: A Transcriptomic Survey. *Plos One,* 7**,** e36366.

SILVA, G. A., PICANCO, M. C., BACCI, L., CRESPO, A. L., ROSADO, J. F. & GUEDES, R. N. 2011. Control failure likelihood and spatial dependence of insecticide resistance in the tomato pinworm, *Tuta absoluta*. *Pest management science,* 67**,** 913-20.

SILVA, W. M., BERGER, M., BASS, C., BALBINO, V. Q., AMARAL, M. H. P., CAMPOS, M. R. & SIQUEIRA, H. A. A. 2015. Status of pyrethroid resistance and mechanisms in Brazilian populations of *Tuta absoluta*. *Pesticide Biochemistry and Physiology*.

SIQUEIRA, H. A. A., GUEDES, R. N. C., FRAGOSO, D. B. & MAGALHAES, L. C. 2001. Abamectin resistance and synergism in Brazilian populations of *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae). *International Journal of Pest Management,* 47**,** 247-251.

SIQUEIRA, H. A. A., GUEDES, R. N. C. & PICANÇO, M. C. 2000a. Cartap resistance and synergism in populations of *Tuta absoluta* (Lep., Gelechiidae). *Journal of Applied Entomology,* 124**,** 233-238.

SIQUEIRA, H. Á. A., GUEDES, R. N. C. & PICANÇO, M. C. 2000b. Insecticide resistance in populations of *Tuta absoluta* (Lepidoptera: Gelechiidae). *Agricultural and Forest Entomology,* 2**,** 147-153.

SMITH, C. W. J. & VALCÁRCEL, J. 2000. Alternative pre-mRNA splicing: the logic of combinatorial control. *Trends in Biochemical Sciences,* 25**,** 381-388.

SMITH, T. J., HYEOCK LEE, S., INGLES, P. J., KNIPPLE, D. C. & M. SODERLUND, D. 1997. The L1014F Point Mutation in the house fly vssc1 sodium channel confers knockdown resistance to pyrethroids. *Insect Biochemistry and Molecular Biology,* 27**,** 807-812.

SOCIETY, THE ROYAL 2009. Reaping the benefits: science and the sustainable intensification of agriculture.

SONODA, S., IGAKI, C., ASHFAQ, M. & TSUMUKI, H. 2006. Pyrethroid-resistant diamondback moth expresses alternatively spliced sodium channel transcripts with and without T929I mutation. *Insect Biochemistry and Molecular Biology,* 36**,** 904-910.

SONODA, S., IGAKI, C. & TSUMUKI, H. 2008a. Alternatively spliced sodium channel transcripts expressed in field strains of the diamondback moth. *Insect Biochemistry and Molecular Biology,* 38**,** 883-890.

SONODA, S., TSUKAHARA, Y., ASHFAQ, M. & TSUMUKI, H. 2008b. Genomic organization of the para-sodium channel α-subunit genes from the pyrethroid-resistant and -susceptible strains of the diamondback moth. *Archives of Insect Biochemistry and Physiology,* 69**,** 1-12.

SPARKS, T. C. 2013. Insecticide discovery: An evaluation and analysis. *Pesticide Biochemistry and Physiology,* 107**,** 8-17.

STRODE, C., WONDJI, C. S., DAVID, J. P., HAWKES, N. J., LUMJUAN, N., NELSON, D. R., DRANE, D. R., KARUNARATNE, S., HEMINGWAY, J., BLACK, W. C. & RANSON, H. 2008. Genomic analysis of detoxification genes in the mosquito *Aedes aegypti*. *Insect Biochemistry and Molecular Biology,* 38**,** 113-123.

TANG, Z. Z., SHARMA, S., ZHENG, S., CHAWLA, G., NIKOLIC, J. & BLACK, D. L. 2011. Regulation of the mutually exclusive exons 8a and 8 in the CaV1.2 Calcium channel transcript by polypyrimidine tract-binding protein. *Journal of Biological Chemistry,* 286**,** 10007-10016.

TGSC, Tribolium Genome Sequencing Consortium. 2008. The genome of the model beetle and pest *Tribolium castaneum*. *Nature,* 452**,** 949-955.

THGC, The Heliconius Genome Consortium . 2012. Butterfly genome reveals promiscuous exchange of mimicry adaptations among species. *Nature,* 487**,** 94-98.

THOMPSON, G. D., DUTTON, R. & SPARKS, T. C. 2000. Spinosad – a case study: an example from a natural products discovery programme. *Pest Management Science,* 56**,** 696-702.

TODA, S. & MORISHITA, M. 2009. Identification of three point mutations on the sodium channel gene in pyrethroid-resistant *Thrips tabaci* (Thysanoptera: Thripidae). *Journal of Economic Entomology* 102, 2296-2300.

TOMIZAWA, M. & CASIDA, J. E. 2001. Structure and diversity of insect nicotinic acetylcholine receptors. *Pest Management Science,* 57**,** 914-922.

ULE, J., ULE, A., SPENCER, J., WILLIAMS, A., HU, J. S., CLINE, M., WANG, H., CLARK, T., FRASER, C., RUGGIU, M., ZEEBERG, B. R., KANE, D., WEINSTEIN, J. N., BLUME, J. & DARNELL, R. B. 2005. Nova regulates brain-specific splicing to shape the synapse. *Nature Genetics,* 37**,** 844-852.

UNTERGASSER, A., CUTCUTACHE, I., KORESSAAR, T., YE, J., FAIRCLOTH, B. C., REMM, M. & ROZEN, S. G. 2012. Primer3--new capabilities and interfaces. *Nucleic Acids Res,* 40**,** e115.

URBANEJA, A., DESNEUX, N., GABARRA, R., ARNÓ, J., GONZÁLEZ-CABRERA, J., MAFRA-NETO, A., STOLTMAN, L., PINTO, A. D. S. & PARRA, J. R. P. 2013.

Biology, ecology, and management of the South American Tomato Pinworm, *Tuta absoluta*. *In:* PEÑA, J. (ed.) *Potential Invasive Pests of Agricultural Crops.* Wallingford: CAB International.

URBANEJA, A., GONZÁLEZ-CABRERA, J., ARNÓ, J. & GABARRA, R. 2012. Prospects for the biological control of *Tuta absoluta* in tomatoes of the Mediterranean basin. *Pest Management Science,* 68**,** 1215-1222.

URBANEJA, A., MONTÓN, H. & MOLLÁ, O. 2009. Suitability of the tomato borer *Tuta absoluta* as prey for *Macrolophus pygmaeus* and *Nesidiocoris tenuis*. *Journal of Applied Entomology,* 133**,** 292-296.

VAIS, H., WILLIAMSON, M. S., DEVONSHIRE, A. L. & USHERWOOD, P. N. R. 2001. The molecular interactions of pyrethroid insecticides with insect and mammalian sodium channels. *Pest Management Science,* 57**,** 877-888.

VAIS, H., WILLIAMSON, M. S., GOODSON, S. J., DEVONSHIRE, A. L., WARMKE, J. W., USHERWOOD, P. N. R. & COHEN, C. J. 2000. Activation of *Drosophila* sodium channels promotes modification by deltamethrin. *The Journal of General Physiology,* 115**,** 305-318.

VAN LEEUWEN, T., DERMAUW, W., VAN DE VEIRE, M. & TIRRY, L. 2005. Systemic use of spinosad to control the two-spotted spider mite (Acari: Tetranychidae) on tomatoes grown in rockwool. *Experimental & Applied Acarology,* 37**,** 93-105.

WANG, D., QIU, X., REN, X., NIU, F. & WANG, K. 2009. Resistance selection and biochemical characterization of spinosad resistance in *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae). *Pesticide Biochemistry and Physiology,* 95**,** 90-94.

WANG, D., QIU, X., WANG, H., QIAO, K. & WANG, K. 2010a. Reduced fitness associated with spinosad resistance in *Helicoverpa armigera*. *Phytoparasitica,* 38**,** 103-110.

WANG, E. T., SANDBERG, R., LUO, S. J., KHREBTUKOVA, I., ZHANG, L., MAYR, C., KINGSMORE, S. F., SCHROTH, G. P. & BURGE, C. B. 2008. Alternative isoform regulation in human tissue transcriptomes. *Nature,* 456**,** 470-476.

WANG, Z., ZHA, X., HE, N., XIANG, Z. & XIA, Q. 2010b. Molecular cloning and expression analysis of Bmrbp1, the *Bombyx mori* homologue of the Drosophila gene rbp1. *Molecular Biology Reports,* 37**,** 2525-2531.

WATSON, G. B., CHOUINARD, S. W., COOK, K. R., GENG, C., GIFFORD, J. M., GUSTAFSON, G. D., HASLER, J. M., LARRINUA, I. M., LETHERER, T. J., MITCHELL, J. C., PAK, W. L., SALGADO, V. L., SPARKS, T. C. & STILWELL, G. E. 2010. A spinosyn-sensitive *Drosophila melanogaster* nicotinic acetylcholine receptor identified through chemically induced target site resistance, resistance gene identification, and heterologous expression. *Insect Biochemistry and Molecular Biology,* 40**,** 376-384.

WEETMAN, D., MITCHELL, S. N., WILDING, C. S., BIRKS, D. P., YAWSON, A. E., ESSANDOH, J., MAWEJJE, H. D., DJOGBENOU, L. S., STEEN, K., RIPPON, E. J., CLARKSON, C. S., FIELD, S. G., RIGDEN, D. J. & DONNELLY, M. J. 2015. Contemporary evolution of resistance at the major insecticide target site gene Ace-1 by mutation and copy number variation in the malaria mosquito *Anopheles gambiae*. *Molecular Ecology,* 24**,** 2656-2672.

WEILL, M., LUTFALLA, G., MOGENSEN, K., CHANDRE, F., BERTHOMIEU, A., BERTICAT, C., PASTEUR, N., PHILIPS, A., FORT, P. & RAYMOND, M. 2003. Insecticide resistance in mosquito vectors. *Nature,* 425**,** 366-366.

WHEELOCK, G. D. & SCOTT, J. G. 1992. The role of cytochrome-p450ipr in deltamethrin metabolism by pyrethroid-resistant and susceptible strains of house-flies. *Pesticide Biochemistry and Physiology,* 43**,** 67-77.

WIERENGA, J. M., NORRIS, D. L. & WHALON, M. E. 1996. Stage-specific mortality of colorado potato beetle (Coleopera: Chrysomelidae) feeding on transgenic potatoes. *Journal of Economic Entomology* 89 , 1047-1052.

WILLIAMSON, M. S., DENHOLM, I., BELL, C. A. & DEVONSHIRE, A. L. 1993. Knockdown resistance *(kdr*) to DDT and pyrethroid insecticides maps to a sodium channel gene locus in the housefly (*Musca domestica*). *Molecular and General Genetics MGG,* 240**,** 17-22.

WILLIAMSON, M. S., MARTINEZTORRES, D., HICK, C. A. & DEVONSHIRE, A. L. 1996. Identification of mutations in the housefly *para*-type sodium channel gene associated with knockdown resistance (*kdr*) to pyrethroid insecticides. *Molecular and General Genetics,* 252**,** 51-60.

WONDJI, C. S., IRVING, H., MORGAN, J., LOBO, N. F., COLLINS, F. H., HUNT, R. H., COETZEE, M., HEMINGWAY, J. & RANSON, H. 2009. Two duplicated P450 genes are associated with pyrethroid resistance in *Anopheles funestus*, a major malaria vector. *Genome Research,* 19**,** 452-459.

WWW.PESTICIDERESISTANCE.ORG 2015. Arthropod Pesticide Resistance Database. Michigan State University.

XIA, Q., ZHOU, Z., LU, C., CHENG, D., DAI, F., LI, B., ZHAO, P., ZHA, X., CHENG, T., CHAI, C., PAN, G., XU, J., LIU, C., LIN, Y., QIAN, J., HOU, Y., WU, Z., LI, G., PAN, M., LI, C., SHEN, Y., LAN, X., YUAN, L., LI, T., XU, H., YANG, G., WAN, Y., ZHU, Y., YU, M., SHEN, W., WU, D., XIANG, Z., GROUP, G. A., YU, J., WANG, J., LI, R., SHI, J., LI, H., LI, G., SU, J., WANG, X., LI, G., ZHANG, Z., WU, Q., LI, J., ZHANG, Q., WEI, N., XU, J., SUN, H., DONG, L., LIU, D., ZHAO, S., ZHAO, X., MENG, Q., LAN, F., HUANG, X., LI, Y., FANG, L., LI, C., LI, D., SUN, Y., ZHANG, Z., YANG, Z., HUANG, Y., XI, Y., QI, Q., HE, D., HUANG, H., ZHANG, X., WANG, Z., LI, W., CAO, Y., YU, Y., YU, H., LI, J., YE, J., CHEN, H., ZHOU, Y., LIU, B., WANG, J., YE, J., JI, H., LI, S., NI, P., ZHANG, J., ZHANG, Y., ZHENG, H., MAO, B., WANG, W., YE, C., LI, S., WANG, J., WONG, G. K.-S. & YANG, H. 2004. A Draft Sequence for the Genome of the Domesticated Silkworm (*Bombyx mori*). *Science,* 306**,** 1937-1940.

YOON, K. S., KWON, D. H., STRYCHARZ, J. P., HOLLINGSWORTH, C. S., LEE, S. H. & CLARK, J. M. 2008. Biochemical and molecular analysis of deltamethrin resistance in the common bed bug (Hemiptera: Cimicidae*). Journal of Medical Entomology* 45 1092-1101.

YOU, M., YUE, Z., HE, W., YANG, X., YANG, G., XIE, M., ZHAN, D., BAXTER, S. W., VASSEUR, L., GURR, G. M., DOUGLAS, C. J., BAI, J., WANG, P., CUI, K., HUANG, S., LI, X., ZHOU, Q., WU, Z., CHEN, Q., LIU, C., WANG, B., LI, X., XU X., LU, C., HU, M., DAVEY, J. W., SMITH, S. M., CHEN, M., XIA, X., TANG, W., KE, F., ZHENG, D., HU, Y., SONG, F., YOU, Y., MA, X., PENG, L., ZHENG, Y., LIANG, Y., CHEN, Y., YU, L., ZHANG, Y., LIU, Y., LI, G., FANG, L., LI, J., ZHOU, X., LUO, Y., GOU, C., WANG, J., WANG, J., YANG, H. & WANG, J. 2013. A heterozygous moth genome provides insights into herbivory and detoxification. *Nat Genet,* 45**,** 220-225.

YOUNG, S. J., GUNNING, R. V. & MOORES, G. D. 2005. The effect of piperonyl butoxide on pyrethroid-resistance-associated esterases in *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae). *Pest Management Science,* 61**,** 397-401.

YU, Q. Y., LU, C., LI, B., FANG, S. M., ZUO, W. D., DAI, F. Y., ZHANG, Z. & XIANG, Z. H. 2008. Identification, genomic organization and expression pattern of glutathione S-transferase in the silkworm, *Bombyx mori*. *Insect Biochemistry and Molecular Biology,* 38**,** 1158-1164.

YU, Q. Y., LU, C., LI, W. L., XIANG, Z. H. & ZHANG, Z. 2009. Annotation and expression of carboxylesterases in the silkworm, *Bombyx mori*. *Bmc Genomics,* 10**,** 14.

ZACHAR, Z., CHOU, T. B. & BINGHAM, P. M. 1987. Evidence that a regulatory gene autoregulates splicing of its transcript. *The EMBO Journal,* 6**,** 4105-4111.

ZHAN, S., MERLIN, C., BOORE, JEFFREY L. & REPPERT, STEVEN M. 2011. The Monarch Butterfly genome yields insights into long-distance migration. *Cell,* 147**,** 1171-1185.

ZHAO, J. Z., LI, Y. X., COLLINS, H. L., GUSUKUMA-MINUTO, L., MAU, R. F. L., THOMPSON, G. D. & SHELTON, A. M. 2002. Monitoring and characterization of diamondback moth (Lepidoptera: Plutellidae) resistance to spinosad. *Journal of Economic Entomology,* 95**,** 430-436.

ZHU, F., PARTHASARATHY, R., BAI, H., WOITHE, K., KAUSSMANN, M., NAUEN, R., HARRISON, D. A. & PALLI, S. R. 2010. A brain-specific cytochrome P450 responsible for the majority of deltamethrin resistance in the QTC279 strain of *Tribolium castaneum*. *Proceedings of the National Academy of Sciences,* 107**,** 8557-8562.

ZUG, R. & HAMMERSTEIN, P. 2012. Still a host of hosts for *Wolbachia* analysis of recent data suggests that 40% of terrestrial arthropod species are infected. *PLoS ONE,* 7**,** e38544.

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Identification of mutations associated with pyrethroid resistance in the voltage-gated sodium channel of the tomato leaf miner (*Tuta absoluta*)

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ABSTRACT

The tomato leaf miner, *Tuta absoluta* (Lepidoptera) is a significant pest of tomatoes that has undergone a rapid expansion in its range during the past six years and is now present across Europe, North Africa and parts of Asia. One of the main means of controlling this pest is through the use of chemical insecticides. In the current study insecticide bioassays were used to determine the susceptibility of five *T. absoluta* strains established from field collections from Europe and Brazil to pyrethroids. High levels of resistance to λ cyhalothrin and tau fluvalinate were observed in all five strains tested. To investigate whether pyrethroid resistance was mediated by mutation of the *para*-type sodium channel in *T. absoluta* the IIS4–IIS6 region of the *para* gene, which contains many of the mutation sites previously shown to confer knock down (kdr)-type resistance to pyrethroids across a range of different arthropod species, was cloned and sequenced. This revealed that three kdr/super-kdr-type mutations (M918T, T929I and L1014F), were present at high frequencies within all five resistant strains at known resistance 'hot-spots'. This is the first description of these mutations together in any insect population. High-throughput DNA-based diagnostic assays were developed and used to assess the prevalence of these mutations in 27 field strains from 12 countries. Overall mutant allele frequencies were high (L1014F 0.98, M918T 0.35, T929I 0.60) and remarkably no individual was observed that did not carry kdr in combination with either M918T or T929I. The presence of these mutations at high frequency in *T. absoluta* populations across much of its range suggests pyrethroids are likely to be ineffective for control and supports the idea that the rapid expansion of this species over the last six years may be in part mediated by the resistance of this pest to chemical insecticides.

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1. Introduction

The tomato leaf miner, *Tuta absoluta* is an economically significant pest of tomatoes that is currently undergoing a rapid expansion in its geographical distribution. The larvae cause damage by feeding on the leaves, stem and fruit leading to significant yield losses of up to 100% if the pest is not controlled (Desneux et al., 2010). *T. absoluta* is native to Central America but has spread to South America and more recently to North Africa and the Middle East. It was first detected in Europe (Spain) in 2006 and has since become a major problem to tomato growers in many European countries (Desneux et al., 2010). The main method of control of *T. absoluta* relies on the application of chemical insecticides (Picancëo et al., 1995). Unfortunately their intensive use has led to the development of resistance with tolerance to organophosphates, carbamates, synthetic pyrethroids, benzoylureas, avermectin and indoxacarb reported in *T. absoluta* populations from Brazil, Chile and Argentina (Guedes et al., 1994; Lietti et al., 2005; Salazar and Araya, 2001; Silva et al., 2011; Siqueira et al., 2001, 2000; Souza et al., 1992).

Pyrethroids are a major class of neurotoxic insecticides that have been used extensively to control a wide range of agricultural and human health pests. They act on the insect nervous system by modifying the gating kinetics of voltage-gated sodium channels to slow activation and inactivation, resulting in continuous nerve stimulation leading to paralysis and death (Soderlund and Bloomquist, 1989). A common mechanism of resistance to pyrethroids, termed knock down resistance (kdr), has been shown to

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arise through alterations (point mutations) in the *para*-type sodium channel protein leading to reduced sensitivity of the insect nervous system to these compounds. The most common mutation identified in a range of arthropod pests, often referred to simply as 'kdr' results from a single point mutation in the S6 segment of domain II of the sodium channel gene usually resulting in a leucine to phenylalanine (L1014F) substitution (Davies et al., 2007; Williamson et al., 1996). This mutation typically confers a 10–20 fold reduction in sensitivity to pyrethroids, however, secondary mutations giving enhanced (super-kdr) resistance have also been identified in pyrethroid resistant arthropods either singly or in combination with kdr (reviewed in Davies and Williamson, 2009). These most commonly occur in domains II or III of the channel protein and include M918T, first described in resistant housefly (*Musca domestica*), and T929I first described in diamondback moth, *Plutella xylostella* (Schuler et al., 1998; Williamson et al., 1996).

To date, the molecular mechanisms underlying pyrethroid resistance in *T. absoluta* have not been characterised. Knowledge of the underlying mechanisms involved and their distribution in world-wide *T. absoluta* populations is essential for effective control of this pest and to minimise the wasteful application of ineffective chemistry. Towards this goal we have carried out leaf-dip bioassays to evaluate the susceptibility of five field strains of *T. absoluta* collected in Europe and Brazil to two pyrethroid insecticides. We describe the cloning and sequencing of a 420 bp fragment (domains IIS4-S6) of the *para* gene from the five *T. absoluta* strains and the identification of three mutations that have previously been reported to confer reduced sensitivity to pyrethroids in several other arthropod pests. We then developed diagnostic tools that allow sensitive detection of these mutations in individual *T. absoluta* larvae and adults. These were used to screen field-collected samples of diverse geographic origin to examine the frequency and distribution of these mutations in global populations of this pest species.

2. Materials and methods

2.1. *Tuta absoluta* strains

Live *T. absoluta* strains were provided by Dr Pablo Bielza (Departamento de Produccion Vegetal, Universidad Politecnica de Cartagena, Spain) and designated TA1 (Spain), TA2 (Spain), TA3 (Italy) and TA4 (Portugal). They were all collected from the field during the year 2010. The strain GA was provided by Bayer CropScience laboratories. This is a mixed field population collected in 2008 from different areas of Brazil (São Paulo and Minas Gerais states) and reared since then without insecticide selection. The five strains were reared on tomato plants inside insect proof cages and maintained under controlled environment conditions (26 ± 2 °C and 16 h daylength). Additionally, field-collected *T. absoluta* stored in 70% ethanol, originating from 27 locations (Table 1), were provided by Dr. Pablo Bielza.

2.2. Insecticide bioassays

A leaf-dip bioassay protocol, as recommended by the Insecticide Resistance Action Committee (IRAC), was used to evaluate the susceptibility of the five strains of *T. absoluta* to λ cyhalothrin (CS 10%, Syngenta) and tau fluvalinate (TEC 91.7%, Makhteshim). Tomato leaflets were immersed in serial dilutions of insecticide or control solutions, allowed to air dry for 1 h and then supplied as the sole food source to larvae. All insecticide solutions were prepared using an aqueous diluent (0.01% Agral, Syngenta); control solutions consisted of the diluent only. Three replicates at each of six different concentrations were used for each insecticide. Replicates consisted of a Petri dish (90 mm × 20 mm) containing a lightly moistened filter paper, onto which one or two tomato leaves (dependent upon size) were placed and inoculated with 15–20

Table 1
Kdr/skdr mutation frequencies in *Tuta absoluta* populations collected from different locations around the world and stored in 70% ethanol for molecular analysis. *N* = number of individuals.

ID N°	Country	Location	Sampled from	*N*	Mutation frequency		
					L1014F	M918T	T929I
1	Canary Islands	Tejina, (La Laguna) Tenerife	Tomato	12	1.00	0.54	0.46
2	Canary Islands	Guía de Isora, Tenerife	Tomato	10	1.00	0.35	0.60
3	Canary Islands	Granadilla, Tenerife	Tomato	10	1.00	0.25	0.60
4	Canary Islands	Arico,Tenerife	Tomato	8	1.00	0.25	0.69
16	Canary Islands	La Palma	Unknown	7	1.00	0.36	0.64
5	Baleares Islands	Teulera, Mallorca	Tomato	3	1.00	0.33	0.67
6	Baleares Islands	San Fangos, Mallorca	Tomato	3	1.00	0.33	0.67
7&8	Algeria	Mostaganem	Tomato under-protection	9	1.00	0.17	0.83
9	Italy	Turín	Unknown	3	1.00	0.33	0.50
15	Italy	Cagliari.S.Margherita di Pula	Tomato under-protection	5	1.00	0.20	0.80
17	Italy	Sicilia	Aubergine	14	1.00	0.43	0.54
28	Italy	Sele valley.Salerno.Campania	Tomato	17	1.00	0.44	0.41
20	Colombia	Antioquía/Rionegro	Tomato	10	0.60	0.50	0.45
10	Ecuador	La Tola, Pichincha	Tomato	11	1.00	0.55	0.41
11	Spain	Tudela, Navarra	Tomato	8	1.00	0.44	0.44
18	Spain	Ramonete/Lorca, MU	Tomato	6	1.00	0.25	0.75
22	Spain	Mazarrón, Murcia	Tomato	16	1.00	0.13	0.81
25	Spain	Valencia	Unknown	9	1.00	0.44	0.44
26	Spain	Maresme, Cataluña	Tomato	12	1.00	0.50	0.50
12	Argentina	Barrancas,Santa Fé	Tomato under-protection	7	1.00	0.00	1.00
13	Argentina	LaPrimavera, Mendoza	Tomato	8	1.00	0.13	0.75
14	Argentina	La Plata, Bs.As	Tomato under-protection	3	1.00	0.00	1.00
21	Crete	Heraklion	wild plants	3	1.00	0.50	0.50
24	Peru	Chulacanas, Piura	Tomato	7	1.00	0.43	0.50
27	Portugal	Silveira. Concello Torres Vedres	Tomato	5	1.00	0.20	0.80
29a	Israel	Beit hashita, Israel valley	Unknown	5	1.00	0.40	0.50
29b	Israel	Ein hmifraz, Western galilee	Unknown	6	1.00	0.50	0.50
Total				217	0.98	0.35	0.60

L2/L3 stage larvae. These were maintained under controlled environmental conditions (26 ± 2 °C, 16 h daylength) and mortality was assessed after 48 h. Larvae were counted as dead if when stimulated with a fine paintbrush, there was either no movement, or if movement was uncoordinated and they were unable to move a distance equal to double their body length. Statistical analysis of data from bioassays was carried out to estimate LC_{50} values (concentration of insecticide required to kill 50% of the tested insects) using GenStat 13th Edition software (VSN International), which corrects for control mortality prior to performing probit transformations.

2.3. Cloning of sequences encoding domain II

To clone and sequence the domain II region of the *T. absoluta* sodium channel gene PCR reactions were initially carried out on cDNA prepared from pools of 15–20 individuals from each strain using degenerate primers designed against conserved motifs within the IIS4 and IIS6/II–III linker regions of the channel protein as described previously (Martinez-Torres et al., 1997). A nested PCR approach was employed using primers DgN1 and DgN3 in a primary PCR and primers DgN2 and DgN3 in a secondary reaction (primer sequences are given in Table 2). Once the *T. absoluta* sodium channel gene sequence had been determined, specific primers were designed to perform direct PCR analysis of genomic DNA. To amplify a PCR fragment encompassing the L1014F mutation position a nested PCR was performed using primers TAF3 and TAR1 in the first reaction and primers TAF4 and TAR2 in the second reaction. To amplify the region containing the M918T and T929I mutations a single PCR using TAF2 and TARouter was performed (Table 2). To determine the positions and sizes of two introns within this region of the sodium channel gene nested PCR was performed using the primers TAF1 and TAR4 followed by TAF2 and TAR3 for the first intron and primers TAF5 and TAR1 followed by TAF4 and TAR2 for the second intron (Table 2).

Total RNA was extracted from pools of 15–20 individuals using Trizol and following the manufacturer's instructions. Genomic DNA was removed by DNase I digestion using DNA-free DNase treatment and removal reagent (Ambion). The quality and quantity of RNA pools were assessed by spectrophotometry (Nanodrop Technologies) and by running an aliquot on a 1.2% agarose gel. A quantity of 4 μg of RNA sample was then used for cDNA synthesis using Superscript III and random hexamers (Invitrogen) according to the manufacturer's instructions.

PCR reactions (20 μl) consisted of 1 μl of template DNA, 1 μl of each primer (10 μM), 10 μl of GreenTaq (Fermentas) and 7 μl of sterile distilled water. Temperature cycling conditions were: 35 cycles of 95 °C for 30 s 48–58 °C for 60 s and 72 °C for 90–120 s. Agarose gel electrophoresis (1.2%) of PCR products was carried out in 1× TBE buffer and the Wizard SV gel and PCR clean up System from Promega was used to recover DNA from gel slices according to manufacturer's recommendations. PCR products were either direct sequenced (using the same primers used in PCR) or cloned using the Strataclone PCR Cloning kit (Stratagene) and plasmids sequenced with standard T3/T7 primers. PCR products or plasmid DNA was sent to Eurofins MWG/Operon for sequencing.

Table 2
Oligonucleotide primers used to amplify the *Tuta absoluta para*-type sodium channel gene and for TaqMan assays, all primers are shown 5′–3′. Degenerate bases are represented using standard IUB codes.

Name	Sequence
TAkdr_F	CTTCTTAGCCACCGTCGTCATT
TAkdr_R	CGCTTTTACTGGTATATTGCAATAAAAAGCT
TAkdr VIC	AACCACAAGATTACC
TAkdr FAM	ACCACAAAATTACC
TAT929I_F	ACGATGGGTGCCTTGGG
TAT929I_R	TGCATACCCATCACGGCAAATAT
TAT929IVIC	CACAATACGAAGGTCAGGTT
TAT929IFAM	CACAATACGAAGATCAGGTT
TAM918T_F	TGGCCGACGTTTAATTTACTCATCT
TAM918T_R	TGCCCAAGGCACCCATC
TAM918TVIC	TCCTACCCATAATCG
TAM918TFAM	TCCTACCCGTAATCG
DgN1	GCNAARTCNTGGCCNACNYT
DgN2	GCNAARTCNTGGCCNAC
DgN3	YTTRTTNGTNTCRTTRTCRGC
TAF1	GAAATCGTGGCCGAC
TAF2	GGCCGACGTTTAATTTACTC
TAF3	AGAATGGATTGAGAGTATGTGG
TAF4	GTATGTGGGACTGTATGTTGG
TAF5	TACCACGATGGAACTTTACG
TAR1	GGTGTCGTTATCGGCAGTAG
TAR2	GTTATCGGCAGTAGGTGTCGA
TAR3	AAGTTCCATCGTGGTAGGTC
TAR4	CGGTGGCTAAGAAGAATGG
TARouter	TGTTTCAACAGAATGACGATACTA

2.4. TaqMan diagnostic assays

Forward and reverse primers and two probes were designed using the Custom TaqMan Assay Design Tool (Applied Biosystems). The primers TAM918T_F + TAM918T_R, TAT929I_F + TAT929I_R and TAkdr_F + TAkdr_R were used to amplify the regions encompassing the M918T, T929I and L1014F sites respectively. For all assays the probe labelled with VIC, was specific for the wild-type allele, while a second probe, labelled with FAM was specific for the mutant allele. Each probe also carried a 3′ non-fluorescent quencher. The probes TAM918TVIC + TAM918TFAM, TAT929IVIC + TAT929IFAM, and TAkdr VIC + TAkdr FAM were used in the M198T, T929I and L1014F assays respectively. The sequences of the primers and probes used in the TaqMan assays are given in Table 2.

PCR reactions (15 μl) contained 2 μl of genomic DNA extracted from individual insects using DNAzol reagent, 7.5 μl of SensiMix DNA kit (Quantace), 800 nM of each primer and 200 nM of each probe. Samples were run on a Rotor-Gene 6000™ (Corbett Research) using the temperature cycling conditions of: 10 min at 95 °C followed by 40 cycles of 95 °C for 10 s and 60 °C for 45 s. The increase in fluorescence of the two probes was monitored in real time by acquiring each cycle on the yellow (530 nm excitation and 555 nm emission) and green channel (470 nm excitation and 510 nm emission) of the Rotor-Gene respectively. In order to optimize and validate the TaqMan method, and before applying it to the screening of *T. absoluta* collections stored in ethanol, genomic DNA templates of known genotype from the five laboratory strains was used to optimise initial PCRs. During all runs template controls (a wild-type homozygous, a mutant homozygous and a mutant heterozygous sample) were included in each run to aid genotype scoring.

3. Results

3.1. Bioassays

Significant variation in susceptibility between the five strains to λ cyhalothrin and tau fluvalinate was observed (Table 3). For λ cyhalothrin GA and TA1 exhibited the highest and lowest LC_{50} values of 1514 mg l^{-1} and 85 mg l^{-1} respectively (17-fold difference). There was no overlap between the confidence intervals of GA or TA1 with the other three strains (TA2, TA3 and TA4), whose LC_{50} values grouped closely with less than 2-fold separation (351–700 mg l^{-1}). For tau fluvalinate TA3 had the lowest LC_{50} value of 821 mg l^{-1}. The other four strains had overlapping confidence

Table 3
Relative toxicity of λ cyhalothrin and tau fluvalinate to five laboratory strains of *Tuta absoluta*.

	Strain	LC50 (mg l^{-1})	Lower 95% CL	Upper 95% CL	Slope	SE
λ cyhalothrin	TA1	85	36.7	159	0.904	0.132
	TA2	351	176	507	2.256	0.577
	TA3	631	456	837	2.452	0.458
	TA4	700	472	957	1.859	0.34
	GA	1514	1137	2106	2.106	0.371
Tau fluvalinate	TA1	2047	1235	4483	1.8	0.5
	TA2	1952	1365	2841	2.4	0.6
	TA3	821	544	1177	1.765	0.358
	TA4	3716	1443	29,334	0.7	0.2
	GA	9259	1403	61,099	2	0.3

intervals with the highest LC_{50} value of 9259 mg l^{-1} being obtained with GA. We were unable to obtain a known susceptible standard strain to use as a reference in these studies, nevertheless, the recommended field rate of λ cyhalothrin is around 25 mg l^{-1} and for tau fluvalinate is around 50 mg l^{-1}. Therefore the resistance exhibited by these strains would compromise control using these insecticides.

3.2. Sequencing of domain II of the voltage-gated sodium channel

Using degenerate primers designed against conserved sequences within the domain II region of several insect *para* sodium channel gene sequences a ~420 bp fragment of the *T. absoluta para* gene was PCR amplified, cloned and sequenced (Genbank accession number JQ701800). The encoded amino acid sequence of this fragment is shown in Fig. 1. As expected this sequence showed high similarity to other insects from the Lepidoptera order including diamondback moth (*P. xylostella*), silkworm (*Bombyx mori*), corn earworm (*Helicoverpa zea*), cotton leafworm (*Spodoptera litura*) and tobacco budworm (*Heliothis virescens*). The sequenced region obtained (IIS4–IIS6) contains many of the mutation sites previously reported to be implicated in conferring kdr-type resistance to pyrethroids across a range of different insects (Davies et al., 2007). Preliminary sequencing of RT-PCR cDNA fragments from pools of 20 individuals of the five strains (four clones per strain) revealed three point mutations within this region that result in a leucine to phenylalanine amino acid substitution in IIS6 (L1014F, all numbering throughout this manuscript is based on the housefly *para* sequence, GenBank accession: X96668), a methionine to threonine substitution upstream of IIS5 (M918T) and a threonine to isoleucine mutation (T929I) within IIS5. In this sequencing of pooled samples the L1014F mutation appeared to be homozygous and present in all strains and was found in combination with either the M918T and/or the T929I mutations. No other nucleotide polymorphisms were observed in the sequence obtained from different clones of the same strain or between strains.

Specific primers were designed based on the obtained cDNA sequence for use in the analysis of *T. absoluta* genomic DNA to determine the positions and sizes of introns within this region of the sodium channel gene. Previous work, characterizing the domain IIS4–S6 region of the *para* gene from different insect species, has shown that this region contains two introns that are highly conserved in their position but vary widely in size. The position and sequence of the two introns is shown in Fig. 2. The size of the introns was 862 and 106 nucleotides respectively with the sequence of both introns highly conserved across the different strains with no polymorphic bases observed. To assess the frequency of the kdr and skdr mutations within the five strains of

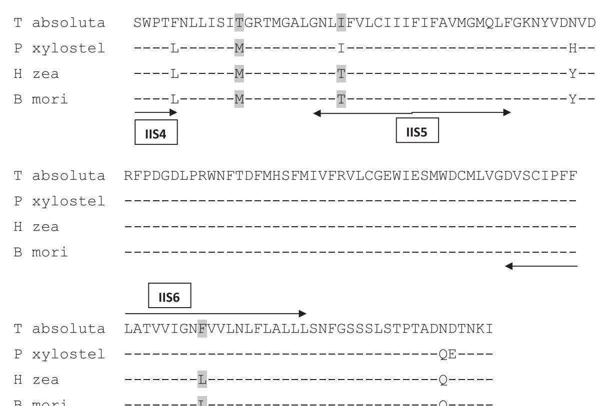

Fig. 1. Amino acid alignment of domains IIS4-IIS6 of the *Tuta absoluta* sodium channel with the corresponding sequence of *Plutella xylostella* (AB265178.2), *Helicoverpa zea* (ADF80418.1) and *Bombyx mori* (NP_001136084). Transmembrane segments (S4, S5 and S6) are indicated by arrows. The positions of the L1014F, M918T and T929I mutations are highlighted.

T. absoluta, genomic DNA was extracted from 10 individual adults of each strain and used as template to amplify the IIS4–IIS6 region of the *para* gene using the specific primers designed from the cDNA sequence. No polymorphic bases were observed in the coding/non-coding sequence obtained between any individuals apart from at the two skdr positions. All ten individuals of all five strains were homozygous for the L1014F mutation. For the two skdr mutations (M918T and T929I), the frequency of the T929I mutation was present at higher frequency than M918T in TA1, TA3 and GA and for TA2 and TA4 the two mutations were present at the same frequency (Table 4). Although for most strains all potential genotypes at each mutation site were present (homozygous wild-type, homozygous mutant and heterozygous), the two mutations were only found in combination in individual larvae when both mutations were in the heterozygous form. To test if the two mutations were on separate alleles or found on the same allele, several individuals of this genotype were cloned and sequenced. Sequencing of ten colonies derived from each individual showed that the two mutations were only ever observed on separate alleles. Therefore two 'resistance alleles' are present in the *T. absoluta* strains the M918T allele which has the amino acid residues T918 + T929 + F1014 and the T929I allele which has the amino acid residues M918 + I929 + F1014.

3.3. TaqMan assays

DNA was extracted from 220 individuals, from 27 locations spanning a substantial part of the geographical range of *T. absoluta* (Table 1). Separate TaqMan assays were performed for each of the three mutations. Using samples of known genotypes as controls, the assays allowed homozygous resistant (R/R), heterozygous (R/S) and homozygous susceptible individuals (S/S) to be distinguished. The TaqMan assays use two probes, one specific for the resistant (mutant) allele labelled with FAM and the other specific for the susceptible (wild-type) allele labelled with VIC. A homozygous resistant individual will display a strong increase in FAM fluorescence, whilst a homozygous wild-type individual will show a strong increase in VIC fluorescence. Heterozygous individuals show an intermediate increase in both channels (Fig. 3). To facilitate genotyping, a scatter plot comparing VIC and FAM fluorescence values at the end of the 40 cycles was created using the Rotor-Gene analysis option. Of the 220 samples analysed three samples failed to amplify a product in PCR, and 17 required confirmation by DNA

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  -S--W--P--T--F--N--L--L--I--S--I--T--G--R--T--M--G--A--L--G-
II TCGTGGCCGACGTTTAATTTACTCATCTCGATTACGGGTAGGACGATGGGTGCCTTGGGC
  -N--L--I--F--V--L--C--I--I--I--F--I--F--A--V--M--G--M--Q--L-
  AACCTGATCTTCGTATTGTGCATCATTATATTCATATTTGCCGTGATGGGTATGCAACTA
  -F--G--K--N--Y--V-
  TTTGGGAAAAATTATGTGggtaagtggattggtgggagtgttgcatgttcattggtgtatcgacgtgttggtgga cggcggtgcccgatggaatatcaag
  tcaatgcacttcaacattatcaa tttcctaaattatacttatatcattacaatgagc accaacttttccaagtgttagttgaggtacat ttactttgtgcaaataggattggggtcaaat
  agtatcgtcattctgttgaaacattctcttaatagtattttatgatttccgaattataatgt catcataaaaatatatgctgtattaatattttattaataatgactgctgacttttaaattt
  tgacactaataaattttaaataagtttcaccatagaatgtcgatactgttttcacctaagcttgtgaatcgggctcgccgcctatactagtgagtattgtgttgatgtgcgtcacactgt
  tatgcatccctgtgttgtaagccactataaaaggcgcctcacttgtgaaacgtttatatgct agcttgttcatatctaactccatctttccc ttgtatttttatatttcctcgaactatttat
  gtattttatgtactccttagtttaatactttatcttcttcttaaggcgctatatcttttgctatattccttagtgtaagtgcagcatggtctataactattagatcgtataattattgcgtgaa
  taatatttaatttaatgtagttgctaatcttgtagaatactatgtcaaaactgcttttagt ataacataatattgttcttaagaatttaaattgc ttctttagaccgttttgctgtttcaaat
                                                    -D--N--V--D--R--F--P--D--G--D--L--P--
  actttaaaactctaggcaaaatactaatataatgtttcttatataca GACAACGTTGATCGTTTTCCCGATGGAGACCTACCAC
  R--W--N--F--T--D--F--M--H--S--F--M--I--V--F--R--V--L--C--G--E
  GATGGAACTTTACGGATTTCATGCATAGCTTCATGATTGTGTTTAGAGTACTCTGCGGAGA
  --W--I--E--S--M--W--D--C--M--L--V--G--D--V--S--C--I--P--F--F-
  ATGGATTGAGAGTATGTGGGACTGTATGTTGGTCGGAGATGTATCGTGTATTCCATTCTTC
  -L--A--T--V--V--I--G--N--F--V-
  TTAGCCACCGTCGTCATTGGTAATTTTGTGgttcgtatcttctttatattcagctttttattgcaatataccagtaaaagcgtttttgtaattg

                                             -V--L--N--L--F--L--A--L--L--L--S--N--F-
  tgtgaatttaaagattcaccaag atcttcattcgcatttcag GTACTTAACCTCTTCTTAGCTCTGTTACTGTCAAACTTT

  -G--S--S--S--L--S--T--P--T--A--D--N--D--T--N--K--I-

  GGTTCGTCGAGTTTATCGACACCTACTGCCGATAACGACACCAATAAGATA
```

Fig. 2. Sequence of domain IIS4-S6 of the *Tuta absoluta para*-type sodium channel gene. Positions of known kdr/super-kdr mutations are boxed. Lower cases indicate intron sequence.

sequencing as the TaqMan results for these samples were ambiguous.

Overall mutation frequencies were high (L1014F 0.98, M918T 0.35, T929I 0.60; Table 1). No individual was observed that did not carry kdr in combination with either M918T or T929I. For the L1014F (kdr) mutation, no individuals were homozygous wild-type, and only eight were heterozygous (from Colombia); samples from all other countries in this study were homozygous mutant. A map comparing mutation frequencies is shown in Fig. 4. Overall no strong geographic trend for the distribution of M198T and T929I was observed, however the frequency of T929I was generally highest and the frequency of M918T correspondingly low where tomato was grown under-protection (see samples 7 and 8, 12, 14 and 15, Table 1).

4. Discussion

Five laboratory strains of *T. absoluta* established from field collections from Europe and Brazil showed significant differences in their susceptibility to two pyrethroid insecticides in leaf-dip bioassays. Although we were unable to source a fully pyrethroid susceptible strain for use as a reference in these experiments (probably for the reasons discussed below) comparison of the LC_{50} values obtained with the recommended field rates of λ cyhalothrin and tau fluvalinate for *T. absoluta* control strongly suggest that all five strains would exhibit resistance to both compounds in the field. In the absence of a known susceptible population, comparisons with the most susceptible strain for each compound (TA1 for λ cyhalothrin and TA3 for tau fluvalinate) gave resistance factors of 4–17-fold for λ cyhalothrin and 2–11-fold for tau fluvalinate. Interestingly, for both pyrethroids the GA strain that was derived from several *T. absoluta* populations collected in Brazil exhibited a higher resistance factor than the four strains collected from Europe. As mentioned above, resistance to pyrethroid insecticides has previously been documented for *T. absoluta*, encompassing multiple reports of decreased susceptibilities to a range of pyrethroid chemistries including deltamethrin, λ cyhalothin, bifenthrin and permethrin (Branco et al., 2001; Guedes et al., 1994; Salazar and Araya, 1997; Silva et al., 2011; Siqueira et al., 2000).

To investigate whether pyrethroid resistance was mediated by mutation of the *para*-type sodium channel in *T. absoluta* we cloned and sequenced the IIS4–IIS6 region of the *para* gene which contains

Table 4
Genotypes of 10 individuals from each of the five laboratory strains of *Tuta absoluta* at mutation positions 918 and 929 and overall mutation frequency in each strain.

Sample N°	TA1		TA2		TA3		TA4		GA	
	M918T	T929I	M918T	T929I	M918T	T929I	M918T	T929I	M918T	T929I
1	S/S	R/R	S/S	R/R	S/S	R/R	R/S	R/S	S/S	R/R
2	S/S	R/R	S/R	S/R	S/S	R/R	R/S	R/S	S/S	R/R
3	R/S	R/S	R/R	S/S	S/S	R/R	R/S	S/S	R/S	R/S
4	R/S	R/S	R/S	R/S	S/S	R/R	S/S	R/R	S/S	R/R
5	R/S	R/S	R/R	S/S	S/S	R/R	R/S	R/S	S/S	R/R
6	R/S	R/S	S/S	R/R	R/S	R/S	R/S	R/S	R/S	R/S
7	S/S	R/R	S/S	R/R	R/S	R/S	R/S	R/S	S/S	R/R
8	S/S	R/R	R/S	R/S	R/S	R/S	R/S	R/S	R/S	R/S
9	R/S	R/S	R/S	R/S	S/S	R/R	R/S	R/S	S/S	R/R
10	R/R	S/S	R/R	S/S	R/S	R/S	R/S	S/S	R/S	R/S
Frequency	0.35	0.65	0.5	0.5	0.2	0.8	0.45	0.45	0.2	0.8

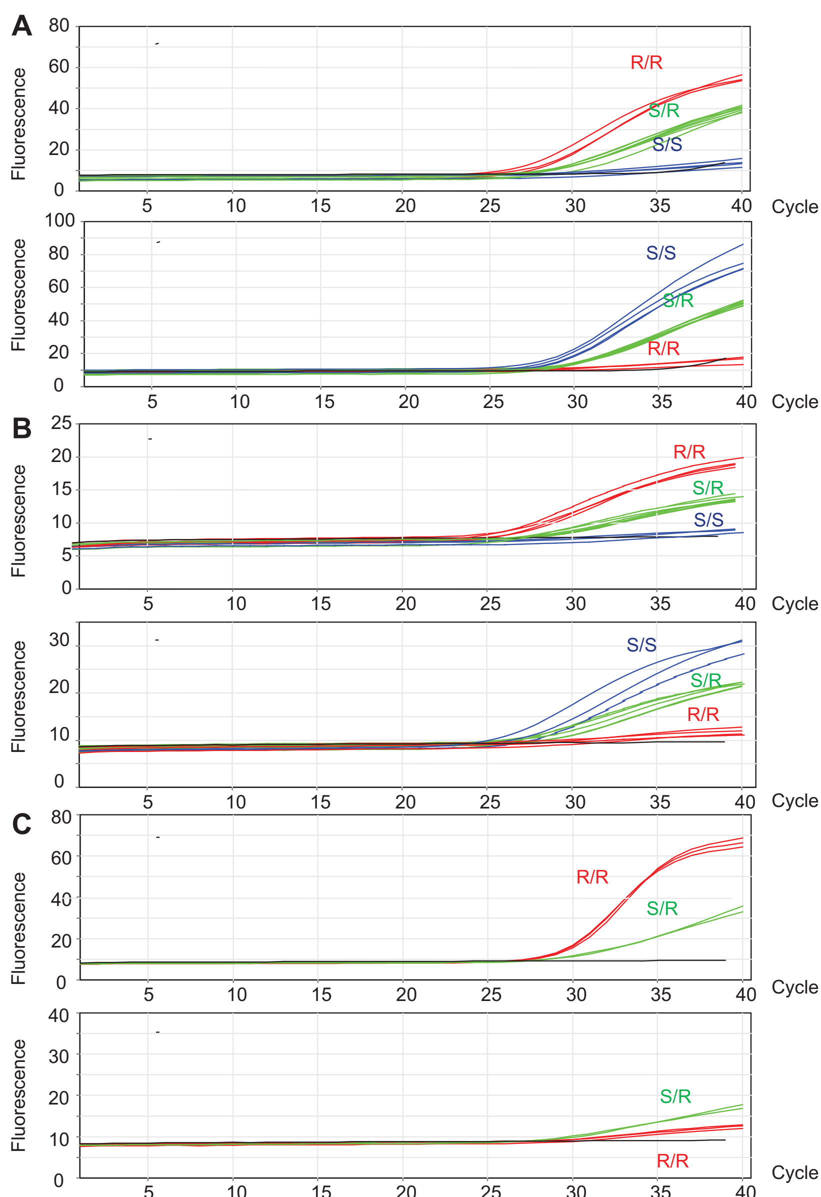

Fig. 3. Real-time TaqMan detection of the kdr and super kdr mutations in *Tuta absoluta*. (A) M918T, (B) T929I, (C) L1014F. For each pair of graphs, the top graph shows the FAM-labelled probe specific for the mutant allele, and the bottom graph shows the VIC-labelled probe specific for the wild-type allele. S: wild-type allele; R: resistant allele.

many of the mutation sites previously reported to be implicated in conferring kdr-type resistance to pyrethroids across a range of different insects. This revealed three kdr/skdr-type mutations within the resistant strains at known resistance 'hot-spots' within this gene region, M918T, T929I and L1014F.

All five strains were fixed for the common kdr mutation L1014F (present at 100% frequency). This mutation, initially identified in *M. domestica* has since been reported in resistant strains of over 20 different arthropod species (Davies and Williamson, 2009). Functional expression studies of cloned insect sodium channels harbouring the L1014F mutation using *Xenopus laevis* oocytes has confirmed the effect of this substitution in conferring up to a17-fold reduction in sensitivity to certain pyrethroids (Soderlund and Knipple, 2003; Tan et al., 2002; Vais et al., 2000). More recently molecular modelling of insect sodium channels (O'Reilly et al., 2006), based on the crystal structure of the rat brain Kv1.2 potassium channel (Long et al., 2005) has revealed that the L1014F mutation does not make physical contact with pyrethroid insecticides and may confer resistance via a conformational effect that makes the sodium channel less prone to open (Davies and Williamson, 2009). Sequencing ten individuals of the five *T. absoluta* strains revealed that the L1014F mutation was always associated with two additional mutations M918T or T929I.

M918T was the first 'skdr'-type mutation identified in insects (*M. domestica*) where it was associated with an enhanced knock down resistant phenotype (Williamson et al., 1996). It has

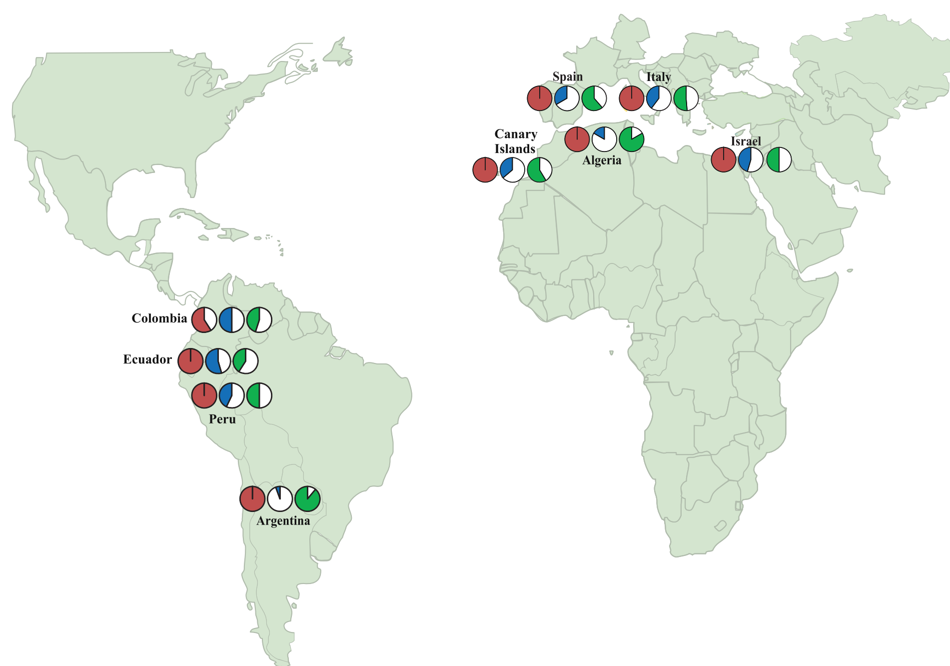

Fig. 4. Map of kdr/skdr mutation frequencies in *T. absoluta* populations from countries where the sample size was >6. The red pie charts show the frequency of L1014F, blue pie charts the frequency of M918T and green pie charts the frequency of T929I. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

subsequently been described for horn fly, *Haematobia irritans*, the peach potato aphid, *Myzus persicae* and the South American leaf miner, *Liriomyza huidobrensis* where it was always associated with L1014F (Davies et al., 2007; Eleftherianos et al., 2008; Guerrero et al., 1997). However, more recently M918T has been found in isolation in bifenthrin resistant tomato red spider mite, *Tetranychus evansi* (Nyoni et al., 2011). The M918T mutation has been shown to confer highest resistance to type II pyrethroids such as λ cyhalothrin and deltamethrin and in *M. domestica* gave 560-fold resistance to the latter compound (in association with L1014F) (Khambay et al., 1994). Functional expression of the *Drosophila melanogaster para* gene containing the M918T + L1014F mutations reduced the sensitivity of sodium channels by approximately 100 fold (Vais et al., 2000).

T929I was first reported in pyrethroid resistant diamondback moth, *P. xylostella* and has since been identified in human headlice, *Pediculosis capitis* and maize weevil, *Sitophilus zeamais* (Araujo et al., 2011; Lee et al., 2000; Schuler et al., 1998). The effect of T929I has also been examined in functional expression studies in oocytes, where in combination with L1014F it was found to make the sodium channel highly insensitive to a range of type I and type II pyrethroid insecticides and DDT (Usherwood et al., 2007; Vais et al., 2001). Modelling suggests that T929 is likely to be a key residue for binding of all pyrethroids as well as DDT as a result of hydrogen bond formation with this amino acid that stabilizes the bound insecticide (Davies and Williamson, 2009).

To date, the three mutations observed in this study have never been identified together within a single insect population and our findings suggests that *T. absoluta* has historically undergone strong selection with a range of pyrethroid insecticides. No correlation was observed between the frequency of the two skdr mutations in the five strains and their response to the two pyrethroids and it is likely that additional resistance mechanisms are present in at least some of these strains. For example the frequency of the two mutations was the same in TA3 and GA but the latter is 2 and 11-fold more resistant to λ cyhalothrin and tau fluvalinate respectively. It would be interesting, in future, to investigate if metabolic mechanisms play a role in the resistance of these strains as previous studies using a range of insecticide synergists on resistant populations in Brazil have implicated increased production of detoxification enzymes as a mechanism of resistance and suggested resistance is multigenic (Siqueira et al., 2001). However, although of academic interest, this may have limited implications for control with pyrethroids as work on other insect species has shown that the level of resistance typically conferred by the L1014F mutation in combination with either M918T or T929I compromises control in the field.

Three diagnostic assays have been developed in this study that can be used for accurate genotyping of large numbers of individual larvae or adults for the three mutations. These are based on TaqMan real-time PCR, a high-throughpout 'closed-tube' approach that requires no post-PCR processing. These tools were used to examine the frequency and distribution of these resistance mutations in *T. absoluta* field strains collected from Europe and South America. This revealed that the mutations are at high frequency throughout the regions surveyed, indeed, no individual was found that did not carry at least two of the three mutations. The kdr mutation appeared to be fixed in all the populations analysed apart from collections from Colombia where individuals were identified with the mutation in the heterozygous form. The overall frequency of the two skdr-type mutations in the field samples tested was lower for M918T (0.35) than T929I (0.6) suggesting the latter may be being preferentially selected in *T. absoluta* populations. As described for the laboratory strains, no allele was observed that carries both skdr mutations. This is consistent with the situation in resistant populations of *Bemisia tabaci* from the Mediterranean basin where the L925I and T929V mutations were never found to occur in combination in the same haplotype (Alon et al., 2006; Roditakis et al., 2006). A common occurrence in both lab and field strains of

T. absoluta were individuals that had one M918T allele and one T929I allele (around 50% of all individuals). An insect of this genotype would have half of their *para*-type sodium channel component modified with the M918T mutation and half modified with the T929I mutation (and no 'susceptible' channels). As kdr and skdr mutations have been shown to be recessive in many resistant insect species heterozygous individuals are susceptible to pyrethroids, however, *T. absoluta* individuals with both the M918T and T929I alleles (in addition to L1014F) would be expected to be strongly resistant. Recently, a genetic study of *T. absoluta* using ribosomal and mitochondrial markers revealed high genetic homogeneity in *T. absoluta* populations from the Mediterranean Basin and South America with a single genetic type identified (Cifuentes et al., 2011). The authors proposed that a single genetically uniform and invasive population has been able to spread through South America, and then to the Mediterranean Basin. They also suggested that this may have occurred as a result of selective pressure resulting from human activities and a replacement of more susceptible populations by one of higher insecticide tolerance, capable of invading crops in new areas. The findings of the current study that the kdr mutation is almost fixed and that the two skdr mutations are both at relatively high frequency in *T. absoluta* populations across its range would support this hypothesis. Indeed, taken together these studies suggest that the rapid expansion of *T. absoluta* over the last six years may have been in part mediated by the resistance of this pest to chemical insecticides.

Clearly our findings have significant implications for the control of *T. absoluta* with pyrethroid insecticides. Both M918T and T929I in combination with L1014F are known to give strong resistance across the entire class of synthetic pyrethroids. The fact that one or other of these mutation combinations are found within all of the field samples that were tested suggests that pyrethroids are likely to be ineffective at controlling *T. absoluta* across its range and alternative control agents should be sought.

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References

Alon, M., Benting, J., Lueke, B., Ponge, T., Alon, F., Morin, S., 2006. Multiple origins of pyrethroid resistance in sympatric biotypes of *Bemisia tabaci* (Hemiptera: Aleyrodidae). Insect Biochem. Mol. Biol. 36, 71–79.
Araujo, R.A., Williamson, M.S., Bass, C., Field, L.M., Duce, I.R., 2011. Pyrethroid resistance in *Sitophilus zeamais* is associated with a mutation (T929I) in the voltage-gated sodium channel. Insect Mol. Biol. 20, 437–445.
Branco, M.C., França, F.H., Medeiros, M.A., Leal, J.G.T., 2001. Use of insecticides for controlling the South American tomato pinworm and the diamondback moth: a case study. Horticultura Brasileira 19, 60–63.
Cifuentes, D., Chynoweth, R., Bielza, P., 2011. Genetic study of Mediterranean and South American populations of tomato leafminer *Tuta absoluta* (Povolny, 1994) (Lepidoptera: Gelechiidae) using ribosomal and mitochondrial markers. Pest Manag. Sci. 67, 1155–1162.
Davies, T.G.E., Williamson, M.S., 2009. Interactions of pyrethroids with the voltage-gated sodium channel. Bayer Crop Sci. J. 62, 159–178.
Davies, T.G., Field, L.M., Usherwood, P.N., Williamson, M.S., 2007. DDT, pyrethrins, pyrethroids and insect sodium channels. IUBMB Life 59, 151–162.
Desneux, N., Wajnberg, E., Wyckhuys, K., Burgio, G., Arpaia, S., Narváez-Vasquez, C., González-Cabrera, J., Catalán Ruescas, D., Tabone, E., Frandon, J., Pizzol, J., Poncet, C., Cabello, T., Urbaneja, A., 2010. Biological invasion of European tomato crops by *Tuta absoluta*: ecology, geographic expansion and prospects for biological control. J. Pest Sci. 83, 197–215.
Eleftherianos, I., Foster, S.P., Williamson, M.S., Denholm, I., 2008. Characterization of the M918T sodium channel gene mutation associated with strong resistance to pyrethroid insecticides in the peach-potato aphid, *Myzus persicae* (Sulzer). Bull. Entomol. Res. 98, 183–191.
Guedes, R.N.C., Picanço, M.C., Matioli, A.L., Rocha, D.M., 1994. Efeito de insecticides e sistemas de condução do tomateiro no control de *Scrobipalpuloides absoluta* (Meyrick) (Lepidoptera: Gelechiidae). An. Soc. Entomol. Bras. 23, 321–325.
Guerrero, F.D., Jamroz, R.C., Kammlah, D., Kunz, S.E., 1997. Toxicological and molecular characterization of pyrethroid-resistant horn flies, *Haematobia irritans*: identification of kdr and super-kdr point mutations. Insect Biochem. Mol. Biol. 27, 745–755.
Khambay, B.P.S., Farnham, A.W., Beddie, D.G., 1994. Relationships between pyrethroid structure and level of resistance in houseflies (*Musca domestica* L.). In: Briggs, C.G. (Ed.), Advances in the Chemistry of Insect Control III. The Royal Society of Chemistry, Cambridge, pp. 117–126.
Lee, S.H., Yoon, K.-S., Williamson, M.S., Goodson, S.J., Takano-Lee, M., Edman, J.D., Devonshire, A.L., Marshall Clark, J., 2000. Molecular analysis of kdr-like resistance in permethrin-resistant strains of head lice, *Pediculus capitis*. Pestic. Biochem. Physiol. 66, 130–143.
Lietti, M.M.M., Botto, E., Alzogaray, R.A., 2005. Insecticide resistance in Argentine populations of *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae). Neotrop. Entomol. 34, 113–119.
Long, S.B., Campbell, E.B., MacKinnon, R., 2005. Crystal structure of a mammalian voltage-dependent shaker family K+ channel. Science 309, 897–903.
Martinez-Torres, D., Devonshire, A.L., Williamson, M.S., 1997. Molecular studies of knockdown resistance to pyrethroids: cloning of domain II sodium channel gene sequences from insects. Pestic. Sci. 51, 265–270.
Nyoni, B.N., Gorman, K., Mzilahowa, T., Williamson, M.S., Navajas, M., Field, L.M., Bass, C., 2011. Pyrethroid resistance in the tomato red spider mite, *Tetranychus evansi*, is associated with mutation of the *para*-type sodium channel. Pest Manag. Sci. 67, 891–897.
O'Reilly, A.O., Khambay, B.P.S., Williamson, M.S., Field, L.M., Wallace, B.A., Davies, T.G.E., 2006. Modelling insecticide-binding sites in the voltage-gated sodium channel. Biochem. J. 396, 255–263.
Picancëo, M.C., Guedes, R.N.C., Leite, G.L.D., Fontes, P.C.R., Silva, E.A., 1995. Incidencia de *Scrobipalpuloides absoluta* (Meyrick) (Lepidoptera: Gelechiidae) em tomateiro sob diferentes sistemas de tutoramento e controle quõÂmico de pragas. Horticultura Brasileira 13, 180–183.
Roditakis, E., Tsagkarakou, A., Vontas, J., 2006. Identification of mutations in the para sodium channel of *Bemisia tabaci* from crete, associated with resistance to pyrethroids. Pestic. Biochem. Physiol. 85, 161–166.
Salazar, E.R., Araya, J.E., 1997. Deteccion de resistencia a insecticidas en la polilla del tomate. Simiente 67, 8–22.
Salazar, E.R., Araya, J.E., 2001. Tomato moth, *Tuta absoluta* (Meyrick), response to insecticides in Arica, Chile. Agricultura. Técnica. 61, 429–435.
Schuler, T.H., Martinez-Torres, D., Thompson, A.J., Denholm, I., Devonshire, A.L., Duce, I.R., Williamson, M.S., 1998. Toxicological, electrophysiological, and molecular characterisation of knockdown resistance to pyrethroid insecticides in the diamondback moth, *Plutella xylostella* (L. Pestic. Biochem. Physiol. 59, 169–182.
Silva, G.A., Picanco, M.C., Bacci, L., Crespo, A.L., Rosado, J.F., Guedes, R.N., 2011. Control failure likelihood and spatial dependence of insecticide resistance in the tomato pinworm, *Tuta absoluta*. Pest Manag. Sci. 67, 913–920.
Siqueira, H.Á.A., Guedes, R.N.C., Picanço, M.C., 2000. Insecticide resistance in populations of *Tuta absoluta* (Lepidoptera: Gelechiidae). Agric. For. Entomol. 2, 147–153.
Siqueira, H.A.A., Guedes, R.N.C., Fragoso, D.B., Magalhaes, L.C., 2001. Abamectin resistance and synergism in Brazilian populations of *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae). Int. J. Pest Manag. 47, 247–251.
Soderlund, D.M., Bloomquist, J.R., 1989. Neurotoxic actions of pyrethroid insecticides. Annu. Rev. Entomol. 34, 77–96.
Soderlund, D.M., Knipple, D.C., 2003. The molecular biology of knockdown resistance to pyrethroid insecticides. Insect Biochem. Mol. Biol. 33, 563–577.
Souza, J.C., Reis, P.R., Salgado, L.O., 1992. Tracea do Tomateiro: Historico, Reconhecimento, Biologia, Prejuõazos e Controle, vol. 20. Belo Horizonte: EPAMIG.
Tan, J., Liu, Z., Tsai, T.D., Valles, S.M., Goldin, A.L., Dong, K., 2002. Novel sodium channel gene mutations in Blattella germanica reduce the sensitivity of expressed channels to deltamethrin. Insect Biochem. Mol. Biol. 32, 445–454.
Usherwood, P.N.R., Davies, T.G.E., Mellor, I.R., O'Reilly, A.O., Peng, F., Vais, H., Khambay, B.P.S., Field, L.M., Williamson, M.S., 2007. Mutations in DIIS5 and the DIIS4–S5 linker of Drosophila melanogaster sodium channel define binding domains for pyrethroids and DDT. FEBS Lett. 581, 5485–5492.
Vais, H., Williamson, M.S., Goodson, S.J., Devonshire, A.L., Warmke, J.W., Usherwood, P.N.R., Cohen, C.J., 2000. Activation of drosophila sodium channels promotes modification by deltamethrin. J. Gen. Physiol. 115, 305–318.
Vais, H., Williamson, M.S., Devonshire, A.L., Usherwood, P.N.R., 2001. The molecular interactions of pyrethroid insecticides with insect and mammalian sodium channels. Pest Manag. Sci. 57, 877–888.
Williamson, M.S., Martinez Torres, D., Hick, C.A., Devonshire, A.L., 1996. Identification of mutations in the housefly *para*-type sodium channel gene associated with knockdown resistance (*kdr*) to pyrethroid insecticides. Mol. Gen. Genet. 252, 51–60.

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Status of pyrethroid resistance and mechanisms in Brazilian populations of *Tuta absoluta*

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Metabolism

A B S T R A C T

The tomato leafminer, *Tuta absoluta*, is a major pest of tomato crops worldwide. This study surveyed the resistance of *T. absoluta* populations from four regions in Brazil to pyrethroid insecticides, the frequencies of L1014F, T929I and M918T Na channel mutations, and the role of detoxification metabolism in the resistance. Resistance ratios varied from 1- to 11-times among populations and insecticides, but control failure likelihood assays showed that all pyrethroids assessed exhibited no efficacy at all (and thus, 98–100% control failure likelihood) against all *T. absoluta* populations. The activity of glutathione *S*-transferase and cytochrome P450-mediated N-demethylation in biochemical assays was significantly correlated with the level of resistance to deltamethrin and permethrin suggesting that these enzymes may play a role in resistance. TaqMan assays were used to screen for the presence of knockdown resistance (*kdr*) mutations and revealed that the L1014F *kdr* mutation was fixed in all populations and associated with two super-*kdr* mutations, M918T and particularly T929I, at high frequency. Altogether, results suggest that control failures are because of mutations in the domain II of the sodium channel, as a prevailing mechanism of resistance to pyrethroids in populations of *T. absoluta* in Brazil. But, enhanced cytochrome P450-dependent monooxygenases and GST activities also play an important role in the resistance of some populations, which reinforce that pyrethroids must not be used overall to control *T. absoluta*.
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1. Introduction

The tomato leafminer, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), is one of the most economically important insect pests of tomato worldwide [1–3]. The larval stages of *T. absoluta* feed on tomato leaves but also damage the flowers, fruits and stems [2,4], resulting in yield losses between 40 and 100% [5,6]. This species is native to South America with its presence confirmed in southern Brazil, in the early 1980s [7]. Since then it has spread to all major tomato-producing regions in Brazil often seriously compromising productivity due to its destructive capacity [4]. This pest has also become a major concern for tomato cultivation in Europe, Africa and the Middle East [8,9]. The control of *T. absoluta* in most settings has relied heavily on the use of chemical insecticides; unfortunately, this has resulted in the development of resistance, with populations now described with resistance to a wide range of compounds [10–17]. Although, only recently, characterisation of resistance mechanisms have had attention, which may be used as tools to survey populations [15,16].

Pyrethroids are an important class of synthetic insecticide widely used to control many arthropod pests, including *T. absoluta*, as a result of their rapid action, high insecticidal activity and low mammalian toxicity [18]. Pyrethroids interact with the voltage-gated sodium channel and modify its kinetic function, leading to nervous system exhaustion and death [19–21]. One of the main mechanisms of pyrethroid resistance is reduction of neuronal sensitivity, known as knockdown resistance (*kdr*) [19,22,23]. This type of resistance was first documented in the housefly (*Musca domestica*) and was subsequently shown to be caused by two amino acid substitutions in domain II of the channel, a leucine to phenylalanine (L1014F) replacement in transmembrane segment IIS6, termed '*kdr*' and a threonine to methionine substitution upstream of segment IIS5, with the latter associated with an enhanced form of resistance termed super-*kdr* [24]. Subsequently, additional mutations associated with pyrethroid resistance, primarily in domains II or III of the channel, have been identified in a range of arthropod species [25].

Pyrethroid resistance in *T. absoluta* has recently been reported to be associated with the presence of the mutations L1014F, M918T and T929I in the sodium channel [16]. These authors found all three

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mutations at high frequency in field strains collected from Europe and South America, although no field populations from Brazil have been screened to date for the presence of those mutations. Furthermore, the role of metabolic detoxification in pyrethroid resistance in this species has not yet been investigated. In other insects metabolic resistance to pyrethroids has been associated with elevated levels of cytochrome P450 monooxygenases (P450s), carboxylesterases (CEs), and glutathione *S*-transferases (GSTs) [26–28].

Metabolism has been in particular assessed only in Chilean populations [15], and it is well known that such mechanism can confer broad resistance to insecticides, which impacts more the agriculture of developing countries. Full characterisation of Brazilian populations of *T. absoluta* regarding resistance has long been a necessity for improving the chemical management of this pest. Here, we provide a survey of resistance of *T. absoluta* populations from different geographical regions of Brazil to three representative pyrethroids using biochemical and molecular approaches as well as toxicological measures.

2. Materials and methods

2.1. Insecticides

Concentration–response curves were estimated for each population of *T. absoluta* through bioassays of larvae mortality using the following insecticides: Deltamethrin (Decis 25 CE, Bayer CorpScience S.A, recommended label rate, 7.5 mg AI/l of water), alpha-cypermethrin (Fastac 100 SC, BASF S.A., recommended label rate, 10 mg AI/l of water) and permethrin (Valon 384 CE, Dow Agrosciences Industrial LTDA, recommended label rate, 49 mg AI/l of water). The efficacy of each insecticide to control *T. absoluta* using the recommend label rate was also assessed.

2.2. Insects

Eight different populations of tomato leaf miner from commercial tomato crops in the Northeast, Midwest, Southeast and South of Brazil were collected in the period between 2010 and 2011 (Table 1). Individual larvae were obtained from various parts of plants, including stems, leaves and fruits. The populations were established and reared individually on leaves of tomato variety "Santa Clara" under ambient conditions as described in Campos et al. [29].

2.3. Bioassays

A toxicological bioassay was conducted using a completely randomised design with two replications, and the whole bioassay was repeated twice. For control failure likelihood [39], bioassays were conducted with the label rates stated above for each insecticide. Full dose–response bioassays were carried out using 7–8 concentrations of each insecticide that resulted in mortality of between 0 and 100%. Distilled water plus Triton X-100 at 0.01% was used as the control treatment. Leaflets of tomato cultivar "Santa Clara" were cleaned using a solution based on sodium hypochlorite 5%. After cleaning the leaflets in tap water, they were immersed horizontally for a minute in insecticide or control solution. The leaflets were kept on paper towels at room temperature until completely dry and then transferred to Petri dishes (80 × 15 mm) containing filter paper misted with distilled water. Each replicate comprised 10 second instar (L2) larvae of *T. absoluta* placed on a treated leaflet in a petri-dish. Petri dishes were sealed and maintained in a climate chamber (BOD) set at an average temperature of 25 ± 1 °C, 65 ± 5% relative humidity and photoperiod of 12 h. Mortality was evaluated after 48 hours with the aid of a light source and magnifying glass (Olympus SZ61, Olympus®, Center Valley, PA, USA). The larvae were considered dead if they could not move at least the extent of their length after touching [30]. Mortality caused by insecticide treatment were corrected for control mortality, using Abbott's formula [31].

2.4. Sample extractions for enzyme assays

For enzyme assays, 10 L2 larvae of each population were transferred to a microfuge tube with three replicates for each assay. For esterase and glutathione *S*–transferase assays, each sample was homogenised in 200 µl of sodium phosphate buffer (0.02 M, pH 7.2) or sodium phosphate buffer (0.1 M, pH 7.5), respectively using a Potter-Elvehjem homogeniser. Homogenates were centrifuged at 15,000 *g* and 4 °C for 15 min and supernatants harvested and stored at –20 °C. For cytochrome P450 assays, samples were homogenised in 500 µl sodium phosphate buffer (0.1M, pH 7.5) + glycerol at 20% and microsomes were prepared in the same buffer. Homogenates were centrifuged at 15,000 *g* and 4 °C for 15 min and the supernatant was ultra-centrifuged at 100,000 *g* for 60 min in an Optima™ L-80 XP ultracentrifuge (Beckman Coulter, Palo Alto, CA) with the resulting microsomal pellet resuspended in homogenisation buffer containing 20% glycerol. Quantitation of protein was determined by the bicinchoninic acid method using bovine serum albumin (BSA) as standard [32].

2.5. Esterase assays

Esterase activity was measured with a method adapted from van Asperen [33]. Stock solutions (250 mM) of α-naphthyl acetate and β-naphthyl acetate substrates were prepared in acetone. For each reaction, 2 µl α-naphthyl acetate at 25 mM, 10 µl of sample diluted to 1:100 and 188 µl of sodium phosphate buffer (0.02M, pH 7.2) were used. The same procedure was carried out for esterase analysis using β-naphthyl acetate as substrate; however, the samples were diluted to 1:10. Reactions were incubated at 30 °C for 15 minutes then stopped using 33.2 µl of 0.3% FAST Blue B. Absorbance was read at 595 ηm on a microtitre plate reader (ELx800, BioTek®, Winooski, VT, USA). Each sample was analysed in triplicate. A standard curve

Table 1
Sites of *Tuta absoluta* populations collected in Brazil.

Populations	Geographic position	Collection date	History*
Anápolis – GO	16° 29′ 46″ S, 49° 25′ 35″ W	Dec/2011	Pyr, IGR, OPs, Avermectins
Guaraciaba do Norte – CE	4° 10′ 01″ S, 40° 44′ 51″ W	Feb/2010	Pyr, OPs, Cartap
Iraquara – BA	12° 14′ 55″ S, 41° 37′ 10″ W	Nov/2011	Pyr, IGR, OPs, Cartap, Spinosyns
Paulínia – SP	22° 45′ 40″ S, 47° 09′ 15″ W	Aug/2010	Pyr, IGR, OPs
Pelotas – RS	31° 46′ 19″ S, 52° 20′ 33″ W	Nov/2011	Pyr, IGR, OP, Cartap
Sumaré – SP	22° 49′ 19″ S, 47° 16′ 01″ W	Sept/2011	Pyr, IGR, OPs,
Tianguá – CE	3° 43′ 56″ S, 40° 59′ 30″ W	Feb/2010	Pyr, IGR, OP, Cartap
Venda Nova – ES	20° 20′ 23″ S, 41° 08′ 05″ W	Aug/2011	Pyr, IGR, OP, Cartap, Bt

* Pyr – pyrethroids, IGR – insect growth regulator, OPs – organophosphates, Bt – *Bacillus thuringiensis*.

was prepared with α-naphtol and β-naphtol. Esterase activity was expressed as ηMol naftol × min^{-1} × mg of $protein^{-1}$.

2.6. Glutathione S-transferase assays

Conjugation activity of reduced glutathione was determined using CDNB (1-chloro-2,4-dinitrobenzene) as substrate in the presence of glutathione *S*–transferase forming 2,4-dinitrophenyl-*S*-glutathione [34]. CDNB solution (150 mM) was prepared in ethanol and reduced glutathione (10 mM) was dissolved in sodium phosphate buffer (0.1M, pH 7.5). For each reaction, 138 µl of sodium phosphate buffer (0.1 M, pH 7.5), 10 µl of sample containing 1 µg of protein, and 150 µl of reduced glutathione (10 mM) were mixed and incubated in a water bath at 30 °C for 5 minutes then 2 µl of CDNB (150 mM) added to the reaction. The formation of 2,4-dinitrophenyl-*S*-glutathione was immediately measured at 340 ηm using a biophotometer (Eppendorf, Hamburg, Germany) with the reaction analysed for 5 minutes using read intervals of 30 s. Each sample was analysed in triplicate. Absorbance data were analysed as a function of reaction time after addition of CDNB. The slope of the line (absorbance/min) was transformed using the extinction coefficient of CDNB (9.6 $mM^{-1} \cdot cm^{-1}$).

2.7. Cytochrome P450 monooxygenase (O-demethylase) assays

Cytochrome P_{450} activity was determined by assessing the *O*-demethylation of the substrate *p*-nitroanisole ($O_2N—C_6H_4—O—CH_3$) to nitrophenol. Reactions were carried out by mixing 178.8 µl of sodium phosphate resuspension buffer (0.1 M, pH 7.5), 56.2 µl of sample, 2.5 µl *p*-nitroanisole (150 mM in ethanol) and 12.5 µl of reduced NADPH (9.6 mM) to each well of a microtitre plate in order. The mix was incubated for 15 minutes at 37 °C with HCl (1 M) added to stop the reaction. The reaction mix was then centrifuged at 14,000 *g* for 10 min, and 200 µl of the supernatant read at 405 ηm on a microtitre plate reader. Each sample was analysed in triplicate. Activity of cytochrome P_{450} per sample was determined based on a standard curve of *p*-nitrophenol and expressed as ηMol *p*-nitrophenol × min^{-1} × mg of $protein^{-1}$.

2.8. Cytochrome P450 monooxygenase (N-demethylation) assays

Assays were performed according to Scharf et al. [35].The substrate 4-chloro-N-methylaniline was used to determine N-demethylation activity. Reactions comprised 50 µl of sodium phosphate buffer with 2% Tween-20 (0.1 M, pH 7.5), 25 µl of sample, 25 µl of 4-chloro-*N*-methylaniline N-(4-CNMA) 7.5 mM diluted in 20% v/v ethanol, and 25 µl of reduced NADPH (9.6 mM). The reaction was processed for 16 min at 37 °C then stopped by the addition of 187.5 µl *p*-dimethylaminobenzaldehyde to 233.33 mM diluted in 3.0 N sulphuric acid. Samples were then centrifuged for 15 min at 10,000 *g* at 4 °C and 200 µl of the supernatant read at 450 ηm on a microtitre plate reader. Activity of cytochrome P450-dependent monooxygenases per sample was determined based on a standard curve of 4-chloroaniline and expressed as ηMol 4-chloroaniline × min^{-1} × mg $protein^{-1}$. Assays were replicated three times using three different protein preparations.

2.9. TaqMan diagnostic assays

Three TaqMan assays developed previously were used to genotype samples for the *kdr*/super-*kdr* mutations L1014F, M918T and T929I [16]. DNA was extracted from individual insects by grinding larvae in a microfuge tube using a micropestle and liquid nitrogen followed by extraction using DNAzol reagent (Life Technologies) following the manufacturer's protocol but reducing reagent volumes by 1/5th. PCR reactions (10 µl) contained 2 µl of genomic DNA, 7.5 µl of SensiMix DNA kit (Quantace, London, UK), 800 nM of each primer and 200 nM of each probe. Samples were run on a Rotor-Gene 6000 (Corbett Research, UK) using temperature cycling conditions of: 10 min at 95 °C followed by 40 cycles of 9 °C for 10 s and 60 °C for 45 s. The increase in fluorescence of the two probes was monitored in real time by acquiring each cycle on the yellow (530 nm excitation and 555 nm emission) and green channel (470 nm excitation and 510 nm emission) of the Rotor-Gene respectively. Reference template controls (a wild-type homozygous, a mutant homozygous and a heterozygous sample) were included in each run to aid genotype scoring.

2.10. Data analysis

Bioassay data were corrected for mortality observed in the control [31] and subjected to Probit analysis [36] using the POLO-Plus program [37] to estimate concentration–response curves for each population and insecticide. Confidence intervals at 95% probability were calculated for both LC_{50} and LC_{80} values. The resistance ratios (RR) were calculated using the LC_{50} value of the most susceptible population to each insecticide as the reference with the 95% confidence limits for each RR calculated using the method of Robertson and Preisler [38]. Differences in mortality of *T. absoluta* populations to the recommended label rate (control failure likelihood) for each insecticide were estimated according to Gontijo et al. [39] by comparing (using Student's paired t-test at $P < 0.05$) the corrected observed mortality with the minimum expected efficacy (80% mortality) required for insecticide registration by the Brazilian Ministry of Agriculture, Livestock and Supplement. Also, the frequency of populations in control failure was estimated as in Gontijo et al. [39] The mean values of esterase, glutathione *S*-transferase and cytochrome P450-dependent monooxygenase activity were subjected to analysis of variance (PROC ANOVA) and Tukey's test (HSD) $P < 0.05$ to identify significant differences using the SAS program [40]. *Pearson* correlation analysis between reduced susceptibility to insecticides and enzymatic activity of third-instar larvae as well as with the genotype frequencies of both *sKdR* mutations were estimated using PROC CORR [40].

3. Results

3.1. Bioassays

All of the pyrethroids assessed through the recommended field rate exhibited no efficacy at all (and thus, 98–100% control failure likelihood) against all of *T. absoluta* populations (Table 2). In full dose response bioassays the Tianguá-CE population had the lowest LC_{50} value for deltamethrin, while the Iraquara-BA population had the lowest LC_{50} values for the insecticides alpha-cypermethrin and permethrin (Table 3). Anápolis-GO was the most resistant population to deltamethrin (LC_{50} 561 mg/l), whereas Venda Nova-ES (LC_{50} 2595 mg/l) and Pelotas-RS (LC_{50} 1417 mg/l) were the most resistant populations to the insecticides alpha-cypermethrin and permethrin respectively (Table 3). The resistance ratios to deltamethrin, alpha-cypermethrin and permethrin ranged from 1.2 to 5.1; from 1.3 to 11.0 and 1.3 to 5.3 times respectively (Table 3) using the most susceptible population as a reference. The LC_{80} values for deltamethrin ranged from 273 (Tianguá-CE) to 1078 mg AI/l (Anápolis-GO), for alpha-cypermethrin 733 (Iraquara-BA) to 6756 mg AI/l (Venda Nova-ES), and for permethrin from 755 (Iraquara-BA) to 3335 mg AI/l (Pelotas-RS) (Table 3).

3.2. Enzyme assays

Biochemical assays of esterase activity differed significantly among populations of *T. absoluta* using the substrate α-naphthyl

Table 2
Corrected mortality (%) (±SE) of *Tuta absoluta* populations exposed to label rate of pyrethroids.

Region	Population	Deltamethrin (7.5 mg/l*)	α-Cypermethrin (10 mg/l*)	Permethrin (49 mg/l*)
Northeast	Guaraciaba – CE	0.0 ± 0.0*	0.5 ± 0.5*	1.0 ± 0.6*
	Iraquara – BA	0.0 ± 0.0*	1.0 ± 0.6*	0.5 ± 0.5*
	Tianguá – CE	1.5 ± 0.8*	0.0 ± 0.0*	0.0 ± 0.0*
Southeast	Paulínia – SP	0.0 ± 0.0*	0.0 ± 0.0*	0.0 ± 0.0*
	Sumaré – SP	0.0 ± 0.0*	0.0 ± 0.0*	0.0 ± 0.0*
	Venda Nova-ES	1.0 ± 0.6*	0.0 ± 0.0*	0.0 ± 0.0*
Central-West	Anápolis – GO	0.0 ± 0.0*	0.0 ± 0.0*	0.0 ± 0.0*
South	Pelotas – RS	0.0 ± 0.0*	0.0 ± 0.0*	0.0 ± 0.0*

* Mortality significantly lower than 80% (Student's paired *t*-test at $P < 0.05$).

acetate but not when using β-naphthyl acetate. The α-esterase activity ranged from 1.35 ± 0.09 mmol/min/mg (Anápolis-GO) to 2.09 ± 0.31 mmol/min/mg (Venda Nova-ES), while the β-esterase activity varied from 1.02 ± 0.06 mmol/min/mg (Anápolis-GO) to 1.30 ± 0.06 mmol/min/mg (Tianguá-CE). Assays of glutathione *S*-transferase (GST) activity showed significant differences between *T. absoluta* populations with variation of up to 3.12-fold observed among populations (Table 4). The Pelotas-RS population had the greatest GST activity (77.67 ± 1.97 μmol/min/mg) while the Tianguá-CE population had the lowest activity (24.83 ± 1.31 μmol/min/mg). The activity of cytochrome P450 monooxygenases using the model substrate 4-chloro-N-methylaniline differed significantly between the populations tested with variation of up to 5.7-fold with N-demethylation activity ranging from 1.03 ± 0.13 ηmol/min/mg for

Table 3
Relative toxicity of pyrethroids to L2 larvae of *Tuta absoluta*.

Insecticide	Population	n[a]	DF[b]	Slope ± SE[c]	LC_{50} (CI95%) (mg AI/l)	LC_{80} (CI95%) (mg AI/l)	χ^2	RR_{50} (CI95%)[d*]
α-Cypermethrin	Iraquara – BA	277	6	1.65 ± 0.22	234 (154–320)	733 (507–1183)	4.77	
	Guaraciaba – CE	308	6	1.96 ± 0.24	298 (224–378)	801 (626–1101).	2.66	1.3 (0.8–1.9)
	Paulínia – SP	277	5	2.37 ± 0.35	382 (236–520)	864 (638–1324)	5.30	1.6 (1.0–2.5)
	Pelotas – RS	292	6	1.72 ± 0.18	409 (552–1735)	1257 (895–1994)	6.28	1.7 (1.1–2.7)
	Anápolis – GO	272	5	3.20 ± 0.49	588 (495–699)	1093 (903–1402)	3.34	2.5 (1.7–3.7)
	Tianguá – CE	261	5	1.96 ± 0.34	656 (397–858)	1732 (1203–2946)	6.60	2.8 (1.8–4.3)
	Sumaré – SP	271	5	2.39 ± 0.24	1493 (1224–1823)	3157 (2534–4178)	1.60	6.1 (4.0–9.2)
	Venda Nova – ES	266	5	2.03 ± 0.21	2595 (1774–3873)	6756 (4440–13200)	8.30	10.8 (7.1–16.2)
Deltamethrin	Tianguá – CE	292	5	2.12 ± 0.21	110 (88–135)	273 (217–366)	2.90	
	Venda Nova – ES	250	5	1.14 ± 0.15	130 (68–213)	705 (400–1948)	6.08	1.2 (0.5–2.6)
	Iraquara – BA	248	5	1.41 ± 0.17	136 (82–212)	552 (350–1144)	3.04	1.3 (0.6–3.0.)
	Paulínia – SP	278	5	1.75 ± 0.18	181 (143–229)	547 (414–790)	1.01	1.6 (0.8–3.4)
	Sumaré – SP	267	5	2.03 ± 0.26	196 (135–281)	517 (351–943)	6.78	2.1 (1.0–4.6)
	Pelotas – RS	258	5	2.00 ± 0.22	269 (195–374)	612 (432–1019)	6.40	2.4 (1.8–3.3)
	Guaraciaba – CE	272	5	2.83 ± 0.34	282 (231–341)	560 (455–737)	4.18	2.5 (1.2–5.2)
	Anápolis – GO	279	5	2.96 ± 0.33	561 (415–780)	1078 (776–1893)	8.20	5.1 (2.5–10.5)
Permethrin	Iraquara – BA	281	6	1.87 ± 0.21	269 (205–342)	755 (582–1052)	5.49	
	Paulínia – SP	261	5	1.90 ± 0.29	338 (215–460)	783 (490–1330)	0.90	1.3 (0.8–2.0)
	Tianguá – CE	270	5	1.46 ± 0.20	455 (293–627)	1716 (1270–2510)	1.65	1.7 (1.0–2.6)
	Guaraciaba – CE	280	5	2.05 ± 0.26	508 (293–737)	1310 (904–2254)	7.54	1.8 (1.3–2.7)
	Venda Nova – ES	271	5	2.82 ± 0.32	659 (534–796)	1180 (851–1803)	1.78	2.4 (1.7–3.3)
	Anápolis – GO	244	4	2.06 ± 0.24	801 (633–991)	2048 (1611–2820)	3.12	3.0 (2.2–4.1)
	Sumaré – SP	271	5	2.28 ± 0.24	1074 (869–1315)	2509 (1999–3358)	0.50	4.0 (2.8–5.5)
	Pelotas – RS	275	5	2.27 ± 0.23	1417 (1059–1871)	3335 (2470–5066)	5.48	5.3 (3.8–7.3)

[a] Total number of insects bioassayed.
[b] Degree of freedom.
[c] Standard ERROR.
[d] Resistance ratio: ratio of LC_{50} estimative between resistance and susceptible populations calculated through Robertson and Preisler's [38] method with confidence interval at 95%.
* Resistance ration significant if confidence interval does not encompass the value 1.0.

Table 4
Mean activity of detoxificative enzymes from *T. absoluta* populations.

Population	α-esterase mmol/min/mg	β-esterase mmol/min/mg	GST μmoles/min/mg	CypO ηmoles/min/mg	CypN ηmoles/min/mg
Anápolis – GO	1.35 ± 0.09 cd*	1.02 ± 0.06 a	76.56 ± 1.10 a	14.75 ± 0.77 ba	5.86 ± 0.17 a
Guaraciaba – CE	2.07 ± 0.07 ab	1.14 ± 0.02 a	71.29 ± 1.81 b	5.57 ± 0.49 dc	4.43 ± 0.30 bc
Iraquara – BA	2.32 ± 0.10 a	1.07 ± 0.06 a	65.00 ± 1.54 c	18.13 ± 1.31 a	3.15 ± 0.29 d
Paulínia – SP	1.88 ± 0.05 abc	1.26 ± 0.04 a	54.97 ± 0.20 e	4.23 ± 0.72 d	1.03 ± 0.13 e
Pelotas – RS	1.88 ± 0.10 d	1.15 ± 0.10 a	77.67 ± 1.97 b	14.39 ± 1.48 ab	4.99 ± 0.39 ab
Sumaré – SP	1.62 ± 0.10 bcd	1.27 ± 0.08 a	68.61 ± 2.24 d	14.56 ± 2.19 ab	2.28 ± 0.23 d
Tianguá – CE	2.10 ± 0.09 ab	1.30 ± 0.06 a	24.83 ± 1.31 c	10.58 ± 1.50 bc	3.30 ± 0.12 cd
Venda Nova – ES	2.09 ± 0.31 ab	1.27 ± 0.07 a	71.25 ± 2.09 b	14.59 ± 1.50 ab	4.48 ± 0.30 b

* Means followed by the same letter are not statistically different by Tukey's test at 5% probability.

Table 5
Kdr genotyping and allelic frequencies (L1014F, M918T, T929I) in *T. absoluta* populations from Brazil determined by TaqMan assays.

Population	L1014F			Frequency	M918T			Frequency	T929I			Frequency
	RR (%)	RS (%)	SS (%)		RR (%)	RS (%)	SS (%)		RR (%)	RS (%)	SS (%)	
Anápolis – GO	100	00	00	100	11	33	56	28	60	30	10	75
Guaraciaba – CE	100	00	00	100	10	50	40	35	20	50	30	45
Iraquara – BA	100	00	00	100	30	20	50	40	40	20	40	50
Paulínia – SP	100	00	00	100	10	40	50	30	60	40	0	80
Pelotas – RS	100	00	00	100	70	20	10	80	10	30	60	25
Sumaré – SP	100	00	00	100	00	30	70	15	70	30	00	85
Tianguá – CE	100	00	00	100	10	50	40	35	40	50	10	65
Venda Nova – ES	100	00	00	100	0	10	90	5	90	10	00	95

S: susceptible allele; R: resistant allele.

the Paulínia-SP population to 5.86 ± 0.17 μmol/min/mg for Anápolis-GO (Table 4). *O*-demethylation activity varied by up to 3.5-fold among the *T. absoluta* populations with activity of 4.23 ± 0.72 ηmol/min/mg for the Paulínia-SP population to 14.75 ± 0.77 ηmol/min/mg for Anápolis-GO (Table 4).

3.3. TaqMan diagnostic assays

TaqMan assays revealed that the L1014F, M918Tand T929I *Kdr* mutations were at high overall frequency in the eight *T. absoluta* populations (Table 5). The frequency of the L1014F mutation was fixed at 100% in all eight populations (Table 5). The overall frequency of T929I was higher (0.65) than M918T (0.37) and this was also the case for each individual population except for Pelotas-RS where M918T was at higher frequency (0.80) than T929I (0.25) (Table 5). All individuals tested carried either M918T or T929I in combination with L1014F apart from a single individual of the Iraquara-BA population which only had the L1014F mutation. As reported previously [16], the M918T and T929I mutations were only found in combination in individual larvae when both mutations were in the heterozygous form. Overall the most common genotype observed was F1014 (homozygous) + M918 (homozygous) + I929 (homozygous) followed by the genotype F1014 (homozygous) + M918T (heterozygous) + T929I (heterozygous).

3.4. Correlations

To investigate whether there is a relationship between the level of resistance to pyrethroids, and the enzymatic activities of different *T. absoluta* populations, enzyme activity was correlated with the LC_{50} values obtained in bioassays (Table 6). Esterase biochemical assays were negatively correlated with resistance using the substrate α-naphthyl acetate for deltamethrin (r = –0.45) and permethrin (r = –0.61). Activity for the substrate β-naphthyl acetate had a significant negative correlation with LC_{50} values for deltamethrin (r = –0.33), for permethrin the correlation was not significant (r = –0.02) and for alpha-cypermethrin there was a low, albeit, significant positive correlation (r = 0.23). A significant positive correlation was observed between the LC_{50} values of deltamethrin and permethrin and the enzymatic activity of GSTs (*r* = 0.50); but there was no correlation between GST_s activity and the insecticide alpha-cypermethrin. P450 enzyme activity using the substrate 4-chloro-N-methylaniline correlated significantly with the LC_{50} values of deltamethrin (r = 0.56) and permethrin (r = 0.36) but not with alpha-cypermethrin whereas P450 activity using 4-nitroanisole as substrate showed a modest significant correlation with the LC_{50} values to alpha-cypermethrin. The LC_{50} values of deltamethrin and permethrin were significantly correlated (r = 0.50); however, the insecticide alpha-cypermethrin showed only a modest negative correlation with deltamethrin (*r* = –0.28) (Table 6). Genotype frequencies correlated only between T929I and alpha-cypermethrin (*r* = 0.78, P = 0.023, N = 8) variables. Also, negative significant correlation was observed between T929I and M918T (*r* = –0.73, P = 0.040, N = 8) variables. No correlation was observed for the other pairwise variables, either using genotype or allelic frequencies.

4. Discussion

Pyrethroids were first registered for control of *T. absoluta* in Brazil in 1980 [7] and resistance to the pyrethroid permethrin was first reported in Brazilian populations in 2000 [41]. Since then resistance to bifenthrin and deltamethrin in populations in Brazil has also been described [14]. To date, recommended label rate of permethrin, deltamethrin and alpha-cypermethrin exhibit total control failure of *T. absoluta* populations collected from eight different regions of Brazil, scenario previously showed in Brazil to pyrethroids [14,39]. Resistance ratios calculated from full dose response bioassays varied only from ~1- to 11-times (compared with the most susceptible population), underestimated because of lack of a fully pyrethroid susceptible strain for use as a reference. A similar

Table 6
Pearson correlation coefficients between reduced susceptibility to insecticides and enzymatic activity of third-instar larvae in field populations of *Tuta absoluta*.

		Deltamethrin	α-Cypermethrin	Permethrin
Insecticides	Deltamethrin	–		
	α-Cypermethrin	r = –0.26*	–	
	Permethrin	r = 0.50****	r = 0.18ns	–
Substrate	α-Naphthyl acetate	r = –0.45****	r = 0.07ns	r = –0.61***
	β-Naphthyl acetate	r = –0.33***	r = 0.23*	r = –0.02ns
	CDNB	r = 0.50****	r = 0.13ns	r = 0.47****
	4-Chloro-*N*-Methylaniline	r = 0.56**	r = 0.05ns	r = 0.36****
	4-Nitroanisole	r = –0.04ns	r = 0.22ns	r = 0.26*

* *P* < 0.05.
** *P* < 0.01.
*** *P* < 0.001.
**** *P* < 0.0001.
ns Not significant.

problem has recently been described by others and likely results from widespread resistance to this chemical class worldwide [12,16,17]. The development of insecticide resistance in Brazilian populations of *T. absoluta* is perhaps unsurprising given the high selection pressure from insecticide use in agricultural regions where tomato growers may apply insecticides up to three times a week during the growing season [42]. For many years, permethrin and deltamethrin along with the nereistoxin-derived cartap were the few insecticides registered for use against *T. absoluta* leading to widespread use [43]. Although pyrethroids are no longer widely used to control *T. absoluta* in Brazil, small and unassisted growers still make use of them because of their cheaper prices. Such practice, not only provides inefficacy, but also worsens the resistance scenario to pyrethroids against other pests and other insecticides against *T. absoluta*.

Three approaches were used in this survey to determine the status of *T. absoluta* resistance to pyrethroids in Brazil: toxicological assessment, biochemical and molecular tools. Biochemical investigation of the major enzymes, most frequently implicated in metabolic resistance (P450s, GSTs and CEs), suggested P450s and GSTs may play a role in resistance to certain pyrethroids, but there was less evidence that CEs contribute to resistance in these strains. The *N*-demethylation activity of microsomal preparations of the different *T. absoluta* populations varied by 5.7-fold and significantly correlated with the level of resistance to deltamethrin and permethrin suggesting enhanced P450 monooxygenases activity may contribute to resistance to these insecticides. In contrast *O*-demethylation activity using the model substrate 4-nitroanisole showed no significant correlation with resistance to any of the pyrethroids. A plausible explanation for the latter result is related to the structure of the pyrethroids in question, which do not have methoxy or alkoxy groups. In other insect pests P450s dependent monooxygenases have been shown to metabolise deltamethrin and permethrin to less toxic secondary metabolites such as 4-hydroxy-deltamethrin and 4-hydroxy-permethrin [44–47]. Further analysis of the metabolic fate of these insecticides in *T. absoluta* is required to confirm if a similar route of P450-mediated detoxification occurs in resistant strains of this species.

Variation among the *T. absoluta* populations in this study was also observed for GST activity with enzyme activity significantly correlating with the level of resistance (LC_{50} values) to permethrin and deltamethrin in dose–response bioassays. These findings suggest a role for this enzyme system in resistance to these two pyrethroids. In other insect species pyrethroids have not been shown to be directly metabolised by GSTs, rather studies have suggested they may sequester pyrethroids until they are metabolised by other detoxification enzymes or protect against lipid peroxidation products and oxidative stress induced by pyrethroid exposure [48,49]. Further work is required to investigate these two possibilities in *T. absoluta*.

In other insects enhanced expression of esterases has been shown to confer modest levels of resistance to pyrethroid insecticides [28]; however, there was little indication of a role for this enzyme class in the resistance of *T. absoluta* populations from Brazil using two substrates for this enzyme family, with only moderate levels of positive correlation herein seen between LC_{50} values of alpha cypermethrin and activity to the substrate β-naphthyl acetate.

One of the primary mechanisms of pyrethroid resistance in many insect species is kdr-type mutation of the voltage-gated sodium channel [50]. Indeed three such mutations have been previously identified at known resistance 'hot spots' in pyrethroid resistant field populations of *T. absoluta* from 12 countries [16], although this did not include populations from Brazil (see introduction). In the current study TaqMan diagnostic assays demonstrated that the same three mutations, L1014F, M918T and T929I, are fixed or at high frequency in *T. absoluta* populations in Brazil. The L1014F mutation was fixed in all populations analysed. In other insects this mutation confers moderate resistance factors of 10 to 30 times to pyrethroids with cross-resistance observed across all pyrethroids [19,50,51]. The 100% frequency of this mutation in Brazilian populations is consistent with results observed in populations of *T. absoluta* from Europe and other South American countries where the L1014F mutation has also undergone fixation [16]. Indeed, to date, only populations from Colombia have been observed that do not carry L1014F at 100% frequency [16].

Almost all individuals tested in the current study carried L1014F in combination with either M918T or T929I, and, as reported previously [16], the M918T and T929I mutations were only found in combination in individual larvae when both mutations were in the heterozygous form. Both these mutations, when found in combination with L1014F, are known to give high levels of resistance to a range of different pyrethroids [50]. The overall frequency of T929I (0.65) was higher than M918T (0.37), consistent with the previous study [16] and suggesting that the T929I mutation may provide a slight selective advantage over M918T. This appears to be the case in *T. absoluta*, because high positive correlation was observed between alpha cypermethrin and the frequency of the T929I mutation. No significant correlation was observed between the genotype or allelic frequency of the M918T mutation and variability in the resistance of the eight populations to permethrin and deltamethrin. Indeed, when mutation frequency and resistance phenotype is compared it is apparent that certain populations with similar mutation frequencies (i.e. Paulínia-SP and Sumaré-SP) display different levels of resistance to certain pyrethroids providing further support to the hypothesis that additional metabolic mechanisms contribute to resistance as discussed above.

In summary the current study shows that target site resistance is widespread in *T. absoluta* populations in Brazil and may be further enhanced by metabolic detoxification. The *kdr* mutation is uniformly fixed and the two *skdr* mutations are at relatively high frequency across Brazilian *T. absoluta* populations as they were in 12 other countries [16]. The presence of these mechanisms confers strong resistance to three representative pyrethroids rendering the use of these insecticides ineffective for control of *T. absoluta* in Brazil. Continued use of pyrethroids by small growers may impose a scenario of resistance for other pests, and thus they must not be used overall to control *T. absoluta*.

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References

[1] R.N.C. Guedes, M.C. Picanço, The tomato borer *Tuta absoluta* in South America: pest status, management and insecticide resistance, Bull. OEPP. 42 (2012) 211–216.
[2] M.C. Picanço, F.G. Faleiro, A. Pallini Filho, A.L. Matioli, Perdas na produtividade do tomateiro em sistemas alternativos de controle fitossanitário, Hortic. Bras. 15 (1997) 88–91.
[3] N. Desneux, E. Wajnberg, K.G. Wyckhuys, G. Burgio, S. Arpaia, C. Narváez-Vasquez, et al., Biological invasion of European tomato crops by *Tuta absoluta*: ecology, geographic expansion and prospects for biological control, J. Pest. Sci. 83 (2010) 197–215.

[4] M.C.F. Coelho, F.H. França, Biologia e quetotaxia da larva e descrição da pupa e adulto da traça-do-tomateiro, Pesqu. Agropecu. Bras. 22 (1987) 129–135.
[5] T.A. Giustolin, J.D. Vendramim, S.B. Alves, S.A. Vieira, R.M. Pereira, Susceptibility of *Tuta absoluta* (Meyrick) (Lep., Gelechiidae) reared on two species of Lycopersicon to *Bacillus thuringiensis* var. kurstaki, J. Appl. Entomol. 125 (2001) 551–556.
[6] F.A. Oliveira, D.J.H. da Silva, G.L.D. Leite, G.N. Jham, M. Picanço, Resistance of 57 greenhouse-grown accessions of Lycopersicon esculentum and three cultivars to Tuta absoluta (Meyrick) (Lepidoptera: Gelechiidae), Sci. Hort. 119 (2009) 182–187.
[7] G.J. Morais, J.A. Normanha Filho, Surto de *Scropipalpula absoluta* (Meyrick) em tomateiro no Trópico Semi-Árido, Pesqu. Agropecu. Bras. 17 (1982) 503–504.
[8] N. Desneux, M.G. Luna, T. Guillemaud, A. Urbaneja, The invasive South American tomato pinworm, *Tuta absoluta*, continues to spread in Afro-Eurasia and beyond: the new threat to tomato world production, J. Pest. Sci. 84 (2011) 403–408.
[9] A. Urbaneja, R. Vercher, V. Navarro, J.L. Porcuna, F. García-Marí, La polilla del tomate, *Tuta absoluta*, Phytoma Esp. 194 (2007) 16–24.
[10] M.M.M. Lietti, E. Botto, R.A. Alzogaray, Insecticide resistance in Argentine populations of *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), Neotrop. Entomol. 34 (2005) 113–119.
[11] E.R. Salazar, J.E. Araya, Respuesta de la polilla del tomate, *Tuta absoluta* (Meyrick), a insecticidas en Arica, Agric. Tecn. 61 (2001) 429–435.
[12] H.A.A. Siqueira, R.N.C. Guedes, M.C. Picanço, Insecticide resistance in populations of Tuta absoluta (Lepidoptera: Gelechiidae), Agric. For. Entomol. 2 (2000) 147–153.
[13] H.A.A. Siqueira, R.N.C. Guedes, D.B. Fragoso, L.C. Magalhaes, Abamectin resistance and synergism in Brazilian populations of *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), Int. J. Pest Manage. 47 (2001) 247–251.
[14] G.A. Silva, M.C. Picanço, L. Bacci, A.L.B. Crespo, J.F. Rosado, R.N.C. Guedes, Control failure likelihood and spatial dependence of insecticide resistance in the tomato pinworm, *Tuta absoluta*, Pest. Manag. Sci. 67 (2011) 913–920.
[15] M. Reyes, K. Rocha, L. Alarcón, M. Siegwart, B. Sauphanor, Metabolic mechanisms involved in the resistance of field populations of *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) to spinosad, Pestic. Biochem. Physiol. 102 (2012) 45–50.
[16] K. Haddi, M. Berger, P. Bielza, D. Cifuentes, L.M. Field, K. Gorman, et al., Identification of mutations associated with pyrethroid resistance in the voltage-gated sodium channel of the tomato leaf miner (Tuta absoluta), Insect Biochem. Mol. Biol. 42 (2012) 506–513.
[17] E. Roditakis, C. Skarmoutsou, M. Staurakaki, Toxicity of insecticides to populations of tomato borer Tuta absoluta (Meyrick) from Greece, Pest. Manag. Sci. 69 (2013) 834–840.
[18] F.D. Rinkevich, Y. Du, K. Dong, Diversity and convergence of sodium channel mutations involved in resistance to pyrethroids, Pestic. Biochem. Phys. 106 (2013) 93–100.
[19] D.M. Soderlund, D.C. Knipple, The molecular biology of knockdown resistance to pyrethroid insecticides, Insect Biochem. Mol. Biol. 33 (2003) 563–577.
[20] A.L. Goldin, Mechanisms of sodium channel inactivation, Curr. Opin. Neurobiol. 13 (2003) 284–290.
[21] T. Narahashi, Neuronal ion channels as the target sites of insecticides, Pharmacol. Toxicol. 79 (1996) 1–14.
[22] K. Dong, Insect sodium channels and insecticide resistance, Invertebr. Neurosci. 7 (2007) 17–30.
[23] D. Soderlund, Molecular mechanisms of pyrethroid insecticide neurotoxicity: recent advances, Arch. Toxicol. 86 (2012) 165–181.
[24] M. Williamson, D. Martinez-Torres, C. Hick, A. Devonshire, Identification of mutations in the housefly para-type sodium channel gene associated with knockdown resistance (kdr) to pyrethroid insecticides, Mol. Gen. Genet. 252 (1996) 51–60.
[25] T.G.E. Davies, L.M. Field, P.N.R. Usherwood, M.S. Williamson, DDT, pyrethrins, pyrethroids and insect sodium channels, IUBMB Life 59 (2007) 151–162.
[26] W.Y. Low, H.L. Ng, C.J. Morton, M.W. Parker, P. Batterham, C. Robin, Molecular evolution of glutathione S-transferases in the genus Drosophila, Genetics 177 (2007) 1363–1375.
[27] H. Ranson, C. Claudianos, F. Ortelli, C. Abgrall, J. Hemingway, M.V. Sharakhova, et al., Evolution of supergene families associated with insecticide resistance, Science 298 (2002) 179–181.
[28] X. Li, M.A. Schuler, M.R. Berenbaum, Molecular mechanisms of metabolic resistance to synthetic and natural xenobiotics, Annu. Rev. Entomol. 52 (2007) 231–253.
[29] M.R. Campos, T.B.M. Silva, W.M. Silva, J.E. Silva, H.A.A. Siqueira, Susceptibility of *Tuta absoluta* (Lepidoptera: Gelechiidae) Brazilian populations to ryanodine receptor modulators, Pest. Manag. Sci. (2014) 10.1002/ps.3835.
[30] B.E. Tabashnik, N.L. Cushing, N. Finson, M.W. Johnson, Field development of resistance to *Bacillus thuringiensis* in diamondback moth (Lepidoptera: Plutellidae), J. Econ. Entomol. 83 (1990) 1671–1676.
[31] W.S. Abbott, A method of computing the effectiveness of an insecticide, J. Econ. Entomol. 18 (1925) 265–267.
[32] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, et al., Measurement of protein using bicinchoninic acid, Anal. Biochem. 150 (1985) 76–85.
[33] K. van Asperen, A study of housefly esterases by means of a sensitive colorimetric method, J. Insect Physiol. 8 (1962) 401–416.
[34] W.H. Habig, M.J. Pabst, W.B. Jakoby, Glutathione S-transferases. The first enzymatic step in mercapturic acid formation, J. Biol. Chem. 249 (1974) 7130–7139.
[35] M.E. Scharf, B.D. Siegfried, L.J. Meinke, R.J. Wright, L.D. Chandler, Cytochrome P450-Mediated N-Demethylation Activity and Induction in Insecticide-Resistant and Susceptible Western Corn Rootworm Populations (Coleoptera: Chrysomelidae), Pestic. Biochem. Physiol. 67 (2000) 137–143.
[36] D.J. Finney, Probit Analysis, London, England, 1971.
[37] L.-S. POLO-Plus, POLO for Windows Computer Program, Version 2.0, LeOra-Software, Petaluma, CA, 2005.
[38] J.L. Robertson, H.K. Preisler, Pesticide Bioassays with Arthropods, CRC Press, Inc, Boca Raton, FL, 1992.
[39] P.C. Gontijo, M.C. Picanço, E.J.G. Pereira, J.C. Martins, M. Chediak, R.N.C. Guedes, Spatial and temporal variation in the control failure likelihood of the tomato leaf miner, *Tuta absoluta*, Ann. Appl. Biol. 162 (2013) 50–59.
[40] S.A.S. Institute, SAS User's Guide: Statistics, Version 8.2, SAS Institute, Cary, NC, 2001.
[41] H.A.A. Siqueira, R.N.C. Guedes, M.C. Picanço, Cartap resistance and synergism in populations of *Tuta absoluta* (Lep., Gelechiidae), J. Appl. Entomol. 124 (2000) 233–238.
[42] R.N.C. Guedes, H.A.A. Siqueira, The tomato borer *Tuta absoluta*: insecticide resistance and control failure, CAB Rev. 7 (2012) 1–7.
[43] J.C. Souza, P.R. Reis, Controle da traça-do-tomateiro em Minas Gerais, Pesqu. Agropecu. Bras. 21 (1986) 343–354.
[44] T. Martin, O.G. Ochou, M. Vaissayre, D. Fournier, Oxidases responsible for resistance to pyrethroids sensitize Helicoverpa armigera (Hübner) to triazophos in West Africa, Insect Biochem. Mol. Biol. 33 (2003) 883–887.
[45] Y. Yang, Y. Wu, S. Chen, G.J. Devine, I. Denholm, P. Jewess, et al., The involvement of microsomal oxidases in pyrethroid resistance in Helicoverpa armigera from Asia, Insect Biochem. Mol. Biol. 34 (2004) 763–773.
[46] B.J. Stevenson, J. Bibby, P. Pignatelli, S. Muangnoicharoen, P.M. O'Neill, L.-Y. Lian, et al., Cytochrome P450 6M2 from the malaria vector Anopheles gambiae metabolizes pyrethroids: sequential metabolism of deltamethrin revealed, Insect Biochem. Mol. Biol. 41 (2011) 492–502.
[47] C.T. Zimmer, C. Bass, M.S. Williamson, M. Kaussmann, K. Wölfel, O. Gutbrod, et al., Molecular and functional characterization of CYP6BQ23, a cytochrome P450 conferring resistance to pyrethroids in European populations of pollen beetle, Meligethes aeneus, Insect Biochem. Mol. Biol. 45 (2014) 18–29.
[48] I. Kostaropoulos, A.I. Papadopoulos, A. Metaxakis, E. Boukouvala, E. Papadopoulou-Mourkidou, Glutathione S–transferase in the defence against pyrethroids in insects, Insect Biochem. Mol. Biol. 31 (2001) 313–319.
[49] J.G. Vontas, G.J. Small, J. Hemingway, Glutathione S-transferases as antioxidant defence agents confer pyrethroid resistance in Nilaparvata lugens, Biochem. J. 357 (2001) 65–72.
[50] T.G.E. Davies, M.S. Williamson, Interactions of pyrethroids with the voltage-gated sodium channel, Bayer. CropSci. J. 62 (2009) 159–178.
[51] H. Vais, M.S. Williamson, S.J. Goodson, A.L. Devonshire, J.W. Warmke, P.N.R. Usherwood, et al., Activation of drosophila sodium channels promotes modification by deltamethrin: reductions in affinity caused by knock-down resistance mutations, J. Gen. Physiol. 115 (2000) 305–318.

Appendix 3A. Contigs with hits to cytochrome p450s, 454 sequencing (Assembly 1)

Contig ID.	Length	NCBI top hit	Accession	Species	E-value	Alignment length
518	1087	cytochrome 9A20	BAI47532.1	*Bombyx mori*	5.03E-152	314
1743	579	antennal cytochrome P450 CYP9	AAR26518.1	*Mamestra brassicae*	2.10E-70	99
1744	752	cytochrome P450	AAV28704.1	*Helicoverpa armigera*	5.74E-34	79
1745	527	cytochrome P450	AAV28704.1	*Helicoverpa armigera*	1.26E-35	80
3943	1638	cytochrome P450	AAP83689.1	*Depressaria*	0.00E+00	515
7511	922	cytochrome P450	ABB69054.1	*Helicoverpa armigera*	4.90E-128	271
8044	428	cytochrome P450 CYP304F2	ACZ97412.2	*Zygaena filipendulae*	4.15E-61	140
10449	618	cytochrome P450	AAT08964.1	*Helicoverpa armigera*	7.69E-77	165
12112	1292	cytochrome P450 9G3	EHJ71541.1	*Danaus plexippus*	4.10E-117	256
12119	657	cytochrome P450 9G3	NP_001108456.1	*Bombyx mori*	1.06E-85	221
12673	280	cytochrome P450 332A4	ADE05587.1	*Manduca sexta*	1.42E-35	87
12674	657	cytochrome P450 332A4	ADE05587.1	*Manduca sexta*	1.65E-60	112
12675	430	cytochrome P450 CYP332A1	NP_001108340.1	*Bombyx mori*	7.75E-50	132
12678	279	cytochrome P450 332A4	ADE05587.1	*Manduca sexta*	1.95E-35	87
15216	1609	cytochrome P450 4g15	BAM17765.1	*Papilio xuthus*	0.00E+00	492
15217	293	cytochrome P450 4g15	BAM17765.1	*Papilio xuthus*	2.30E-30	65
15221	293	cytochrome P450 4g15	BAM17765.1	*Papilio xuthus*	6.14E-30	65
15876	766	cytochrome P450 4c3	BAM19419.1	*Papilio xuthus*	8.62E-42	167
17597	713	cytochrome P450 9G3	NP_001108456.1	*Bombyx mori*	1.71E-78	146
19047	1660	cytochrome P450 332A5	EHJ67573.1	*Danaus plexippus*	1.27E-165	482
21608	563	cytochrome P450 6a8	BAM19219.1	*Papilio polytes*	2.23E-79	188
21609	1043	cytochrome P450 6B45	ADE05578.1	*Manduca sexta*	1.51E-14	63

21610	602	cytochrome P450 6B45	ADE05578.1	*Manduca sexta*	1.86E-15	63
21794	1377	CYP6AB4	ABJ97708.1	*Bombyx mandarina*	0.00E+00	419
21795	373	cytochrome P450 6a13	BAM18744.1	*Papilio xuthus*	3.40E-29	70
21796	529	cytochrome P450	AEL87782.1	*Spodoptera litura*	6.08E-73	173
21803	1028	cytochrome P450	AAV28704.1	*Helicoverpa armigera*	4.80E-75	156
22301	1508	cytochrome P450	AAP83689.1	*Depressaria*	1.15E-102	282
22302	177	cytochrome CYP6AE50	AFP20594.1	*Spodoptera littoralis*	6.23E-07	40
23831	155	CYP6AB3v2	ABL60878.1	*Depressaria*	5.33E-07	51
23832	779	cytochrome P450 6AB13	ADE05584.1	*Manduca sexta*	2.92E-99	203
23833	752	cytochrome P450 6AB13	ADE05584.1	*Manduca sexta*	4.15E-99	203
24776	664	CYP6AB4	ABJ97708.1	*Bombyx mandarina*	6.49E-98	221
24777	378	cytochrome P450 6a13	BAM18744.1	*Papilio xuthus*	2.15E-40	118
24778	244	CYP6AB4	EHJ69768.1	*Danaus plexippus*	9.91E-39	81
26114	412	cytochrome P450 333B11	ADE05592.1	*Manduca sexta*	1.06E-38	106
26405	160	cytochrome 6AB4	BAI47531.1	*Bombyx mori*	1.28E-08	54
26406	779	cytochrome P450	CAZ65618.1	*Cnaphalocrocis*	2.73E-30	108
28222	1753	gossypol-induced cytochrome P450	ADW23116.1	*Helicoverpa armigera*	5.37E-109	363
28356	1594	microsomal cytochrome P450	ABY47596.1	*Helicoverpa armigera*	0.00E+00	473
28748	1376	cytochrome P450 CYP4L4	AAL48300.1	*Mamestra brassicae*	5.87E-104	262
29661	1149	cytochrome P450 CYP302A1	ACM46003.1	*Spodoptera littoralis*	1.66E-137	213
29969	1109	CYP6AB3v2	ABL60878.1	*Depressaria*	1.21E-42	116
30104	1094	cytochrome P450 12a5, partial	BAM20328.1	*Papilio polytes*	3.63E-44	119
30125	1090	cytochrome P450 CYP333B8	ACZ97415.1	*Zygaena filipendulae*	9.14E-52	147
30174	1085	cytochrome P450	EHJ76072.1	*Danaus plexippus*	1.14E-38	98
30493	1045	CYP6AB7	ABL60877.1	*Depressaria*	4.55E-127	290
30820	997	cytochrome P450 332A5	EHJ67573.1	*Danaus plexippus*	2.48E-64	280

31463	903	cytochrome P450	CAZ65618.1	*Cnaphalocrocis*	3.48E-57	234
31610	924	cytochrome P450	ABB69054.1	*Helicoverpa armigera*	4.42E-115	288
31830	895	cytochrome P450 4CG1	ADE05577.1	*Manduca sexta*	1.86E-88	204
32038	885	cytochrome P450, partial	AEY75584.1	*Helicoverpa armigera*	2.09E-29	86
32097	881	cytochrome P450 CYP306A1	ACM45975.1	*Spodoptera littoralis*	2.01E-66	122
33008	811	cytochrome P450	AET11927.1	*Helicoverpa armigera*	1.34E-05	42
34016	729	cytochrome P450 333B11	EHJ70454.1	*Danaus plexippus*	8.71E-44	65
34454	700	cytochrome P450 4c3	BAM19419.1	*Papilio xuthus*	1.82E-49	155
34746	674	cytochrome P450 333B10	ADE05591.1	*Manduca sexta*	1.32E-21	60
34862	669	cytochrome P450 CYP4	AAS67285.1	*Helicoverpa armigera*	4.48E-61	157
35240	645	cytochrome P450 354A5	ADE05589.1	*Manduca sexta*	7.51E-63	160
35376	635	cytochrome P450, partial	AEY75580.1	*Helicoverpa armigera*	1.24E-51	122
35441	629	cytochrome P450 4c3	BAM19419.1	*Papilio xuthus*	9.25E-31	170
35712	616	cytochrome CYP341A13	AFP20605.1	*Spodoptera littoralis*	9.81E-06	55
36161	581	PREDICTED: cytochrome P450 20A1-like	XP_003207553.1	*Meleagris gallopavo*	7.16E-74	124
37246	353	cytochrome P450	AEY75585.1	*Helicoverpa armigera*	1.46E-24	73

Appendix 3B. Contigs with hits to cytochrome p450s, Illumina sequencing (Assembly 2)

Contig ID	Length	NCBI top hit	Accession	Species	E-Value	Alignment length
92247_c0_seq1	253	cytochrome p450 4v2-like	XP_004922118.1	*Bombyx mori*	7.57E-09	79
109953_c0_seq1	760	cytochrome p450 cyp314a1	ABD18735.1	*Manduca sexta*	2.20E-50	109
109953_c0_seq2	774	cytochrome p450 cyp314a1	ABD18735.1	*Manduca sexta*	2.31E-50	109
117079_c1_seq1	331	cytochrome p450 cyp341b2	AID54868.1	*Helicoverpa armigera*	3.67E-35	111
120244_c0_seq1	459	cytochrome p450 4c3	BAM19419.1	*Papilio xuthus*	6.93E-21	143
124031_c0_seq1	214	cyp450 family 4	ACM16804.2	*Ruditapes philippinarum*	2.06E-06	66
124031_c1_seq1	269	cytochrome p450 cyp341b2	AID54868.1	*Helicoverpa armigera*	5.16E-13	86
124230_c0_seq1	222	cytochrome p450	ABW34434.1	*Plutella xylostella*	8.26E-24	74
124230_c1_seq1	397	cytochrome p450 cyp4m10v2	AID54880.1	*Helicoverpa armigera*	1.83E-10	55
128022_c0_seq1	871	cytochrome p450 cyp6ct1	EHJ78442.1	*Danaus plexippus*	8.94E-108	274
128022_c1_seq1	283	cytochrome p450 cyp6ct1	EHJ78442.1	*Danaus plexippus*	2.87E-32	93
128022_c2_seq1	342	cytochrome p450 cyp6ct1	EHJ78442.1	*Danaus plexippus*	3.50E-46	112
129237_c0_seq1	753	cytochrome p450 cyp341b2	AID54868.1	*Helicoverpa armigera*	2.06E-56	218
129237_c0_seq2	804	cytochrome p450 cyp341b2	AID54868.1	*Helicoverpa armigera*	1.61E-81	271
129237_c0_seq3	781	cytochrome p450 cyp341b2	AID54868.1	*Helicoverpa armigera*	1.17E-65	235
129237_c0_seq4	776	cytochrome p450 cyp341b2	AID54868.1	*Helicoverpa armigera*	2.82E-72	254
129237_c1_seq1	261	cytochrome p450 cyp341b2	AID54868.1	*Helicoverpa armigera*	7.72E-22	80
130095_c0_seq1	359	cytochrome p450 4cg1	ADE05577.1	*Manduca sexta*	1.15E-04	87
130095_c1_seq1	470	cytochrome p450 4cg1	ADE05577.1	*Manduca sexta*	4.10E-58	156
130095_c1_seq2	829	cytochrome p450 4cg1	ADE05577.1	*Manduca sexta*	3.66E-84	235
130450_c0_seq1	1756	gossypol-induced cytochrome p450	ABI84381.1	*Helicoverpa armigera*	1.56E-67	278
130489_c1_seq3	995	cytochrome p450 4c3	BAM19419.1	*Papilio xuthus*	4.70E-61	325
131809_c0_seq1	379	cytochrome p450 9a20	NP_001077079.1	*Bombyx mori*	1.84E-39	127

131809_c0_seq2	238	cytochrome p450 9a20	NP_001077079.1	*Bombyx mori*	1.00E-16	80
132416_c0_seq1	1997	cytochrome p450 6b45	ADE05578.1	*Manduca sexta*	1.37E-163	502
132438_c1_seq2	1159	cytochrome p450 cyp367b2	AID54872.1	*Helicoverpa armigera*	2.02E-141	369
132438_c1_seq3	1235	cytochrome p450 cyp367b2	AID54872.1	*Helicoverpa armigera*	7.10E-156	394
132745_c3_seq1	1978	cytochrome p450 301b1	EHJ78479.1	*Danaus plexippus*	1.13E-226	468
132745_c3_seq2	1680	cytochrome p450 301b1	EHJ78479.1	*Danaus plexippus*	9.08E-227	468
133301_c0_seq2	264	cytochrome p450 cyp341d1	AID54869.1	*Helicoverpa armigera*	4.69E-14	57
133576_c0_seq1	722	cytochrome p450 cyp341d1	AID54869.1	*Helicoverpa armigera*	6.15E-60	240
133576_c0_seq2	1588	cytochrome p450 cyp341d1	AID54869.1	*Helicoverpa armigera*	2.55E-106	486
133731_c0_seq1	530	cytochrome p450	EHJ63183.1	*Danaus plexippus*	2.84E-30	140
133786_c0_seq1	732	cytochrome p450 cyp9aj3	AID54908.1	*Helicoverpa armigera*	8.77E-94	246
133786_c0_seq2	664	cytochrome p450 cyp9aj3	AID54908.1	*Helicoverpa armigera*	3.83E-76	191
133786_c0_seq3	931	cytochrome p450 cyp9aj3	AID54908.1	*Helicoverpa armigera*	1.16E-103	231
133786_c0_seq4	718	cytochrome p450 9e2-like	XP_004926685.1	*Bombyx mori*	7.79E-92	216
133786_c0_seq5	678	cytochrome p450 cyp9aj3	AID54908.1	*Helicoverpa armigera*	4.29E-78	196
133871_c0_seq1	1986	cytochrome p450 6ab13	ADE05584.1	*Manduca sexta*	2.61E-215	495
134106_c2_seq4	2208	cytochrome p450	BAM73813.1	*Bombyx mori*	1.54E-179	512
134763_c0_seq1	303	cytochrome p450 cyp405a2	ACZ97406.2	*Zygaena filipendulae*	1.23E-06	52
134763_c0_seq2	1530	cytochrome p450 cyp405a3	ACZ97409.2	*Zygaena filipendulae*	5.90E-129	484
134763_c0_seq3	241	cytochrome p450 cyp405a2	ACZ97406.2	*Zygaena filipendulae*	1.22E-06	52
134769_c0_seq1	233	cytochrome p450	NP_001140197.1	*Bombyx mori*	2.92E-24	76
134769_c1_seq1	1133	cytochrome p450	NP_001140197.1	*Bombyx mori*	1.59E-135	344
135169_c0_seq1	1669	cytochrome p450 4cg1	ADE05577.1	*Manduca sexta*	1.98E-141	482
135233_c3_seq1	1909	cytochrome p450 cyp6ae12v1	AID54888.1	*Helicoverpa armigera*	1.21E-145	430
135233_c3_seq2	607	cytochrome p450 cyp6ae12v1	AID54888.1	*Helicoverpa armigera*	4.44E-56	190
135233_c3_seq3	297	cytochrome p450	AAP83689.1	*Depressaria pastinacella*	3.35E-20	89

135233_c3_seq4	2104	cytochrome p450 6ae32	ADE05581.1	*Manduca sexta*	8.08E-130	413
135233_c3_seq5	1972	cytochrome p450	AAP83689.1	*Depressaria pastinacella*	9.32E-165	515
135233_c3_seq6	3093	cytochrome p450	ABB69054.1	*Helicoverpa armigera*	1.72E-142	437
135233_c3_seq7	2843	cytochrome p450	ABB69054.1	*Helicoverpa armigera*	1.15E-161	494
135262_c2_seq1	1920	nadph cytochrome p450 reductase	ADK25060.1	*Helicoverpa armigera*	2.08E-270	513
135262_c2_seq2	2324	nadph cytochrome p450 reductase	ADK25060.1	*Helicoverpa armigera*	0.00E+00	687
135856_c0_seq1	495	cytochrome p450 4c3	BAM19419.1	*Papilio xuthus*	5.10E-16	106
136751_c0_seq2	1445	cytochrome p450	ABB69054.1	*Helicoverpa armigera*	1.13E-174	472
136751_c0_seq3	2054	cytochrome p450	ABB69054.1	*Helicoverpa armigera*	1.00E-193	514
136751_c0_seq4	2013	cytochrome p450	ABB69054.1	*Helicoverpa armigera*	2.23E-137	393
137134_c0_seq1	468	cytochrome p450	EHJ73975.1	*Danaus plexippus*	2.21E-11	51
137134_c0_seq2	445	cytochrome p450	EHJ73975.1	*Danaus plexippus*	2.19E-11	51
137134_c0_seq3	1796	cytochrome p450 6d5	BAM18499.1	*Papilio xuthus*	1.60E-83	206
137134_c0_seq5	1819	cytochrome p450 6d5	BAM18499.1	*Papilio xuthus*	1.63E-83	206
137379_c1_seq1	1932	cytochrome p450 4v2-like	XP_004926524.1	*Bombyx mori*	1.39E-189	490
137406_c4_seq1	727	cytochrome p450	AAT08964.1	*Helicoverpa armigera*	2.23E-65	184
137406_c5_seq1	1000	cytochrome p450 cyp4l5	AID54879.1	*Helicoverpa armigera*	2.95E-95	298
137574_c0_seq1	2316	cytochrome p450 cyp339a1	NP_001121192.1	*Bombyx mori*	1.86E-183	524
137574_c0_seq4	2042	cytochrome p450 cyp339a1	NP_001121192.1	*Bombyx mori*	6.54E-169	458
137941_c0_seq1	2483	cytochrome p450	CAJ31114.1	*Spodoptera littoralis*	2.43E-253	540
138141_c0_seq1	1715	cytochrome p450 332a4	ADE05587.1	*Manduca sexta*	2.50E-171	508
138606_c0_seq2	907	cytochrome p450 cyp315a1	ABC96070.1	*Manduca sexta*	7.48E-60	173
138606_c0_seq4	2265	cytochrome p450 cyp315a1	ABC96070.1	*Manduca sexta*	2.63E-174	481
138842_c1_seq1	1608	cytochrome p450 cyp4m10v2	AID54880.1	*Helicoverpa armigera*	5.69E-154	488
138842_c1_seq2	1461	cytochrome p450 monooxygenase	NP_001103833.1	*Bombyx mori*	5.91E-147	453
139011_c1_seq2	2093	cytochrome p450 337b2	AFO72904.1	*Helicoverpa armigera*	2.65E-149	475

139166_c0_seq1	233	cytochrome p450 cyp4g48	ACZ97414.1	*Zygaena filipendulae*	2.47E-23	73
139166_c2_seq1	1628	cytochrome p450 4g49	ADE05583.1	*Manduca sexta*	2.91E-230	471
139383_c1_seq1	729	cytochrome p450 4c21-like	XP_004922122.1	*Bombyx mori*	6.22E-15	82
139383_c1_seq3	1318	cytochrome p450 4c3	BAM19419.1	*Papilio xuthus*	2.57E-82	437
139383_c1_seq4	480	cytochrome p450 4c21-like	XP_004922122.1	*Bombyx mori*	2.56E-15	82
139561_c0_seq2	2096	cytochrome p450 cyp306a1	AID54855.1	*Helicoverpa armigera*	3.72E-252	539
139686_c6_seq1	1646	cytochrome p450 cyp4l5	AID54879.1	*Helicoverpa armigera*	5.66E-149	448
140082_c1_seq1	229	cytochrome p450 4c3	BAM19419.1	*Papilio xuthus*	3.98E-05	36
140082_c1_seq3	368	cytochrome p450 4c3	BAM19419.1	*Papilio xuthus*	5.83E-25	122
140082_c1_seq4	642	cytochrome p450 4c3	BAM19419.1	*Papilio xuthus*	1.13E-50	191
140082_c1_seq5	267	cytochrome p450 4c3	BAM19419.1	*Papilio xuthus*	3.23E-15	89
140082_c1_seq6	357	cytochrome p450 4c3	BAM19419.1	*Papilio xuthus*	3.82E-24	120
140082_c1_seq8	995	cytochrome p450 4c3	BAM19419.1	*Papilio xuthus*	3.85E-55	168
140082_c1_seq9	332	cytochrome p450 4c3	BAM19419.1	*Papilio xuthus*	1.65E-35	109
140082_c1_seq10	669	cytochrome p450 4c3	BAM19419.1	*Papilio xuthus*	2.43E-09	50
140082_c1_seq11	501	cytochrome p450 4c3	BAM19419.1	*Papilio xuthus*	3.26E-39	150
140082_c1_seq12	927	cytochrome p450 4c3	BAM19419.1	*Papilio xuthus*	3.41E-47	145
140082_c1_seq13	660	cytochrome p450 4c3	BAM19419.1	*Papilio xuthus*	2.92E-52	201
140082_c1_seq14	856	cytochrome p450 4c3	BAM19419.1	*Papilio xuthus*	1.90E-38	120
140082_c1_seq15	266	cytochrome p450 4c3	BAM19419.1	*Papilio xuthus*	2.03E-17	80
140082_c1_seq17	471	cytochrome p450 4c3	BAM19419.1	*Papilio xuthus*	3.35E-52	157
140230_c0_seq1	777	cytochrome p450 cyp302a1	AID54852.1	*Helicoverpa armigera*	1.47E-60	157
140230_c0_seq2	1624	cytochrome p450 cyp302a1	AID54852.1	*Helicoverpa armigera*	1.50E-194	440
140230_c0_seq3	1009	cytochrome p450 cyp302a1	AID54852.1	*Helicoverpa armigera*	5.83E-99	235
140230_c0_seq4	1386	cytochrome p450 cyp302a1	AID54852.1	*Helicoverpa armigera*	3.71E-111	261
140230_c0_seq5	2001	cytochrome p450 cyp302a1	AID54852.1	*Helicoverpa armigera*	7.71E-207	466

140230_c0_seq6	1392	cytochrome p450 cyp302a1	AID54852.1	*Helicoverpa armigera*	4.98E-156	362
140493_c0_seq1	2072	cytochrome p450 cyp324a1	AID54859.1	*Helicoverpa armigera*	1.87E-155	481
140608_c1_seq1	1910	cytochrome p450	BAM73852.1	*Bombyx mori*	1.29E-171	486
140827_c3_seq3	861	cytochrome p450 cyp341b2	AID54868.1	*Helicoverpa armigera*	1.42E-57	266
140914_c0_seq1	2087	cytochrome p450 cyp304f1	AID54853.1	*Helicoverpa armigera*	3.43E-173	509
140914_c0_seq2	671	cytochrome p450 partial	BAM20450.1	*Papilio polytes*	3.95E-52	172
141079_c1_seq1	1049	cytochrome p450 6an5	ADE05585.1	*Manduca sexta*	7.76E-118	324
141079_c1_seq2	675	cytochrome p450 cyp6an4	AGO62003.1	*Spodoptera frugiperda*	2.37E-60	176
141079_c1_seq3	927	cytochrome p450 6an5	ADE05585.1	*Manduca sexta*	1.73E-99	284
141079_c1_seq4	1208	cytochrome p450 6an5	ADE05585.1	*Manduca sexta*	7.73E-115	310
141327_c1_seq1	1867	cytochrome p450 6d4	BAM18151.1	*Papilio xuthus*	2.00E-169	495
141374_c3_seq1	446	cytochrome p450 cyp4l4	EHJ76614.1	*Danaus plexippus*	4.87E-19	113
141374_c3_seq2	1430	cytochrome p450 cyp4l5	AID54879.1	*Helicoverpa armigera*	3.51E-112	311
141374_c3_seq3	1992	cytochrome p450 cyp4l5	AID54879.1	*Helicoverpa armigera*	4.85E-169	464
141374_c3_seq5	1512	cytochrome p450 cyp4l5	AID54879.1	*Helicoverpa armigera*	3.78E-112	311
141374_c3_seq6	2038	cytochrome p450 cyp4l5	AID54879.1	*Helicoverpa armigera*	5.00E-169	464
141497_c1_seq1	3278	cytochrome p450 cyp4s1	ABU88427.1	*Helicoverpa armigera*	1.31E-164	471
141497_c1_seq2	3256	cytochrome p450 cyp4s1	ABU88427.1	*Helicoverpa armigera*	1.30E-164	471
141497_c1_seq3	3111	cytochrome p450 cyp4s1	ABU88427.1	*Helicoverpa armigera*	1.23E-164	471
141497_c1_seq5	3263	cytochrome p450 cyp4s1	ABU88427.1	*Helicoverpa armigera*	1.30E-164	471
141497_c1_seq6	3241	cytochrome p450 cyp4s1	ABU88427.1	*Helicoverpa armigera*	1.29E-164	471
141497_c1_seq7	3096	cytochrome p450 cyp4s1	ABU88427.1	*Helicoverpa armigera*	1.23E-164	471
141615_c1_seq1	1612	cytochrome p450	EHJ68958.1	*Danaus plexippus*	1.23E-148	372
141615_c1_seq2	1571	cytochrome p450	EHJ68958.1	*Danaus plexippus*	2.95E-123	306
141615_c1_seq3	1555	cytochrome p450	EHJ68958.1	*Danaus plexippus*	2.91E-123	306
141615_c1_seq6	1410	cytochrome p450	EHJ68958.1	*Danaus plexippus*	2.69E-141	442

141615_c1_seq7	1946	cytochrome p450 cyp4au1	AID54874.1	*Helicoverpa armigera*	1.27E-190	470
141668_c2_seq1	1539	cytochrome p450	BAM73846.1	*Bombyx mori*	1.91E-252	494
141668_c2_seq2	547	cytochrome p450 cyp301a1	AGU36304.1	*Spodoptera littoralis*	5.06E-65	147
141668_c2_seq3	1562	cytochrome p450	BAM73846.1	*Bombyx mori*	2.30E-261	508
141950_c0_seq1	1754	cytochrome p450 cyp324a1	AID54859.1	*Helicoverpa armigera*	1.38E-148	488
141950_c0_seq2	977	cytochrome p450 cyp324a1	AID54859.1	*Helicoverpa armigera*	1.08E-78	278
141950_c0_seq3	1019	cytochrome p450 cyp324a1	AID54859.1	*Helicoverpa armigera*	5.17E-79	278
141950_c0_seq4	1796	cytochrome p450 cyp324a1	AID54859.1	*Helicoverpa armigera*	6.38E-149	488
142031_c0_seq2	2654	cytochrome p450 6j1	XP_004930467.1	*Bombyx mori*	1.33E-188	471
142034_c1_seq1	1974	cytochrome p450 cyp49a1	AID54873.1	*Helicoverpa armigera*	3.73E-222	542
142034_c1_seq2	1764	cytochrome p450 cyp49a1	AID54873.1	*Helicoverpa armigera*	1.01E-191	487
142151_c0_seq1	1943	cytochrome p450 cyp333b3	AID54862.1	*Helicoverpa armigera*	4.70E-161	508
142151_c0_seq4	1573	cytochrome p450 cyp333b3	AID54862.1	*Helicoverpa armigera*	1.01E-123	420
142351_c1_seq1	1937	cytochrome p450 cyp9a14v2	AID54902.1	*Helicoverpa armigera*	2.65E-172	485
142351_c1_seq2	642	cytochrome p450	EHJ65260.1	*Danaus plexippus*	3.96E-35	122
142351_c1_seq3	320	cytochrome p450	AAV28704.1	*Helicoverpa armigera*	5.04E-13	49
142755_c0_seq1	573	cytochrome p450 cyp6au1	NP_001104826.1	*Bombyx mori*	1.22E-09	76
142755_c0_seq2	1901	cytochrome p450	CAZ65618.1	*Cnaphalocrocis*	3.77E-131	498
142755_c0_seq3	1955	cytochrome p450	CAZ65618.1	*Cnaphalocrocis*	3.91E-131	498
142914_c1_seq1	1779	cytochrome p450	BAM73809.1	*Bombyx mori*	3.99E-119	376
142914_c1_seq3	1210	cytochrome p450 monooxygenase	ABP99018.1	*Bombyx mori*	8.92E-95	312
142914_c1_seq4	576	cytochrome p450	ABC72321.2	*Spodoptera litura*	5.60E-39	149
142914_c1_seq5	1659	cytochrome p450 cyp4m10v2	AID54880.1	*Helicoverpa armigera*	1.77E-166	484
142914_c1_seq6	1909	cytochrome p450	ABC72321.2	*Spodoptera litura*	2.97E-168	486
142914_c1_seq8	1275	cytochrome p450	ABC72321.2	*Spodoptera litura*	6.41E-115	324
142914_c1_seq9	960	cytochrome p450	BAM73809.1	*Bombyx mori*	5.17E-94	309

142914_c1_seq10	2115	cytochrome p450 monooxygenase	ABP99018.1	*Bombyx mori*	2.10E-85	296
142914_c1_seq11	2029	cytochrome p450 monooxygenase	ABP99018.1	*Bombyx mori*	1.62E-119	379
142914_c1_seq12	1395	cytochrome p450 4m1	ADE05575.1	*Manduca sexta*	2.15E-66	216
142914_c1_seq13	520	cytochrome p450 cyp4m10v2	AID54880.1	*Helicoverpa armigera*	9.55E-39	119
142914_c1_seq14	1995	cytochrome p450	ABC72321.2	*Spodoptera litura*	1.55E-82	295
142978_c0_seq1	3250	cytochrome p450 cyp333b3	AID54862.1	*Helicoverpa armigera*	5.35E-118	447
142978_c0_seq2	2692	cytochrome p450 cyp333b3	AID54862.1	*Helicoverpa armigera*	4.29E-118	447
142978_c0_seq3	2991	cytochrome p450 cyp333b3	AID54862.1	*Helicoverpa armigera*	1.24E-105	342
142978_c0_seq4	2433	cytochrome p450 cyp333b3	AID54862.1	*Helicoverpa armigera*	9.72E-106	342
142978_c0_seq5	2468	cytochrome p450 cyp333b3	AID54862.1	*Helicoverpa armigera*	9.54E-117	390
142978_c0_seq6	3026	cytochrome p450 cyp333b3	AID54862.1	*Helicoverpa armigera*	1.21E-116	390
143106_c0_seq1	345	cytochrome p450 protein	ACJ05915.1	*Bombyx mandarina*	2.18E-40	113
143106_c0_seq2	1169	cytochrome p450 9a20	NP_001077079.1	*Bombyx mori*	3.76E-111	321
143106_c0_seq3	1763	cytochrome p450 cyp9a19	ABQ08710.1	*Bombyx mandarina*	1.28E-186	465
143106_c0_seq4	1083	cytochrome p450 cyp9a19	ABQ08710.1	*Bombyx mandarina*	4.54E-100	241
143106_c0_seq5	2099	cytochrome p450 9a20	NP_001077079.1	*Bombyx mori*	8.16E-199	513
143106_c0_seq6	833	cytochrome p450 cyp9a19	ABQ08710.1	*Bombyx mandarina*	1.55E-98	273
143266_c1_seq3	2413	cytochrome p450 4g49	ADE05583.1	*Manduca sexta*	1.80E-245	562
143266_c1_seq8	1519	cytochrome p450 4g49	ADE05583.1	*Manduca sexta*	9.82E-185	432
143282_c1_seq1	355	cytochrome p450	EHJ65260.1	*Danaus plexippus*	7.73E-09	40
143282_c1_seq2	2007	cytochrome p450	AAY21809.1	*Helicoverpa armigera*	3.04E-203	528
143282_c1_seq3	244	cytochrome p450	EHJ65260.1	*Danaus plexippus*	6.01E-22	78
143282_c1_seq4	508	cytochrome p450	AAV28704.1	*Helicoverpa armigera*	1.40E-21	62
143282_c1_seq5	1358	cytochrome p450	ACB30273.2	*Helicoverpa armigera*	1.60E-143	381
143282_c1_seq6	2262	cytochrome p450	AAY21809.1	*Helicoverpa armigera*	1.58E-203	530
143282_c1_seq7	1996	cytochrome p450	ACB30273.2	*Helicoverpa armigera*	4.90E-145	381

143282_c1_seq8	763	cytochrome p450	AAV28704.1	*Helicoverpa armigera*	9.83E-22	63
143282_c1_seq9	1828	cytochrome p450	ACB30273.2	*Helicoverpa armigera*	4.38E-145	381
143282_c1_seq10	707	cytochrome p450 cyp9a19	ABQ08709.1	*Bombyx mori*	3.25E-50	156
143282_c1_seq12	1792	cytochrome p450	AAY21809.1	*Helicoverpa armigera*	6.48E-202	530
143282_c1_seq13	931	cytochrome p450	AAV28704.1	*Helicoverpa armigera*	1.46E-21	63
143282_c1_seq14	1573	cytochrome p450	ACB30273.2	*Helicoverpa armigera*	7.99E-145	379
143282_c1_seq15	2430	cytochrome p450	AAY21809.1	*Helicoverpa armigera*	1.72E-203	530
143330_c3_seq1	1577	cytochrome p450 cyp9a17v2	AID54904.1	*Helicoverpa armigera*	5.15E-176	475
143330_c3_seq2	2253	cytochrome p450 cyp9a17v2	AID54904.1	*Helicoverpa armigera*	5.41E-204	532
143330_c3_seq3	2297	cytochrome p450 cyp9a17v2	AID54904.1	*Helicoverpa armigera*	3.23E-204	533
143330_c3_seq4	438	cytochrome p450	EHJ65260.1	*Danaus plexippus*	9.90E-12	75
143330_c3_seq6	457	cytochrome p450	EHJ65260.1	*Danaus plexippus*	1.00E-11	75
143330_c3_seq7	1163	cytochrome p450 cyp9a17v2	AID54904.1	*Helicoverpa armigera*	1.16E-112	337
143330_c3_seq8	752	cytochrome p450	EHJ65260.1	*Danaus plexippus*	1.41E-65	200
143330_c3_seq9	821	cytochrome p450	EHJ65260.1	*Danaus plexippus*	8.10E-52	168
143389_c1_seq1	3020	cytochrome p450 cyp333a1	AID54861.1	*Helicoverpa armigera*	1.04E-168	460
143389_c1_seq2	3031	cytochrome p450 cyp333a1	AID54861.1	*Helicoverpa armigera*	1.04E-168	460
143389_c1_seq3	2877	cytochrome p450 cyp333a1	AID54861.1	*Helicoverpa armigera*	9.86E-169	460
143389_c1_seq5	2866	cytochrome p450 cyp333a1	AID54861.1	*Helicoverpa armigera*	9.81E-169	460
143664_c4_seq1	1135	cytochrome p450 9g3	NP_001108456.1	*Bombyx mori*	1.66E-15	82
143664_c4_seq2	1117	cytochrome p450 9g3	NP_001108456.1	*Bombyx mori*	9.83E-114	345
143664_c4_seq3	1251	cytochrome p450 9g3	NP_001108456.1	*Bombyx mori*	5.21E-138	391
143664_c4_seq4	1442	cytochrome p450 9g3	NP_001108456.1	*Bombyx mori*	2.35E-148	432
143664_c4_seq5	1227	cytochrome p450 9g3	NP_001108456.1	*Bombyx mori*	2.20E-133	383
143664_c4_seq6	1160	cytochrome p450 9g3	NP_001108456.1	*Bombyx mori*	1.71E-15	82
143664_c4_seq7	1466	cytochrome p450 9g3	NP_001108456.1	*Bombyx mori*	5.55E-153	440

143664_c4_seq8	222	cytochrome p450 9g3	NP_001108456.1	*Bombyx mori*	5.54E-28	73
143664_c4_seq10	1332	cytochrome p450 9g3	NP_001108456.1	*Bombyx mori*	1.09E-128	394
143664_c4_seq11	1283	cytochrome p450 9g3	NP_001108456.1	*Bombyx mori*	1.59E-121	379
143664_c4_seq12	1068	cytochrome p450 9g3	NP_001108456.1	*Bombyx mori*	1.41E-106	330
144521_c0_seq1	1928	cytochrome p450 304a1	BAM20370.1	*Papilio polytes*	2.92E-38	107
144521_c0_seq6	1745	cytochrome p450 304a1	BAM20370.1	*Papilio polytes*	2.57E-38	107
144521_c0_seq8	1615	cytochrome p450 304a1	BAM20370.1	*Papilio polytes*	2.32E-38	107
144521_c0_seq9	2961	cytochrome p450 cyp304f1	AID54853.1	*Helicoverpa armigera*	1.04E-189	510
144521_c0_seq10	3274	cytochrome p450 cyp304f1	AID54853.1	*Helicoverpa armigera*	1.18E-189	510
144521_c0_seq11	3091	cytochrome p450 cyp304f1	AID54853.1	*Helicoverpa armigera*	1.10E-189	510

Appendix 4. Amino acid sequences of insecticide target sites assembled from Illumina transcriptomes

T. absoluta acetylcholinesterase-1 (partial CDS)

SPHEHRGRHHAPDRQPHFPAPVPPQPYRGHGEAVRYNPELDTILPRIEDHETSSKRSKIE
DETSSKRVKFDTYYSNHERAEEVLMADDPNLGPEEDDPLVVRTRKGRVRGITLTAAT
GKKVDAWFGIPYAQKPIGDLRFRHPRPIEGWGEEILNTTTLPHSCVQIIDNVFGDFPGA
MMWNPNTDMQEDCLYINIVVPKPRPKNAAVMLWVFGGGFYSGTATLDVYDPKILVS
EEKVVYVSMQYRVASLGFLFFDTPDVPGNAGLFDQLMALQWVKDNIAYFGGNPHNV
TLFGESSGAASVSLHLLSPLSRNYFSQAIMQSGAATLPWAIISREESILRGIRLAEAVHCP
YSRNDVGPMIECLRKKTPEELVNNEWGTLGICEFPFVPIIDGSFLDEMPARSLAHQNFK
KTNLLMGSNTEEGYYFILYYLTEMMPKEENVGISREQYLQAVKELNPYVNDIVRQAIV
YEYTDWLNPNDPVKNRNALDKMVGDYHFTCSVNEFAHRYAETGNNVYTYYYKHRS
KNNPWPSWTGVLHADEINYVFGEPLNPGKNYSPEEVEFSKRIMRYWSNFARTGNPSM
NPNGELTNPVWPLHSPLGREYLALGVNESSVGQGVRVKECAFWQKYLPQLIAATSKP
DPPKNCTSSASSQWLSFDVLSLSVATIGLTHSMLSKYII

T. absoluta acetylcholinesterase-2 (complete CDS)

MVCNSKIVLTKLLLCCFVTSVWGRSWANHHDTTTSTTQTTPTTTLPPKNFHNDPLIVE
TKSGLVKGYAKTVMGREVHIFTGIPFAKPPLGPLRFRKPVPIEPWHGVLEATAMPNSC
YQERYEYFPGFEGEEMWNPNTNISEDCLYLNIWVPQHLRVRHHQDKPLTERPKVPILV
WIYGGGYMSGTATLDIYKADIMASSSDVIVASMQYRVGAFGFLYLNKYFSPGSEEAPG
NMGLWDQQLAVRWIKENARAFGGDPELVTLFGESAGGGSVSLHMLSPEMKGLFRRG
ILQSGTLNAPWSWMTGERAQVIGNVLIDDCNCNSSLLTTDPMLVMDCMRGVDAKTIS
VQQWNSYTGILGFPSAPTVDGVFLPKDPDTLMKEGNFHNTEVLLGSNQDEGTYFLLY
DFLDYFEKDGPSFLQREKFLEIIDTIFKDFSKIKREAIVFQYTDWEEITDGYLNQKMIADI
VGDYFFVCPTNLFAEVMADSGVEVYYYYFTHRTSTSLWGEWMGVMHADEIEYVFGH
PLNMSLQYHTRERDLAAHIMQTFTRFALTGKPHKPDEKWPLYSRASPHYYTYTADGP
SGPAGPRGPRASACAFWNDFLNKLNELEHVPCDGAVTGPYSSVAGTTLPILLLTTLAT
TVAL

T. absoluta ecdysone receptor (complete CDS)

MRRRWSNNGGFQTLRMLEESSSEVTSSSALGLPPAMVMSPESLASPEYGALELWGYD
DGINSYNATQLLQANACNMPPQQPQQTLPSMPLPMNPQTPKSENESISSGREELSPASS
VNGCSTDGDARRQKKGPAPRQQEELCLVCGDRASGYHYNALTCEGCKGFFRRSVTK
NAVYICKFGHACEMDMYMRRKCQECRLKKCLAVGMRPECVVPETQCQIKRNEKKK
QREKDKLPVSTTTVDDHMPPIMQCDPPPPEAARILECLQHEVVPRFLPEKLLEQNRAK
NIPPLTANQQFLIARLVWYQDGYEHPSEEDLKRVTQQAAEEEEGSSDLPFRQITEMTIL
TVQLIVEFAKGLPGFSKISQPDQITLLKACSSEVMMLRVTRNYDAATDSVMFATNQAY
TRDNYRKAGMDYVIEDLLHFCRCMHAMAMDNVHYALLIAIVIFSDRPGLEQPQLVEEI
QRYYLNTLRMYILNQHSASPRCAIIYGKMLSILSELRTLGMQNSNMCISLKLKNRKLPP
FLEEIWDVADVSSAQTTTPPVLRDPSEL

T. absoluta GABA-gated chloride channel (complete CDS)

MQTSRPRGVHSIALLLALAIAWLPHADHAAGAGGGGMFGDVNISAILDSFSISYDKRV
RPNYGGPPVEVGVTMYVLSISSLSEVKMDFTLDFYFRQFWTDPRLAYKKRPGVETLSV
GSEFIKNIWVPDTFFVNEKQSYFHIATTSNEFIRIHYSGSITRSIRLTITASCPMNLQYFPM
DRQLCHIEIESFGYTMRDIRYKWNEGPNSVGVSSEVSLPQFKVLGHRQRAMEISLTTG
NYSRLACEIQFVRSMGYYLIQIYIPSGLIVIISWVSFWLNRNATPARVALGVTTVLTMTT
LMSSTNAALPKISYVKSIDVYLGTCFVMVFASLLEYATVGYMAKRIQMRKQRFVAIQ
KIASEKKMPPLDCPPGVGDPHTLSKMSTLGRCPPGRPSVSYSEVRFKVHDPKAHSKGG
TLENTINGGRSGAEDENPGPPPHILHPGKDISKLLGMTPSDIDKYSRIVFPVCFVCFNLM
YWIIYLHVSDVVADDLVLLGEDK

T. absoluta Glutamate-gated chloride channel (complete CDS)

MELRLPSCASISLLLLCLLQLTQCMNAKINFREKEKQILDQILGPGRYDARIRPSGINGT-
DGPAVVSVNIFVRSISKIDDVTMEYSVQLTFREQWLDERLKFNNLGGRLKYLTLTEAN
RVWMPDLFFSNEKEGHFHNIIMPNVYIRIFPNGNVLYSIRISLTLSCPMNLKLYPLDKQT
CSLRMASYGWTTDDLVFLWKEGDPVQVVKNLHLPRFTLEKFLTDYCNSKTNTGEYSC
LKVDLLFKREFSYYLIQIYIPCCMLVIVSWVSFWLDQGAVPARVSLGVTTLLTMATQSS
GINASLPPVSYTKAIDVWTGVCLTFVFGALLEFALVNYASRSDMHRENMKKTRREME
AAAQMDAASDLLDTDSNATFAMMRQCEIHISPPRKNCCRLWMSKFPTRSKRIDVISRI
TFPLVFALFNLAYW

T. absoluta nAChR α1 subunit (complete CDS)

MVLAIVVGVLCVWGRLTDANPEAKRLYDDLLSNYNRLIRPVGNNSDRLTVKMGLRL
SQLIDVNLKNQIMTTNVWVEQEWNDYKLKWNPDDYGGVDTLHVPSEHIWLPDIVLY
NNADGNYEVTIMTKAILHHDGKVVWKPPAIYKSFCEIDVEYFPFDEQTCFMKFGSWS
YDGYTVDLRHLKQTPDSDHIGMGIDLSEYYISVEWDIMRVPATRNEKFYSCCEEPYPDI
IFNLTLRRKTLFYTVNLIIPCVGISFLSVLVFYLPSDSGEKISLCISILLSLTVFFLLLAEIIPP
TSLTVPLLGKYLLFTMMLVTLSVVVTIVVLNVNFRSPVTHHMAPWVRKVFIDFLPKIL
CIQRPDKPPDEEDDENDKPTEVLTDVFGGDDMDGKFKEWGCEEYELPGMPPSPPPPPG
GDDELFSPPPGSPCRLDLDDGSPSLEKPYVREMEKTIEGSRFIAQHVKNKDKFESVEDD
WKYVAMVLDRIFLFLFTIACVLGTALIIFRAPTFYDNSKPIDILYSKIAKKKLELLKMGS
EGDPGL

T. absoluta nAChR α2 subunit (complete CDS)

MSRVCLFVLLGLCGVCLANPDAKRLYDDLLSNYNRLIRPVDKNNNTVLVKLGLRLSQ
LIDLNLKDQILTTNVWLEHEWEDHKFKWDPSEYGGQRELYVPSEHIWLPDIVLYNNA
DGEYVVTTMTKAVLHFTGKVLWTPPAIFKSSCEIDVRYFPFDQQTCFLKFGSWSYDGD
QIDLKHINQKKGDMVEVGIDLREYYPSVEWDILGVPAERHEKYYPCCQEPYPDIFFNIT
LRRKTLFYTVNLIVPCVGISYLSVLVFYLPADSGEKIALSISILLSQTMFFLLISEIIPSTSL
ALPLLGKYLLFTMLLVGLSVVITIIILNVHYRKPSTHKMAPWVRKFFITKLPKLLLMRV
PKDLLRDLAAQKIAGRSMKNKNKFKDALAAVEQTNSNASSPDSLRHHLPGGCNGLHT
TTATNRFSGLVGALGSLGAGYNGLPSVMSGLDDSLSDVAPRKKYPFELEKAIHNVMFI
QHHMQRQDEFNAEDQDWGFVAMVLDRLFLWIFTIASIVGTFAILCEAPSLYDDTKPID
MILSSVAQQQFLPVDSGDS

T. absoluta nAChR α3 subunit (partial CDS)

GNPDAKRLYDDLLSNYNKLVRPVLNVSDALTVRIKLKLSQLIDVNLKNQIMTTNLWV
EQSWYDYKLSWEPREYGGVEMLHVPSDHIWRPDIVLYNNADGNFEVTLATKATLNY
TGRVEWRPPAIYKSSCEIDVEYFPFDQQTCVMKFGSWTYDGFQVDLRHIDEARGTNV
VELGVDLSEFYTSVEWDILEVPAVRNEKFYTCCDEPYLDITFNITMRRKTLFYTVNLIIP
CMGISFLTVLVFYLPSDSGEKVSLSISILLSLTVFFLLLAEIIPPTSLVVPLLGKFVLFTMIL
DTFSICVTVVVLNVHFRSPQTHTMAPWVRRVFIHVLPRLLVMRRP

T. absoluta nAChR α4 subunit (partial CDS)

MGALVWWLAAAFLVRAATAGNPDAKRLYDDLLSNYNKLVRPVVNTTDVLRVCIKL
KLSQLIDVNLKNQIMTTNLWVEQSWYDYKLRWEPKEYGGVHMLHVPSDHIWRPDIV
LYNNADGNFEVTLATKATIYHQGLVEWKPPAIYKSSCEIDVEYFPFDEQTCVLKFGSW
TYDGFKVDLRHMDEQAGSNVVSVGVDLSEFYMSVEWDILEVPAVRNEKFYTCCDEP
YLDITFNITMRRKTLFYTVNIIIPCMGISFLTVLTFYLPSDSGEKVTLSISILISLHVFFLLV
VEIIPPTSLVVPLLGKYLIFAMILVSISICVTVVVLNVHFRSPQTHRMAPWVKRVFIHILP
RLLFMKRPQYKFDTTSLSRSRYTACGMVVRCSGTARPLYPYRLAAADDDCCAPG

T. absoluta nAChR α5 subunit (partial CDS)

YDGFQLDLKKQFDEGDTTNYQTNGEFDLVSFDAIRHNQYYSCCVEPYPDITYVIKLRR
RPMFYVFNLILPCLLINGIALLVFYVPSESGEKVTLGISALLSMTVFLMTIRDTLPPTEKT

T. absoluta nAChR α6 subunit (partial CDS)

SEQGPHEKRLLNALLNSYNTLERPVANESEPLEVKFGLTLQQIIDVDEKNQILTTNVWL
NLEWNDYNLRWNDSEYGGVKDVRITPNKLWKPDVLMYNSADEGFDGTYQTNVVVR
NGGSCQYVPPGIFKSTCKMDIXWFPFDDQHCDMKFGSWTYDGXQLDLVLKDENGGD
LSDFITNGEWYLIGMPGKKNTISYACCPEPYVDVTFTIRIRRRTLYYFFNLIVPCVLISSM
ALLGFTLPPDSGEKLTLGVTIMLSMTVFXNLVAEXXPNTSDXXXXXGTYFNCIMFMV
AXSVVLXVVVLNYHHRTADIHEMPQWIKSVFLQWLPWILRMSRPGKKITRKTIMMSN
RMRELELKERSSKSLLANVLDIDDDFRHAPPPNSTASTGNLGPGCSIFRTDFRRSFVRP
STMEDVGGGLGGHHRELHLILTELKFITARMRKADEEAELISDWKFAAMVVDRFCLF
VFTLFTIIATVAVLLSAPHIIVQ

T. absoluta nAChR α7 subunit (complete CDS)

MCGERARRALTAAPAALFLLLGLLWPRGACGGYHEKRLLHHLLDHYNVLERPVVNE
SDPLQLSFGLTLMQIIDVDEKNQLLITNIWLKLEWNDMNLRWNTSDFGGVKDLRVPPH
RLWKPDVLMYNSADEGFDSTYPTNVVVRNNGSCLYVPPGIFKSTCKIDITWFPFDDQR
CEMKFGSWTYGGYQLDLQLQDEAGGDISNFVTNGEWELIGVPGKRNEINYNCCPEPYI
DITFAVVIRRKTLYYFFNLIVPCVLIASMALLGFTLPPDSGEKLSLGVTILLSLTVFLNM
VAETMPATSDAVPLLGTYFNCIMFMVASSVVSTILILNYHHRHADTHEMSDWIRCVFL
YWLPWILRMSRPGSAATPPPARAPPPPDLELRERSSKSLLANVLDIDDDFRHAQQQPPC
CRYYRSLDDLHEHYSPGAEENGAGLAAHSCFGVDYELSLILKEVRVITDQMRKDDED
ADISRDWKFAAMVVDRLCLIIFTLFTIIATLAVLLSAPHIMVS

T. absoluta nAChR α8 subunit (complete CDS)

MKLGLVWLFLSVLRSAVGIKFLEANPDVKRLYDDLLSNYNRLIRPVTNVSEILTVRLG
LKLSQLMEVNLKNQVMTTNLWVEQKWFDYKLTWNPEEYGGVEMLYVPSEHIWLPDI
VLYNNWDGNYEVTLMTKATLKYTGEVNWKPPAIYKSSCEINVEYFPFDEQTCFMKFG
SWTYNGAQVDLKHMDQSPGSSLVHVGIDLSEFYLSVEWDILEVPATRNEEYYPCCAE
PFSDITFKLTMRRKTLFYTVNLIIPCVGLTFLTVLVFYLPSDSGEKISLCISILVSLTVFFL
GLAEIIPPTSLAIPLLGKYLLFTMILVSLSVWVTVCILNVHFRSPSTHTMSPWMKKLFLQ
LMPKVLMMRRTKYSLPDYDDTFHSNGYTNELEMSRESLTDAFDKNSDNGDYRKSPA
PEDDMLGAGVHQRPSVTESENMLPRHLSPEVAAALQSVRFIAQHIKDADKDNEIVED
WKFMSMVLDRFFLWLFTIACFVGTFGIIFRSPSLYDTRVPVDQQLSSIPMKKNNFFYPK
DVDVVGIIN

T. absoluta nAChR α9 subunit (complete CDS)

MSTLICLCALLAATVQVRGDDACPADRKQSLHDEGKLHYHLTCNYNSNYRPVKDHK
TSIQVKIRFALKYLSFDSLEETFTVHSWVAMTWKDEFLTWTPSDYNNIKEIQVESHEIW
SPRMALFNADASLYQSDSMYTTCLVSHDGVVKCVPHITHSGICRTTLRRWPYDSQNC
TLYFGSWMHTGEQVNFTFYNKDPVMYEQYQDGPGWKLLKVTNERLPGRYECCPNAT
YPMLKYTFQMEREASGPAAIVVVPSVLIVLLTLTSLLLDVKNNVRLMLICFSFFGHYTF
LSEIGYDIPKHGSETPIILMFVRDSMIITMVAILETLFLMSIMKRTVPAPNWVVRVTRLA
TSGPGKYVVFTEFDPTDATDKRNITEDPTSSFNEEKARVESDWVQFANLLNSCLFILSC
LIYLVLIFVYIPYN

T. absoluta nAChR β1 subunit (complete CDS)

MSGGSRAVLLAAALLTILYSGWCSEDEERLVRDLFRGYNKLIRPVQNMTQKVDVRFG
LAFVQLINVNEKNQIMKSNVWLRLVWMDYQLMWDEADYGGIGVLRLPPDKVWKPD
IVLFNNADGNYEVRYKSNVLIYPNGEVLWVPPAIYQSSCTIDVTYFPFDQQTCIMKFGS
WTFNGDQVSLALYNNKNFVDLSDYWKSGTWDIIEVPAYLNIYEGNHPTETDITFYIIIR
RKTLFYTVNLILPTVLISFLCVLVFYLPAEAGEKVTLGISILLSLVVFLLLVSKILPPTSLV
LPLIAKYLLFTFIMNTVSILVTVIIINWNFRGPRTHRMPLWIRSVFLHYLPAALLMRRPR
KTRLRWMMEMPGMGAPPHATTAPHDLPKHLSKMEAMELSDLHHPNCKINRAAGGG
GEMGALGGLGALGGLGLGERRESESSDSLLLSPEAAKATEAVEFIAEHLRNEDLYIQT
REDWKYVAMVIDRLQLYIFFIVTTAGTVGILMDAPHIFEYVDQDRIIEIYRGK

T. absoluta nAChR β3 subunit (complete CDS)

MAPSTCILVSLLVLLKNSFCDDCPANRYGEINYEEKLRNHLKTDCKQTINSPPNNGDG
KPVEVNVIMKQLSFDFNDAEEEIMVELLLSFMWRDKRLTWKLEDYGHIETVTILSIHM
WTPFLKHYNTRNDFDGFEKGKYFCELYYRGRVACHLIKTYNAICSTKLRNWPFDYQQ
CVFHFGTWDGENTTVLFKYNLVETKRKDIFDAFNSAGWHIFSNQIVNNENTTKQQMS
LILNFKRVSEYLESILFIPVILSCVLTVVSFILKLDNDRLLLSCLSLLIHFWALLETSDKIP
KNSSEPPNILLFLRNSMVLTSFSIVLTLYLKYLITFTKPISLRMKSVLNFVYDCKYNRYF
GRDGKTKIVILLIQMTEWNGFILQVY

T. absoluta ryanodine receptor (complete CDS)

MAEPEGGASEQDDVSFLRTEDMVCLSCTATGERVCLAAEGFGNRHCFLENIADKNIPP
DLSQCVFVIEQALSVRALQELVTAAGSETGKENLGKGTGSGHRTLLYGNAILLRHLNS
DMYLACLSTSSSQDKLAFDVGLQEHSQGEACWWTLHPASKQRSEGEKVRVGDDLILV
SVATERYLHTTKENEVSIVNASFHVTHWSVQPYGTGISRMKYVGYVFGGDVLRFFHG
GDECLTIPSTWAKDGGQNIVVYEGGSVMSQARSLWRLELARTKWAGGFINWYHPMR
IRHITTGRYLGVNDQNELYLVSREEATTSSCAFCLRQEKDDQKVVLEDKDLEVIGAPII
KYGDSTVIMQHSETGLWLSYKSYETKKKGLGKVEEKQAILHEEGKMDDGLDFSRSQE
EESRTARVIRKCSSLFTKFINGLETLQENRRHSMFFASVNLGEMVMCLEDLINYFAQPD
EDMEHEEKQNKFRALRNRQDLFQEEGILNLILEAIDKINVITSQGFLAGFLASDESGHC
WDMISGYLYQLLAAIIKGNHTNCAQFANSNRLNWLFSRLGSQASGEGTGMLDVLHCV
LIDSPEALNMMRDEHIKVIISLLEKHGRDPKVLDVLCSLCVGNGVAVRSSQNNICDYLL
PGKNLLLQTALVDHVSSVRPNIFVGRVEGSAVYQKWYFEVTMDHIEKTTHMMPHLRI
GWANTSGYVPYPGGGEKWGGNGVGDDLYSFGFDGAYLWSGGRRTPVTRAHVDEPFI
RKGDVIGCALDLTVPIINFMFNGIRVTGSFTNFNLEGMFFPVISCSSKLSCRFLLGGEHG
RLRYAAPEGYSPLVESLLPQQILNLEPCFYFGNLAKRALAGPPLVQDDTAFVPTPVDT
MAITLPSYVEQIRDKLAENIHEMWAMNKIEAGWVYGEQREDMHKIHPCLVPFERLPQ
AEKRYDIQLAVQTLKTILALGYYISLDKPPARIRNIRLPNEQFMQSNGYKPAPLDLSAV
TLTPKMDELVDQLAENTHNLWARERIQQGWTYGLNEDPDMQRSPHLVPYPKVDDAI
KKANRDTASETVRTLLVYGYMLDPPTGEQHEALLAEASKLKQADFRTYRAEKNYAV
SSGKWYFEFEILTAGPMRVGWAHADMAPGMMLGQDENSWAFDGYNEEKVYSGNSE
SFGKQWAVGDVVGVFLDLIDKTISFSLNGELLMDALGGETTFADVQGDNFVPACTLG
VGQKARLTYGQDVNTLKYFTTCGLQEGYEPFCVNMKRDVTHWYTKDQPIFENTDDM
ADTRIDVTRIPAGSDTPPCMKISHNTFETMEKANWEFLRLSLPVICQAEFIDEREKARR
WVDIKGRQQVLMREHVDAQMPAHIDQIMRSGFTINDIKGLHYDENQEEATSSKMKRL
PSRPPRKGSISQSRNYNLSPGQTNGMHRTTSEAEMSKYELGAQSVASEEKKDKRGRSP
FKFFKSRRGESSDRKARSGKSKTPDPLSDAETSPERATVRRPNPQIRVSQTNLSVPSPQL
QDRKQMTTATLAQSTTETVGNEIFDAECLRLINEYFYGVRIFPGQDPTHVYIGWVTTQ
YHLHSKDFNQNKVTKSSVIITDEHDRIIESVNRQSCYMVRADELYNEVMAEATAKGAS
QGMFIGCSVDTSTGTVAFTCEGKDTSIKFKMEPETKLFPAIFVEATSKEILQIELGRSATS
LPLSAAVLPTSDKHVIPQFPPRLKVQCLKPHQWARVPNQALQVHALKLSDIRGWSML

CEDAVSMLALHIPEEDRCIDILELIEMDKLLSFHSHTLTLYAALCYQSNYRAAHALCQH
VDQKQLLYAIQSQYMSGPLRQGFYDLLIALHLESHATTMEVCKNEYVIPLGPELKALY
EDPEMGHSLRSLQTESVRPQMKMTDIAENISDISNLYSPYFPLEVVREFVMQALAEAV
ETNQVHNRDPVGGSNENLFLPLIKLTDRLLLVGMMRDEDVEKLLIMSNPETWDPTFD
KDGKDEHRKGLLHMKMAEGAKLQMCYLLQHLNDIQLRHRVEAIISFAHDFVGDLQT
DQLRRYVEIKASDLPSAVAAKKTREFRCPPREQMNAILSFKHMAEDEEVDNFPCGEDL
IQRMNEFHESLMARVSLAALQEPETDENAEPETKKGAFSKLYNIINTVKELEEEPKAIE
EPPKKTPEEKFRKVLIQTIVSWAEESQIETPKLVREMFSLLVRQYDAVGELIRALEKTY
VINAKTKQDVAEMWVGLSQIRALLPVQMSQEEEELMRKRLWKLVNNHTFFQHPDLIR
VLRVHENVMAVMMNTLGRRAQAQSDAQPSSQPAAEEGKEKDTSHEMVVACCRFLC
YFCRSGRLNQKAMFDHFDFLLENSNILLSRPSLRGSTPLDVAYSSLMENTELALALREH
YLEKIAVYLSRCGLQSNSELVEKGYPDLGWDPVEGERYLDFLRFCVWVNGESVEENA
NLVIRLLIRRPECLGPALRGEGEGLLKAIVDANKMSERIADRRKMRELEGEGDVSFTHP
LPESDDDEDYIDTGAAILNFYCTLVDLLGRCAPDAAVIALGKNESLRARAILRSLVPLE
DLQGVLSLRFTLNNPAAGEERPKSDMPSGLIPGHKQSVGLFLERVYGIETQELFFRLLE
EAFLPDLRAATMLDRNDGCESDMALSMNRYIGNSILPLLIKHAYFYNEAENYASLLDA
TLHTVYRLSKNRMLTKGQREAVSDFLVALTSAMQPAMLLKLLRKLTVDVSQLSEYTT
VALRLLTLHYERCAKYYGSTGAGSGVYGASSDEEKRLTMMLFSNIFDSLSKMDYEPE
LFGKALPCLIAIGCALPPDYSLSKNYDDEFYSKEPQATGEPANPQYDPQPINTTSVALN
NDLNTIVQKFSEHYHDAWASRKIENGWVYGESYSESQKAHPRLKPYNMLNDYEKER
YKEPVRESLKALLAIGWSVEHSEVDIPSTNRSSMRRQSKSGGRPESLVTDSATPFNYNP
HPVDMTNLTLSREMQNMAERLAENAHDIWAKKKKEELVTNGGGIHPQLVPYDLLTD
KEKKKDRERSQEFLKYLQYQGYKLHRPSKATQSETEQTATGVAIELRFAYSLLEKLIQ
YIDRATINMKLLKPSTTFSRRTSFKTSTRDIKFFSKVVLPLMEKYFSTHRNYFIAVATAT
NNVGAASLKEKEMVASLFCKLASLLRSRLAAFGPDVRITVRCLQVLVKGIDAKSLVK
NCPEFIRTSMLTFFNNVADDLGHTILNLQEGKYSHLRGTHLKTSTSLAYINAVVLPILTS
LFDHLANCEYGADLLLDEIQVASYKMLGSLYALGTDATLTHDRKYLKTEIERNKPAL
GSCLGAFSSTFPVAFLEPHLNKHNQFSLLNRIADHSLEAQDIMAKMEQTMPTLETILGE
VDQFAESDKTYLDAPHIIDVVMPLLCSYLPFWWAQGPDNVTPTAGNHVTMVTAEHM
NQLLKNVLKLIKKNIGNETAPWMTRIATYTQQIIINSSEDLLRESFLPLAERVRKRTDN
MFHKEESLRGFIKSSTDDTSQVESQIQEDWQLLVRDIYSFYPLLIKYVDLQRNHWLRN
NVSEAEELYNHVAEIFNIWSKSQYFLKEEQNFISANEIDNMVLIMPTATRRVTAVTDGA
PSGGGKKKKKHRDKKRDKDKEVQASLMVACLKRLLPVGLNLFAGREQELVQHCKD
RFLKKMSEQDVSEFAKTQLTLPDKIDPADEMSWQHYLYSKLGSKSRTNMTVEGAENK
AKIIDDTVERIVAMSKVLFGLHMIDHPQQMSKKAYRSVVSIQRKRAVIQCFRHLSLHSL
PRHRCCNIFARTYYELWLEEENVGQEVMIEDLTQSFEDAELKKSDAVEEEGKPDPLTQ
LVTTFCRGAMTERSGALQEDPLYMSYAFIIAKSCGEEEEEGGDEEEEGGGEEVAEDEG
KASIHEQEMEKQKLLFHQARLADRGVAEMVLLHISASKGVPSDMVMKTLQLGNSILR
GGNIDIQMGMLNHLKDKKDVGFFTSIAGLMNSCSVLDLDAFERNTKAEGLGVGLEGA
AGEKNMHDAEFTCALFRFIQLTCEGHNLEWQNYLRTQAGNTTTVNVVICTVDYLLRL

QESIMDFYWHYSSKELIDPAGKANFFKAIGVASQVFNTLTEVIQGPCTQNQQALAHSR
LWDAVGGFLFLFSHMQDKLSKHSSQVDLLKELLNLQKDMITMMLSMLEGNVVNGTI
GKQMVDTLVESASNVELILKYFDMFLKLKDLTSSPSFQEIDGNSDGWVAPKDFREKM
EQQKSYTSEEIEFLLACCETNHDGKLDYIGFCDRFHEPAKEIGFNLAVLLTNLSEHMPN
EPRLARFLETAGSVLNYFEPFLGRIEIMGGSKRIERVYFEIKESNIEQWEKPQIKESKRAF
FYSIVTEGGDKEKLEAFVNFCEDAIFEMTHASGLMAASEDSSSGPKNREAAYMYLGD
DDDENSRKDPFRRGLQAIKDAIAMAFSSLSPANIKQRVADMQQMPPQELAVGFFKMF
FYMFYYIGYGALVVVRYIFGVLLGLMRGPQVEEPPPEPTEEEKIGQLRHRLLTQQSSPS
RHLPALPPPDDTGQPQVSAFGLDIAKEDNGQIQLKPHEKTPTASTPSSGEEGGETSPEEG
ATEGGEQQQPPSLIDLLGGEQKKKEVQERMEAQAAQQAAMSAIEAESKKAAQGITQP
SAVSQIDLSQYTKRAVSFLARNFYNLKYVALVLAFCINFVLLFYKVSTLDSEDGEGSGL
GDLISGSGSGRDGSGGGSGDGGSGESGEEDDPLEIVHIDEDYFYMEHVINIAAALHSIV
SLAILIGYYHLKVPLAIFKREKEIARKLEFDGLYIAEQPEDDDLKSHWDKLVISAKSFPV
NYWDKFVKKKVRAKYSETYDFDSISNMLGMEKTSFTAQEDEGSKGLFKYIITIDWRY
QVWKAGVTFTDNSFLYSLWYFSFSVMGNFNNFFFAAHLLDVAVGFKTLRTILQSVTH
NGKQLVLTVMLLTIIVYIYTVIAFNFFRKFYVQEEDDEVNRNCHDMLTCFVFNLYKGV
RAGGGIGDELEPPDGDESEVWRIIFDITFFFFIIVILLAILQGLIIDAFGELRDQLESVKED
MESNCFICGIGKDYFDKVPHGFDTHVAREHNLANYMFFLMHLINKPDTEYTGQETYV
WNMYTQRCWDFFPVGDCFRKQYEDAMGE

T. absoluta voltage-gated sodium channel (partial CDS)

MSEDLDSVSEEEVSLFRPFTRESLAAIEARIAEEHAKQKELEKKRAEGEVRYDDEDEDE
GPQPDATLEQGLPLPVRMQGNFPPELASTPLEDIDPYYHNQKTFVVISRGRDIFRFSAT
DAMWMLDPFNPIRRVAIYILVHPLFSFFIITTILVNCILMIMPSTPTVESTEVIFTGIYTFES
AVKLMARGFILQPFTYLRDAWNWLDFVVIALAYVTMGIDLGNLAALRTFRVLRALKT
VAIIPGLKTIVGAVIESVKNLRDVIILTMFSLSVFALMGLQIYMGVLTQKCIKVFPEDGS
WGNLTDENWERFCQNETNWYMENNDYPLCGNSSGAGTCEPGYICLQGYGPNPNYGY
TSFDTFGWAFLSAFRLMTQDYWENLYQLVLRSAGSWHVLFFVVIIFLGSFYLVNLILAI
VAMSYDELQKKAEEEEAAEEEALREAEQKAAAKADRQEAREAHARQVADAAAAAA
YAEAHPELALAAKSPSDTSCQSYELFVNQERGNQDDNTRERMSLRSDPFADSVSTQPT
HKPTADTHHEARRQRKVSMVPHPERINKYGQLSYGPLREGSQASLSLPGSPFNLRRGS
RGSHQMALRPNGRPRYPPGADRKPLVLSTYLDAQEHLPYADDSNAVTPMSEENGAIII
PVYYANLGSRHSSYTSHQSRLSYTSHGDLLGGGRNQTKEAKLRSRTASRNHSVTSQPH
AYPLPRQDSSLASRPLREYDPSTTECTDEAGKVLKPGSNDNPFIESSQQPNVVDMRDV
MVLNEIIEQAGRQSRASEQNVSVYYFPTAEDDEDGPTVKERLLECLMKGIDIFCVWDC
CWLWLEFQKYVALLVFDPFVELFITLCIVVNTLFMALDHHDMDRDMERALKSGNYFF
TATFGIEALFKLIAMSPKYYFQEGWNIFDFIIVALSLLELGLEGVQGLSVLRSFRLLRVF
KLAKSWPTLNLLISIMGRTMGALGNLIFVLCIIIFIFAVMGMQLFGKNYVDNVDRFPDG
DLPRWNFTDFMHSFMIVFRVLCGEWIESMWDCMLVGDVSCIPFFLATVVIGNFVVLN
LFLALLLSNFGSSSLSTPTADQDTNKIAEAFNRISRFNAWVKKNINEFLKMLKNKLTNQ
IAIHAPERVDNELELGTDLENAILYEDKKLKDQVEVAIGDGMEFTIPGDNNKYKKGKN
ILMNNINAITDNHRDNRLDCEINHHGYSIQDDDTISQKSYGSHKIRSFKDESHKGSADTI
DGEEKKDASKEELGLEEEIEAEEDIGELGKADIIVAADEDVVDDSPADCCPEPCYVKFP
FLAGDDESPFWQGWAMLRLKTFRLIENTYFETAVITMILLSSLALALEDVHLPHRPILQ
DILYYMDRIFTVIFFLEMLIKWLALGFQKYFTNAWCWLDFVIVMVSLINFVAALCGAG
GIQAFKTMRTLRALRPLRAMSRMQGMRVVVNALVQAIPSIFNVLLVCLIFWLIFAIMG
VQLFAGKYFKCVDLNHTTLSHEIIPDRNACILENYTWENSPMNFDHVGKAYLCLFQVA
TFKGWIQIMNDAIDSREVGRQPIRETNIYMYLYFVFFIIFGSFFTLNLFIGVIIDNFNEQK
KKAGGSLEMFMTEDQKKYYNAMKKMGSKKPLKATPRPKWRPQAIVFEIITDKKFDM
LIMLFIGFNMLTMTLDHYQMEETYSVVLDYLNMIFIVIFSSECLLKIFALRYHYFAEPW
NLFDFVVVTFSILTLVVSDVIEKYFVSPTLLRVVRVAKVGRVLRLVKGAKGIRTLLFAL
AMSLPALFNICLLLFLVMFIFAIFGMSFFMHVKNKGGLDDVYNFKTFVQSMILLFQMS
TSAGWDGVLDGIINEEECDLPDNERGYPGNCGSATIGITYLLSYLVISFLIVINMYIAVIL
ENYSQATEDVQEGLTDDDYDMYYEIWQRFDPDGTQYIRYDQLSDFLDVLEPPLQIHKP
NKYKIISMDIPICRGDMMFCVDILDALTKDFFARKGNPIEETGDLEVGRPDEVGYEPVS
STLWRQREEYCARLIQHAWRRHRRAHSEPATTDEGGADEGAPTAVLLDA

Appendix 5. Alignment of Spin, SpinSel and TA4 genomic DNA sequence. Nucleotides which differ between populations are highlighted. Exons 3A (red) and 3B (blue) are boxed.

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Spin consensus      CCTACTAGGGGTAGTGGGCAGTT-------GAAAACTCTGACACCA-------------
SpinSel consensus   CTGACTGTAAAGAATACGAAATTTTTCGCGGATGAACTGTGATGTCATATTAAAATAATTT
TA4 consensus       CTGACTGTAAAGAATACGAAATTTTTCGCGGATGAACTGTGATGTCATATTAAAATAATTT

Spin consensus      ------------------------------------------------------------
SpinSel consensus   GCAAGCTAGGTAGGTACTTATCCAACACATTAAAACAATTCTTATATTTATTACACTTATT
TA4 consensus       GCAAGCTAGGTAGGTACTTATCCAACACATTAAAACAATTCTTATATTTATTACACTTATT

Spin consensus      ------------------------------------------------------------
SpinSel consensus   CCTTGATATTTCGAATATAAGCAATCAAATTAATTTTTCCAATGGCGTAGCGGATAGTCCG
TA4 consensus       CCTTGATATTTCGAATATAAGCAATCAAATTAATTTTTCCAATGGCGTAGCGGATAGTCCG

Spin consensus      ---------------------GGGTTCA--------------------------------
SpinSel consensus   TGCCTGCTACGCCGAGGGTCGTGGATTCGATCCTCACAAGACACAGTTTTTTTAAAAATAT
TA4 consensus       TGCCTGCTACGCCGAGGGTCGTGGATTCGATCCTCACAAGACACAGTTTTTTTAAAAATAT

Spin consensus      TCGGGTT--GTTGGACGACT---------TGATGTCCCAC------ACGAAAAGAG----
SpinSel consensus   TTGGGTTTAGTCAGAGTGTTTTCTATGAATGACATACCATGATTTGACAAAAACAGTATAT
TA4 consensus       TTGGGTTTAGTCAGAGTGTTTTCTATGAATGACATACCATGATTTGACAAAAACAGTATAT

Spin consensus      ---AGTTATCTAGTTCCCATAGTACAAGCTATGCTTAGTTTAGGACTCGAGGATTTTATGT
SpinSel consensus   ATCAGTTATCTGGTTCCCATAGTACAAGCTACGCTTAGTTTAGGACTCAAGGATTTTATGT
TA4 consensus       ATCAGTTATCTGGTTCCCATAGTACAAGCTACGCTTAGTTTAGGACTCAAGGATTTTATGT

Spin consensus      GTATTACAATTTGAGGCGATGGGCTGACAACATGTCGCT-------------ATTTTCTGA
SpinSel consensus   GTATTGTAATTTGAGGCAATGGGCTGACAACATGTCGCTATGGCGATACACAATTTTCTGA
TA4 consensus       GTATTGTAATTTGAGGCAATGGGCTGACAACATGTCGCTATGGCGATACACAATTTTCTGA

Spin consensus      CTAATCCTGAAATGTTTGCGAGATGTGGCGTAAATGTTCTTTTTTACGGCTTTTTACGCTC
SpinSel consensus   ------CTGAAATGTTTGCCAGATGTGGCGTAAATGTTCGTTTTTACGGCCTTTTACGCTC
TA4 consensus       ------CTGAAATGTTTGCCAGATGTGGCGTAAATGTTCGTTTTTACGGCCTTTTACGCTC

Spin consensus      TGCCTACTCCATAAGGGATATATGTATGTATATAACTTTCAGTTTAATCAGGTTGAAAGCT
SpinSel consensus   TGCCTACTCCACAAGGGATATATGTATGTATATAACTTTCAGTTTAATCAGGTTGAAAGCT
TA4 consensus       TGCCTACTCCACAAGGGATATATGTATGTATATAACTTTCAGTTTAATCAGGTTGAAAGCT

Spin consensus      CATAGCCGCAGAACGGGTTAACTTATAATGGGATGAGTCATAAAACGCATTATAATATCAC
SpinSel consensus   CATAGCCCCAGAACGGGCTAATTTATAATGGGATGAGTCATAAAACGCATTATAATATCAC
TA4 consensus       CATAGCCCCAGAACGGGCTAATTTATAATGGGATGAGTCATAAAACGCATTATAATATCAC

Spin consensus      AAAGTTATTATAAAAGACATCAGTCATAAACAGAGCGCTGTTATTATATCTGTGATGTCTA
SpinSel consensus   AAAGTTATTATAAAAGACATCAGTCATAAACAGAGCGCTGTTATTATATCTGTGATGTCTA
TA4 consensus       AAAGTTATTATAAAAGACATCAGTCATAAACAGAGCGCTGTTATTATATCTGTGATGTCTA

Spin consensus      TTTGATTGCTCCTTTAACGATATTGAAGAGGATTACTGGCAGATGCTCGGGAAATTATTGC
SpinSel consensus   TTTGATTGCTCCTTTAACGATATTGAAGAGGATTACTGGCAGATGCTCGGGAAATTATTGC
TA4 consensus       TTTGATTGCTCCTTTAACGATATTGAAGAGGATTACTGGCAGATGCTCGGGAAATTATTGC

Spin consensus      TTCGGTACACTGACTAAATAGTAGGAGAAGACTTGTGTACATTCATACATATATCACGTGT
SpinSel consensus   TTCGATACACTGACTAAAGAGTAGGAGAAGACTTGTGTACATTCATACATATATCACGTGT
TA4 consensus       TTCGATACACTGACTAAAGAGTAGGAGAAGACTTGTGTACATTCATACATATATCACGTGT

Spin consensus      ATATCCCTTGCGGGGTATGCAGAGCGCGAACGGCCGTAAAAACAACATTTGATTGTAATTC
SpinSel consensus   ATATCCCTTGCGGGTTAGGCAGAGCGCGAACGGCCGTAAAAACAACATTTGATTGTAATTC
TA4 consensus       ATATCCCTTGCGGGTTAGGCAGAGCGCGAACGGCCGTAAAAACAACATTTGATTGTAATTC

Spin consensus      GGAAAATTGTGT------------ATTGACGTAGTGATATGTTGTCAACCTATCGCCTAAA
SpinSel consensus   GGAAAATTACGTAATAATTACGTAATTGACGTAATGATATGTTGTCACCCTATCGCCTAAA
TA4 consensus       GGAAAATTACGTAATAATTACGTAATTGACGTAATGATATGTTGTCACCCTATCGCCTAAA

Spin consensus      ATTTCAATACAAATGTTAAACTCGAGTTCCTAAGTAGCCTCTTACAACACCAACGGGAAAG
SpinSel consensus   ATTTGGATACAAATGTTAAACTCGAGTTCCTAAGTAGCCTCTTACAACACCAACGGGAAAG
TA4 consensus       ATTTGGATACAAATGTTAAACTCGAGTTCCTAAGTAGCCTCTTACAACACCAACGGGAAAG

Spin consensus      AAATGGGGGATGCTATTCACACGGCACCGTAAAAATAACAATAAAAAAAAGAAATTCATT
SpinSel consensus   AAATGGGGGATGCTATTCACTCGGCAATACAA----TACAAT-AATAAAAGGAAATTCATT
TA4 consensus       AAATGGGGGATGCTATTCACTCGGCAATACAA----TACAAT-AATAAAAGGAAATTCATT

Spin consensus      ATTTAATTAAAATAAATATAATGATAAAAAGAAACTAAAAGTTTTACACGAGCGGGATTCG
SpinSel consensus   ATTTAATTAAAATAATAATAATAATAAAAAGAAACTAAAAGTTTTACACGAGCGGGATTCG
TA4 consensus       ATTTAATTAAAATAATAATAATAATAAAAAGAAACTAAAAGTTTTACACGAGCGGGATTCG

Spin consensus      AAACCACTAAGCTGTCATTTCCTTCAATCGGGTTTTTCGTTACCAACCTCAAGAACAATCC
SpinSel consensus   AAACAACGAAGCTGTCATTTCCTTCAATCGGGTTGACGTTACCAACCTCAAGAACAATCT
TA4 consensus       AAACAACGAAGCTGTCATTTCCTTCAATCGGGTTGACGTTACCAACCTCAAGAACAATCT

Spin consensus      TTGCAAAAATTGTATTACCGATTTATTATTTACAGAAAATACTTAATTTATTACACTAG--
SpinSel consensus   TTGCAAAAATTGTATTACCGATTTATTATTTACAGAAAATACTTAATTTATTGCACTAGAT
TA4 consensus       TTGCAAAAATTGTATTACCGATTTATTATTTACAGAAAATACTTAATTTATTGCACTAGAT

Spin consensus      --ATAATCAATTATTTGGAGTACTAATTTATATAAATTATAGTTTAAATGTTAAAGGTGTC
SpinSel consensus   ACATAATCAATTATTTGGATTACTGATTTATATAAGTTATAATTTTAATGTTAAAGGTGTC
TA4 consensus       ACATAATCAATTATTTGGATTACTGATTTATATAAGTTATAATTTTAATGTTAAAGGTGTC

Spin consensus      TCCCG--TGTTATGGTCTATGCGTTTAAAATACAGACTAGAGATTAAACTTACCTAAAGTT
SpinSel consensus   TCCTGTTTTTTATGGTCTATGCATTTAAAATACAGACTAGAGATTAAACTTACCTAAAGTT
TA4 consensus       TCCTGTTTTTTATGGTCTATGCATTTAAAATACAGACTAGAGATTAAACTTACCTAAAGTT

Spin consensus      TTTACATAAATAAGAGTAATGCCTGTAGCTTTGGTTATTAAACGA-TTTTATTACAATAAG
SpinSel consensus   ATTACATAAATAAGAGTAATGCCTGTAGCTTTGGTTATTAAACGATTTTTATTACAATAAG
TA4 consensus       ATTACATAAATAAGAGTAATGCCTGTAGCTTTGGTTATTAAACGATTTTTATTACAATAAG

Spin consensus      AGAATAAAATTATTGATACCATTGGAGAAGCAAACATTTGCGAGTTTGCG-TA-ATGTGGA
SpinSel consensus   AG-----AGTTATTGATACGATTGAAGAAGCAGACATTTGCGAGTTTGCGATATATGTGGA
TA4 consensus       AG-----AGTTATTGATACGATTGAAGAAGCAGACATTTGCGAGTTTGCGATATATGTGGA

Spin consensus      TGATATTATGATTTCAGAATTGATTGTAATAGAGTTTGTGATAGAATTCTTACGGTATAGT
SpinSel consensus   TGATATTATGATTTCAGAATTGATTGTAATAGAGTTTGTGATAAAATTCTTACGGTGTAAT
TA4 consensus       TGATATTATGATTTCAGAATTGATTGTAATAGAGTTTGTGATAAAATTCTTACGGTGTAAT
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Spin consensus      TACAAAAAAAAAGTTTAACAATGTCTAAGTCGTTTGACAACGTCAAATCTTATGG------
SpinSel consensus   CAC----AAAAAGTTAAGCAATGCCAAAGTCGTTGGACAACGTCAAATCTTATGGAAAAGT
TA4 consensus       CAC----AAAAAGTTAAGCAATGCCAAAGTCGTTGGACAACGTCAAATCTTATGGAAAAGT

Spin consensus      ------------------------------------------------------------
SpinSel consensus   AGCTAAGTAGCTTGCTGCGTTCCGTCAGTGCGTGGGAGTGCCAGTGCCTTAACAGGTTTCT
TA4 consensus       AGCTAAGTAGCTTGCTGCGTTCCGTCAGTGCGTGGGAGTGCCAGTGCCTTAACAGGTTTCT

Spin consensus      ------------------------------------------------------------
SpinSel consensus   TCTGGGAGAGCTCGCTCCATTTTAGACTCTTTCTCTGTCCTATGACCTAGACTAGAGTCAA
TA4 consensus       TCTGGGAGAGCTCGCTCCATTTTAGACTCTTTCTCTGTCCTATGACCTAGACTAGAGTCAA

Spin consensus      -----------------------AAAAGTTATTTTAACAGCGTTTCAATGCGCGTCTAAAT
SpinSel consensus   GAGCAAACCCATCATTAATAAAAAAAAGTTATTTTAACAGCGTTTCAATGCGCGTC----T
TA4 consensus       GAGCAAACCCATCATTAATAAAAAAAAGTTATTTTAACAGCGTTTCAATGCGCGTC----T

Spin consensus      GTTTTGTCATAACACGGATAGAGATAAAACATACTATATCGTGTACAGTCAATTCTGAAAT
SpinSel consensus   GTTTTGTGAAAACACAGATAGAGATAAAACATACTATTTCGTGTACAGTCAATTCTGAAAT
TA4 consensus       GTTTTGTGAAAACACAGATAGAGATAAAACATACTATTTCGTGTACAGTCAATTCTGAAAT

Spin consensus      GAATATAATTTTTTCTTTCTTTATTATATCAATAAGATTAAGTGATGGTAATATGGGTAAA
SpinSel consensus   GAATATAACTTTTTCTTTCTTTATCATATCAATAAGATTAAGTGATGGTAATATGGGTAAA
TA4 consensus       GAATATAACTTTTTCTTTCTTTATCATATCAATAAGATTAAGTGATGGTAATATGGGTAAA

Spin consensus      AGCCAGTACAACCCAACAACCTGATGTTTATTATGTTTTTCGTTCCTTTCTTGCGACGCCG
SpinSel consensus   AGCCAGTACAACCCAACAACCTGATGTTTATTATGTTTTCCGTTCCTTTCTTGCGACGCCG
TA4 consensus       AGCCAGTACAACCCAACAACCTGATGTTTATTATGTTTTCCGTTCCTTTCTTGCGACGCCG

Spin consensus      TGCGTTCTATTAACAAAATAAGGATGAGAAAAATCAAATCCTGACTACAAACGTCTGGCTT
SpinSel consensus   TGCGTTCTATTAACAAAATAAGGATGAGAAAAATCAAATCCTGACTACAAACGTCTGGCTT
TA4 consensus       TGCGTTCTATTAACAAAATAAGGATGAGAAAAATCAAATCCTGACTACAAACGTCTGGCTT

Spin consensus      AATTTGGTAAGTGGTAGGTGNCGGGCCCGGCGCCTCGCGTCCCGCCCCCGCTCCCGTCGTC
SpinSel consensus   AATTTGGTAAGTGGTAGGTG-CGGGCCCGGCGCCTCGCGTCCCGCCCCCGCTCCCGTCGTC
TA4 consensus       AATTTGGTAAGTGGTAGGTG-CGGGCCCGGCGCCTCGCGTCCCGCCCCCGCTCCCGTCGTC

Spin consensus      AACTTTCACTGGTAACATCAAGTTTTCCCTAACAAACACTTCGACAGTCAATTTTATCTAT
SpinSel consensus   AACTTTCACTGGTAACATCAAGTTTTCCCTAACAAACACTTCGACAGTCAATTTTATCTAT
TA4 consensus       AACTTTCACTGGTAACATCAAGTTTTCCCTAACAAACACTTCGACAGTCAATTTTATCTAT

Spin consensus      CACCACACAGTACTTTCGACTGCACCCTCTGAATGCGGTTTTGTATCGGCTAGGATGCGAT
SpinSel consensus   CACCACACAGTACTTTCGACTGCACCATCTGAATGCGGTTTTGTATCGGCTAGGATGCGAT
TA4 consensus       CACCACACAGTACTTTCGACTGCACCATCTGAATGCGGTTTTGTATCGGCTAGGATGCGAT

Spin consensus      GGGCGAGGCAAGAATTGGAAAATTGAGATTGACTGAGCGTAAATTGCATTTTGCTAGACAG
SpinSel consensus   GGGCGAGGCAAGAATTGGAAAATTGAGATTGACTGAGCGTAAATTGCATTTTGCTAGACAG
TA4 consensus       GGGCGAGGCAAGAATTGGAAAATTGAGATTGACTGAGCGTAAATTGCATTTTGCTAGACAG

Spin consensus      CACTGCGAATTTACTTATTTCACTGTGTATTGGGAATCATTGAATGTGATAGATGGAATAT
SpinSel consensus   CACTGCGAATTTACTTATTTCACTGTGTATTAGGAATCATTGAATGTGATAGATGGAATAT
TA4 consensus       CACTGCGAATTTACTTATTTCACTGTGTATTAGGAATCATTGAATGTGATAGATGGAATAT

Spin consensus      ATAATGTACCTACTAATATGATAGACTAGAATGAAAATTAGCACCTTTAGAATTGTAGTGA
SpinSel consensus   ATAACGTACCTACTAATATGATAGACTGGAATGAAAATTAGCACCTTTAGAATTTTAGT--
TA4 consensus       ATAACGTACCTACTAATATGATAGACTGGAATGAAAATTAGCACCTTTAGAATTTTAGT--

Spin consensus      TCTATATTAAAAGCTAGAAAACTTTGTAAAACCAAGTGA----TACTTCATCAATTTATTC
SpinSel consensus   ----------AAGCTAAGAAACGTTGTAAAAGCAAATGATAAGTACTTCATCAATTTATTC
TA4 consensus       ----------AAGCTAAGAAACGTTGTAAAAGCAAATGATAAGTACTTCATCAATTTATTC

Spin consensus      GTAAAAAA--------ACAGCCCCAAATCTTGCCTCAACTCGTCAGAAATCCAGGCTGGTA
SpinSel consensus   GTAGAAAAACGAAAGTACAGCCCCAAATCTTGCCTCAACTTGTTAGAAATCCTGGCTGGTA
TA4 consensus       GTAGAAAAACGAAAGTACAGCCCCAAATCTTGCCTCAACTTGTTAGAAATCCTGGCTGGTA

Spin consensus      ACAAAATAGCACAAATTTTAAAGAGTCTTAAACATTTCAGCAAAAGGGTGATGGTGGCGGG
SpinSel consensus   ACAAAATAGCACAAATTTTAAAGAGTCTTAAACATTTCAGCAAAAGGGTGATGGTGGCGGG
TA4 consensus       ACAAAATAGCACAAATTTTAAAGAGTCTTAAACATTTCAGCAAAAGGGTGATGGTGGCGGG

Spin consensus      AGGTAGAGCGCGCCAAGCGGCGGTTGTCGCATGGCCGTCGGTGTTATGTTGTCACTCGCTC
SpinSel consensus   AGGTAGAGCGCGCCAAGCGGCGGTTGTCGCATGGCCGTCGGTGTTATGTTGTCACTCGCTC
TA4 consensus       AGGTAGAGCGCGCCAAGCGGCGGTTGTCGCATGGCCGTCGGTGTTATGTTGTCACTCGCTC

Spin consensus      GGTATAGGTGCCGTGCGGGATACATTGATTGCGCCACTTCGTACAAGCTTTAAGACGCAAC
SpinSel consensus   GGTATAGGTGCCGCGCGGGATACATTGATTGCGCCACTTCGTACAAGCTTTAAGACGCAAC
TA4 consensus       GGTATAGGTGCCGCGCGGGATACATTGATTGCGCCACTTCGTACAAGCTTTAAGACGCAAC

Spin consensus      CACTACTGCTGGTTTAAAGTGTTGCAATTTTATTCAAAAGGCTTTTACTAACTGGCGCTTT
SpinSel consensus   CACTACTGCGGGTTTAAAGTATTGCAATTTTATTCAAAAGGCTTTTACTAACTGGCGCTTT
TA4 consensus       CACTACTGCGGGTTTAAAGTATTGCAATTTTATTCAAAAGGCTTTTACTAACTGGCGCTTT

Spin consensus      TATGAGCCCGCTAGGTTCCGTTAGCTCTGTGTGGCGGCTCGCGGGGACTGCTCGATCGCAT
SpinSel consensus   TATGAGCCCGCAAGGTTCCGTTAGCTCTGTGTGGCGGCTCGCGGGGACTGCTCGATCGCAT
TA4 consensus       TATGAGCCCGCAAGGTTCCGTTAGCTCTGTGTGGCGGCTCGCGGGGACTGCTCGATCGCAT

Spin consensus      GTTGGCAGCTCCTGTGTGCCGCTGGTCCTCGTAATGTGTCATCCAGGTGTCGATCACAGTG
SpinSel consensus   GTTGGCAGCTCCTGTGTGCCGCTGGTCCTCGTAATGTGTCATCCAGGTGTCGATCACAGTG
TA4 consensus       GTTGGCAGCTCCTGTGTGCCGCTGGTCCTCGTAATGTGTCATCCAGGTGTCGATCACAGTG

Spin consensus      TTTAGATCATTATACTGACTGCGCCCTTTCTTTCTGTTTTTGTTGACATCGTCTAACCCAA
SpinSel consensus   TTTAGATCATTATACTGACTGCGCCCTTTCTTTCTGTTTTTGTTGACATCGTCTAACCCAA
TA4 consensus       TTTAGATCATTATACTGACTGCGCCCTTTCTTTCTGTTTTTGTTGACATCGTCTAACCCAA

Spin consensus      TAGGATGAGAAGAATCAACTACTTATAACCAATATATGGCTGTCATTGGTAAGTGTGTATA
SpinSel consensus   TAGGATGAGAAGAATCAACTACTTATAACCAATATATGGCTGTCATTGGTAAGTGTGTATA
TA4 consensus       TAGGATGAGAAGAATCAACTACTTATAACCAATATATGGCTGTCATTGGTAAGTGTGTATA

Spin consensus      AAAGAACGACCTAAATTAATAATTAAAACTTCTAATGTTCCGTTCTTTACTTACTTTCTTT
SpinSel consensus   AAAGAACGACCTAAATTAATAATTAAAACTTCTAATGTTCCGTTCTTTACTTACTTTCTTT
TA4 consensus       AAAGAACGACCTAAATTAATAATTAAAACTTCTAATGTTCCGTTCTTTACTTACTTTCTTT
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Spin consensus      CTGTTGTGATTCAAACTCTTCTTCATTTTGTTTGTCTCCGATTGAGTTATCTTCTTCTCTG
SpinSel consensus   CTGTTGTGATTCAAACTCTTCTTCTTTTTGTTTGTCTCCGATTGAGTTATCTTCTTCTCTG
TA4 consensus       CTGTTGTGATTCAAACTCTTCTTCTTTTTGTTTGTCTCCGATTGAGTTATCTTCTTCTCTG

Spin consensus      TATGTTATATTTGGAAATTGCATAGCATTGTGGAATATGCGGCAGTTACAAGATTTTCTCA
SpinSel consensus   TATGTTATATTTGGAAATTGCATAGCATTGTGGAATATGCGGCAGTTACAAGATTTTCTCA
TA4 consensus       TATGTTATATTTGGAAATTGCATAGCATTGTGGAATATGCGGCAGTTACAAGATTTTCTCA

Spin consensus      AATTGTTATTGGCTGATCTCGTTTTGCCAGTAGCTAGTGTAGACCCTGGTTCCTGTTATGC
SpinSel consensus   AATTGTTATTGGCTGATCTCGTTTTGCCAGTAGCTAGTGTAGACCCTGGTTCCTGTTATGC
TA4 consensus       AATTGTTATTGGCTGATCTCGTTTTGCCAGTAGCTAGTGTAGACCCTGGTTCCTGTTATGC

Spin consensus      ATCCATCGTATCTGATACTATACCTTGCTGTAGTTACTTTTTTATCCTTGGCTATTTAAAG
SpinSel consensus   ATCCATCGTATCTGATACTATACCTTGCTGTAGTTACTTTTTTATCCTTGGCTATTTAAAG
TA4 consensus       ATCCATCGTATCTGATACTATACCTTGCTGTAGTTACTTTTTTATCCTTGGCTATTTAAAG

Spin consensus      ATTTAAATCGTCAATAGTCTGTCAACTAGAGATTCTCTGACTTTCACATACACACTCGTAC
SpinSel consensus   ATTTAAATCGTCAATAGTCTGTCAACTAGAGATTCTCTGACTTTCACATACACACTCGTAC
TA4 consensus       ATTTAAATCGTCAATAGTCTGTCAACTAGAGATTCTCTGACTTTCACATACACACTCGTAC

Spin consensus      ACGAAGTTCTATCTAGATGGCATTGGATCTATTTGTGATTTGTCATCTTGACGTCGCGTTG
SpinSel consensus   ACGAAGTTCTATCTAGATGGCGTTGGATCTATTTGTGATTTGTCATCTTGACGTCGCGTTG
TA4 consensus       ACGAAGTTCTATCTAGATGGCGTTGGATCTATTTGTGATTTGTCATCTTGACGTCGCGTTG

Spin consensus      CTGGAAGACATCTGTATTTTGAGCGATGCGATATATCTAATGTACAGCTATTGATCCATCC
SpinSel consensus   CTGGAAGACATCTGTATTTTGAGCGATGCGATATATCTAATGTACAGCTATTGATCCATCC
TA4 consensus       CTGGAAGACATCTGTATTTTGAGCGATGCGATATATCTAATGTACAGCTATTGATCCATCC

Spin consensus      TGTTGCTTAAAGTGTATCTTTGCTCTATTGAAATACATTTAAGAAAATAGTCCAATATACA
SpinSel consensus   TGTTGCTTAAAGTGTATCTTTGCTCTATTGAAATACATTAAAGAAAATAGTCCAATATACA
TA4 consensus       TGTTGCTTAAAGTGTATCTTTGCTCTATTGAAATACATTAAAGAAAATAGTCCAATATACA

Spin consensus      CGAACTGCCGTGTATGCCGCAATGCGATAACAATATGTACTAATGGTTGACGATAAAAAGC
SpinSel consensus   CGAACTGCCGTGTATGCCGCAATGCGATAACAATATGTACTAATGGTTGACGATAAAAAGC
TA4 consensus       CGAACTGCCGTGTATGCCGCAATGCGATAACAATATGTACTAATGGTTGACGATAAAAAGC

Spin consensus      TCTAAACATAAAACAAGTAAATATCTGGGATTGTGGTACATCTACAATCTATATAACATAT
SpinSel consensus   TCTAAACATAAAACAAGTAAATATCTGGGATTGTGGTACATCTACAATCTATATAACATAT
TA4 consensus       TCTAAACATAAAACAAGTAAATATCTGGGATTGTGGTACATCTACAATCTATATAACATAT

Spin consensus      GATTAATTGTACTTAAGTGATATAGGGATTCTAGGTTCAGACTGTCAATTAAAAGTAACAT
SpinSel consensus   GATAAATTGTACTTAAGTGATATAGGGATTCTAGGTTCAGACTGTCAATTAAAAGTAACAT
TA4 consensus       GATAAATTGTACTTAAGTGATATAGGGATTCTAGGTTCAGACTGTCAATTAAAAGTAACAT

Spin consensus      ATTGCTGGGATGTTTCTATGTCGTCTGTATTATTGATACTTGTAAAACCAGCTTAATTTAT
SpinSel consensus   ATTGCTGGGATGTTTCTATGTCGTCTGTATTATTGATACTTGTAAAACCAGCTTAAATTAT
TA4 consensus       ATTGCTGGGATGTTTCTATGTCGTCTGTATTATTGATACTTGTAAAACCAGCTTAAATTAT

Spin consensus      TTAAAGGGATAATGT---TCTCATAAAACTGAATATTGCAAAGAGACTGATCTTTTAAATA
SpinSel consensus   TTAAAGGGTAAATGTGACTTTTATAAAACTGAAAATTGCAAAGAGACTGATCTTTAAAATA
TA4 consensus       TTAAAGGGTAAATGTGACTTTTATAAAACTGAAAATTGCAAAGAGACTGATCTTTAAAATA

Spin consensus      CTGTGTACACAAATTCAGCAACAGCAATTCGATCTTTTTAGAAGGTCTTTGGAATATAACA
SpinSel consensus   CTGTGTACACAAATTCAGCAACAGCAATTCTATATTTTTAGAAGCTCTTTGGAATATTACA
TA4 consensus       CTGTGTACACAAATTCAGCAACAGCAATTCTATATTTTTAGAAGCTCTTTGGAATATTACA

Spin consensus      TCAGTTTTACGAATACGTTTACCACTTTTATGATGTAGTTGGTTTTGTAAGTGTCACTCAT
SpinSel consensus   TCAGTTTTACGAATGCGTTTACCATTTTTATGATGTAGTTGGTTTTGTAAGTGTCACTCAT
TA4 consensus       TCAGTTTTACGAATGCGTTTACCATTTTTATGATGTAGTTGGTTTTGTAAGTGTCACTCAT

Spin consensus      ACACATATTGTTTTATTGATATGTGATTCACTGTACATTGACTATGCACTTCCCGGGTACC
SpinSel consensus   ACACATATTGTTTTATTGATATGTGATTCACTGTACATTGACTATGCACTTCCCGGGTACC
TA4 consensus       ACACATATTGTTTTATTGATATGTGATTCACTGTACATTGACTATGCACTTCCCGGGTACC

Spin consensus      GCTGTTTTTTTGTTTGGCTAAACCAATAGTGTTTTTCTCTGCATGGCCATGGAATGTTACA
SpinSel consensus   GCTGTTTTTTTGTTTGGCTAAACCAATAGTGTTTTTCTCTGCATGGCCATGGAATGTTACA
TA4 consensus       GCTGTTTTTTTGTTTGGCTAAACCAATAGTGTTTTTCTCTGCATGGCCATGGAATGTTACA

Spin consensus      TTATTCTATGGGAATGAGGTGCCTACCATGGTATGTAGATGTCTGCAGAAGGTTTCCCTTA
SpinSel consensus   TTATTCTATGGGAATGTGGTGCCTACCATGGTATGTAGATGTCTGCAGAAGGTTTCCCTTA
TA4 consensus       TTATTCTATGGGAATGTGGTGCCTACCATGGTATGTAGATGTCTGCAGAAGGTTTCCCTTA

Spin consensus      AGACGAAACAAAGGATTCTAAAACTTCTCCGTAACAAACTTTTTAAAACTTTTGTAGTTTC
SpinSel consensus   AGACGAAACAAAGGATTATAAAACTTCGCCATAATAAACTTTTTAAAACTTTTGTAGTTTC
TA4 consensus       AGACGAAACAAAGGATTATAAAACTTCGCCATAATAAACTTTTTAAAACTTTTGTAGTTTC

Spin consensus      TAGATCTTTTGATAGAGGGAGCTATTTAGAGAGATTGCAATTTCAGGATTCAAGGGAAGAG
SpinSel consensus   TAGATCTTTTGATAGAGGGAGCTATTTAGAGAGATTGCAATTTCAGGATTCAAGGGAAGAG
TA4 consensus       TAGATCTTTTGATAGAGGGAGCTATTTAGAGAGATTGCAATTTCAGGATTCAAGGGAAGAG

Spin consensus      TTACGAGACAATTTTAATTTAAACTTCATTTACTATTGAAGAAGAATTTAAAACTTTTTGT
SpinSel consensus   TTACGAGACGATTTTAATTTAAACTTCATTTACTATTGAAGAAGAATTTAAAACTTTTTGT
TA4 consensus       TTACGAGACGATTTTAATTTAAACTTCATTTACTATTGAAGAAGAATTTAAAACTTTTTGT

Spin consensus      TTTATTTGTTTGTTGGTAATGACTTTTATT
SpinSel consensus   TTTATTTGTTTGTTGGTAATGACTTTTATT
TA4 consensus       TTTATTTGTTTGTTGGTAATGACTTTTATT
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Appendix 6A. Differentially expressed transcripts in Assembly 5. FDR = False discovery rate; FC = Fold change estimated by DEseq2. List filtered by FDR <0.05 by both EdgeR and DEseq2 analyses.

Contig ID	NCBI top hit	EdgeR FDR	DEseq2 FDR	FC
72410_c0_seq2	---NA---	5.27E-14	6.30E-25	258.23
63590_c1_seq17	uncharacterized protein	1.88E-08	6.35E-18	140.93
68931_c0_seq5	---NA---	2.83E-12	1.12E-15	108.14
67838_c0_seq20	hypothetical protein KGM_11873	9.64E-08	4.73E-13	96.40
63687_c0_seq4	---NA---	4.97E-07	2.36E-16	95.11
68794_c0_seq6	---NA---	1.75E-09	6.74E-12	93.49
72670_c0_seq2	---NA---	1.51E-07	5.86E-14	87.04
66260_c0_seq2	PREDICTED: twinfilin-like	5.76E-06	2.95E-12	81.12
73371_c0_seq2	hypothetical protein KGM_02829	9.48E-05	3.79E-12	72.99
66260_c0_seq11	PREDICTED: twinfilin-like	2.28E-06	1.99E-09	62.24
69919_c0_seq3	hypothetical protein KGM_08754	3.72E-05	5.39E-10	62.14
66685_c2_seq16	hypothetical protein	2.56E-05	8.44E-10	61.51
72367_c0_seq3	hypothetical protein KGM_16827	3.72E-04	1.14E-10	61.23
71303_c0_seq13	protein arginine n-	5.49E-05	1.17E-09	59.13
66685_c2_seq13	hypothetical protein	2.26E-07	9.60E-09	58.71
67838_c0_seq5	hypothetical protein KGM_11873	8.60E-05	3.32E-09	55.22
65946_c0_seq3	---NA---	1.30E-03	5.12E-10	54.29
68928_c0_seq1	PREDICTED: uncharacterized	1.57E-05	1.99E-08	52.26
63395_c0_seq5	fumarylacetoacetase	1.36E-06	4.16E-08	51.82
73260_c0_seq8	protein real-time-like isoform x1	6.37E-05	1.35E-08	51.65
71971_c0_seq13	vacuolar atp synthase subunit s1	1.76E-07	9.80E-08	47.31
68853_c0_seq14	PREDICTED: uncharacterized	3.35E-03	6.87E-09	46.16
69840_c2_seq2	cathepsin l-like protease	2.26E-05	4.26E-09	45.84
61770_c1_seq2	---NA---	2.07E-03	1.80E-08	45.01
65435_c0_seq8	neural cell adhesion molecule 1-	3.61E-04	6.99E-08	44.68
71257_c0_seq11	bcl2 adenovirus e1b 19 kda	9.41E-04	4.67E-08	44.17
64381_c0_seq9	ring finger protein 181	6.86E-03	3.92E-08	40.56
63226_c1_seq1	---NA---	1.33E-05	9.72E-07	39.78
59853_c0_seq3	---NA---	1.14E-03	2.09E-08	39.22
72484_c0_seq2	---NA---	9.95E-03	8.25E-08	38.15
72951_c0_seq14	hypothetical protein KGM_15294	8.80E-03	1.18E-07	37.74
71583_c0_seq1	protein fam49b-like	2.60E-03	5.39E-07	36.76
61004_c0_seq2	PREDICTED: uncharacterized	5.98E-03	4.12E-07	36.09
63096_c0_seq1	hypothetical protein NP_c11	9.38E-03	2.68E-07	36.01
71477_c0_seq1	pi-plc x domain-containing	9.94E-03	2.68E-07	35.87
54204_c0_seq1	dopamine d2-like receptor-like	8.91E-03	3.58E-07	35.51
72601_c0_seq4	synaptic vesicle glycoprotein 2c-	6.84E-04	2.26E-06	34.82
73362_c0_seq2	histone-lysine n-	7.75E-04	2.27E-06	34.66
71884_c0_seq8	---NA---	2.72E-03	1.31E-06	34.62
65762_c4_seq10	---NA---	1.77E-04	4.81E-06	33.52
72802_c0_seq22	---NA---	6.83E-03	8.78E-08	33.22
71977_c0_seq6	c-type lectin partial	1.65E-02	5.99E-07	33.14
65006_c0_seq18	dullard-like protein	7.38E-04	4.03E-06	33.14
64002_c1_seq16	---NA---	1.67E-02	6.44E-07	32.96
56722_c0_seq9	zinc finger bed domain-	1.16E-02	1.28E-06	32.42
71039_c0_seq10	---NA---	6.97E-03	1.08E-08	32.15
66906_c0_seq3	g protein alpha subunit	9.23E-04	5.51E-06	32.10
69755_c0_seq25	triacylglycerol pancreatic	9.94E-03	3.08E-06	30.90
62319_c0_seq1	---NA---	1.95E-02	1.87E-06	30.60
68925_c1_seq13	stromal interaction molecule 1	1.13E-02	4.21E-06	30.03
66385_c1_seq9	endonuclease-reverse	5.10E-03	7.45E-06	29.83
72264_c0_seq4	---NA---	1.99E-02	2.86E-06	29.79
66821_c0_seq42	PREDICTED: uncharacterized	6.59E-03	7.45E-06	29.58

71707_c0_seq21	---NA---	2.27E-02	2.84E-06	29.54
71033_c0_seq6	hypothetical protein KGM_12398	1.55E-02	4.24E-06	29.50
68244_c0_seq6	---NA---	1.04E-03	1.42E-05	29.39
68119_c1_seq40	glycine receptor beta precursor	2.27E-02	3.20E-06	29.25
63435_c0_seq5	zinc finger protein 91	9.78E-03	6.85E-06	29.25
72174_c0_seq4	hemocyte-specific integrin alpha	4.34E-03	1.12E-05	29.00
65536_c4_seq3	ras-related protein rab-9b-like	2.52E-02	5.95E-06	27.83
69078_c0_seq3	---NA---	2.36E-02	7.06E-06	27.66
60733_c0_seq6	hypothetical protein KGM_22419	3.26E-02	4.60E-06	27.63
58134_c2_seq3	---NA---	8.79E-03	1.62E-05	27.41
65874_c0_seq3	probable 3 -cyclic	1.22E-02	1.42E-05	27.27
68925_c1_seq8	stromal interaction molecule 1	1.33E-02	1.37E-05	27.25
73558_c0_seq1	---NA---	2.91E-02	3.74E-07	27.15
67954_c0_seq7	leptin receptor gene-related	4.08E-03	2.48E-05	27.09
58938_c1_seq12	---NA---	1.97E-02	1.15E-05	27.05
72415_c0_seq1	---NA---	3.47E-02	6.61E-06	26.92
58107_c0_seq3	---NA---	2.77E-02	1.22E-05	26.21
51219_c0_seq2	---NA---	3.73E-02	1.26E-05	25.50
63437_c0_seq10	PREDICTED: uncharacterized	3.27E-02	1.21E-06	25.47
72711_c0_seq14	hypothetical protein KGM_15424	2.69E-02	2.05E-05	25.28
72906_c0_seq1	---NA---	7.22E-03	5.45E-06	24.93
62555_c1_seq1	rna-directed dna polymerase from	3.72E-02	1.55E-06	24.86
64078_c0_seq21	PREDICTED: uncharacterized	1.19E-02	4.95E-05	24.67
71571_c0_seq3	PREDICTED: interaptin-like	3.41E-02	2.41E-05	24.58
68832_c1_seq10	---NA---	2.04E-02	4.06E-05	24.48
68992_c1_seq20	---NA---	2.64E-02	3.87E-05	24.16
65831_c0_seq2	hypothetical protein	4.13E-02	2.80E-05	23.93
63274_c3_seq27	---NA---	1.69E-02	5.98E-05	23.89
68753_c0_seq10	reverse transcriptase ( )	2.55E-02	5.10E-05	23.67
58361_c4_seq2	---NA---	3.05E-02	1.15E-06	23.64
66601_c1_seq1	---NA---	4.94E-02	2.96E-05	23.44
63565_c0_seq1	hypothetical protein KGM_22404	2.07E-02	8.22E-05	23.02
67039_c1_seq5	---NA---	3.73E-02	6.17E-05	22.73
63607_c0_seq1	unknown	2.48E-02	1.04E-04	22.27
69981_c0_seq3	---NA---	6.79E-03	3.71E-05	21.31
67354_c1_seq23	hypothetical protein	7.10E-03	3.02E-05	20.19
70957_c0_seq5	beta- -galactosyltransferase	3.27E-02	1.29E-06	19.51
64647_c0_seq6	beta-adaptin	4.87E-02	3.27E-04	19.29
71623_c0_seq7	hypothetical protein KGM_09042	2.06E-02	1.18E-04	19.10
72702_c0_seq26	mannose-1-phosphate	1.61E-02	5.85E-04	18.87
51318_c0_seq1	---NA---	3.60E-02	1.03E-04	18.02
62459_c1_seq8	pol polyprotein	4.32E-02	7.91E-04	17.63
71747_c0_seq42	---NA---	2.41E-02	1.11E-03	17.21
67207_c1_seq4	---NA---	3.78E-02	1.12E-03	16.95
65912_c0_seq3	btb poz domain-containing	3.64E-02	1.29E-03	16.70
70810_c0_seq8	---NA---	5.63E-04	8.60E-04	16.63
69994_c0_seq26	non-muscle myosin heavy chain	5.85E-03	8.07E-04	16.04
56722_c0_seq10	---NA---	3.26E-02	1.01E-03	15.60
59684_c0_seq13	hypothetical protein KGM_22605	4.05E-02	2.80E-03	15.03
70976_c0_seq2	probable bifunctional	3.00E-05	5.46E-06	14.52
70810_c0_seq4	---NA---	1.15E-04	2.90E-03	14.39
60557_c0_seq1	hypothetical protein KGM_10094	2.03E-02	3.10E-04	11.91
67329_c0_seq10	---NA---	2.72E-03	1.23E-02	11.13
72476_c1_seq10	farnesyl diphosphate synthase-	3.78E-02	1.25E-02	10.53
68420_c0_seq4	calcium-activated potassium	4.83E-02	9.11E-03	10.39
62333_c0_seq3	histone h1	9.50E-03	1.96E-02	10.24
72914_c0_seq16	c-myc promoter-binding	4.70E-02	2.87E-02	10.05
67519_c1_seq13	---NA---	1.74E-02	2.19E-02	10.00
61850_c2_seq1	---NA---	2.41E-02	8.94E-03	9.18
68936_c1_seq18	hypothetical protein	1.98E-02	2.66E-02	8.00
62327_c1_seq6	aldo-keto reductase	1.10E-02	1.93E-02	7.36

68983_c0_seq1	actin related protein 2 3 complex	2.80E-02	7.53E-03	6.83
50811_c0_seq4	hypothetical protein KGM_08118	4.15E-02	5.64E-09	6.50
62773_c0_seq1	serine protease	8.52E-03	1.80E-02	6.32
71608_c0_seq6	PREDICTED: uncharacterized	3.11E-02	4.24E-02	5.97
64333_c0_seq2	copper-zinc superoxide dismutase	2.29E-02	4.24E-02	5.47
67881_c1_seq10	---NA---	3.01E-02	1.10E-02	4.57
73387_c0_seq1	muscle-specific protein 300	3.99E-02	2.43E-02	0.22
70667_c0_seq2	v-type proton atpase subunit b	2.46E-02	3.62E-02	0.21
66044_c1_seq7	---NA---	1.47E-02	9.81E-04	0.21
69445_c0_seq1	zinc transporter zip1-like	2.20E-02	1.22E-05	0.20
70707_c0_seq10	hypothetical protein KGM_19125	4.51E-02	9.93E-03	0.20
67590_c1_seq8	---NA---	4.70E-02	3.73E-04	0.19
54215_c0_seq5	---NA---	1.93E-02	3.11E-03	0.17
68838_c0_seq1	en protein binding engrailed	2.37E-02	2.66E-02	0.17
68060_c2_seq1	eukaryotic translation initiation	1.46E-02	5.12E-03	0.17
71213_c0_seq2	sodium-bile acid cotransporter	1.47E-02	2.11E-02	0.16
46506_c0_seq1	hypothetical protein KGM_05412	9.95E-03	2.51E-04	0.16
61767_c0_seq3	---NA---	1.25E-02	1.32E-02	0.15
71213_c0_seq5	sodium-bile acid cotransporter	1.16E-02	5.05E-04	0.15
72191_c0_seq7	maternal effect protein staufen-	5.05E-03	1.32E-05	0.14
66428_c0_seq1	PREDICTED: uncharacterized	1.13E-02	2.83E-05	0.14
67452_c0_seq4	PREDICTED: uncharacterized	1.48E-02	1.97E-02	0.14
70270_c0_seq18	sulfate transporter	6.74E-03	1.04E-04	0.14
63375_c1_seq4	juvenile hormone epoxide	9.41E-04	9.61E-05	0.14
72296_c0_seq7	hypothetical protein	1.33E-02	2.24E-03	0.14
67452_c0_seq5	PREDICTED: uncharacterized	6.88E-03	1.31E-03	0.13
62590_c0_seq3	cuticular protein hypothetical 4	1.52E-02	3.77E-02	0.13
68266_c0_seq21	hypothetical protein	1.04E-02	4.18E-02	0.12
62836_c0_seq17	probable very-long-chain enoyl-	9.73E-03	3.67E-02	0.12
67652_c0_seq4	hypothetical protein KGM_14251	4.76E-02	1.34E-02	0.12
65493_c0_seq3	PREDICTED: uncharacterized	2.94E-02	1.29E-09	0.12
68375_c0_seq18	hypothetical protein KGM_21879	2.64E-02	1.40E-02	0.11
71061_c0_seq1	PREDICTED: uncharacterized	7.54E-03	4.60E-02	0.11
62691_c1_seq5	---NA---	1.55E-02	4.83E-02	0.11
70679_c0_seq7	diphosphoinositol polyphosphate	3.78E-02	7.68E-04	0.11
72220_c0_seq26	hypothetical protein KGM_17358	6.87E-03	2.28E-02	0.11
69585_c2_seq41	PREDICTED: uncharacterized	3.26E-02	3.35E-02	0.11
44960_c0_seq1	hypothetical protein	1.18E-02	1.09E-03	0.11
64031_c0_seq1	PREDICTED: uncharacterized	3.65E-02	4.77E-07	0.11
66840_c0_seq3	cuticular protein rr-3 motif 148	9.48E-05	5.40E-05	0.11
54255_c1_seq1	---NA---	9.78E-03	1.87E-02	0.10
68206_c0_seq18	zinc finger protein xfin	1.57E-02	1.06E-02	0.10
50156_c1_seq3	orf2-encoded protein	4.89E-02	6.44E-03	0.10
67534_c0_seq2	rna-directed dna polymerase from	7.88E-03	1.06E-04	0.10
72456_c0_seq2	extracellular domains-containing	2.26E-02	1.33E-02	0.09
53481_c0_seq4	elongation factor 1 delta	2.07E-03	1.29E-02	0.09
62892_c0_seq2	unknown secreted protein	4.34E-03	2.54E-03	0.09
68180_c2_seq2	---NA---	3.05E-02	1.32E-02	0.09
66645_c0_seq5	endonuclease-reverse	2.60E-03	2.27E-03	0.09
71578_c0_seq15	cytochrome b5-like isoform x2	1.03E-03	4.20E-03	0.09
71850_c0_seq2	simila to cg6762	4.43E-05	1.70E-16	0.08
61890_c2_seq4	hypothetical protein M514_20469	1.64E-03	4.76E-03	0.08
62539_c2_seq4	---NA---	2.74E-06	7.78E-08	0.08
43832_c0_seq1	---NA---	5.73E-04	3.62E-03	0.07
70061_c0_seq3	hypothetical protein KGM_01470	4.95E-03	3.40E-04	0.07
63646_c1_seq4	---NA---	3.51E-02	8.08E-04	0.07
66151_c2_seq11	---NA---	6.10E-03	1.93E-03	0.07
69561_c0_seq6	PREDICTED: uncharacterized	3.35E-03	8.86E-05	0.07
71969_c1_seq9	heterotrimeric guanine	3.76E-02	2.43E-03	0.07
71750_c0_seq2	f-box wd repeat-containing	4.05E-02	2.21E-03	0.06
59236_c0_seq9	---NA---	3.48E-02	1.97E-03	0.06

67783_c2_seq2	s-adenosylmethionine synthetase	1.43E-13	2.63E-03	0.06
67613_c0_seq2	rna-directed dna polymerase from	8.41E-04	2.78E-04	0.06
71492_c0_seq7	PREDICTED: epsin-1-like	3.79E-02	1.37E-03	0.06
55380_c0_seq2	---NA---	3.53E-02	1.27E-03	0.06
67220_c0_seq29	GH10059	2.69E-02	1.30E-03	0.06
64100_c0_seq2	hypothetical protein	1.30E-03	5.69E-04	0.06
64002_c1_seq14	---NA---	3.86E-02	9.81E-04	0.06
69011_c0_seq2	cytochrome b-c1 complex subunit	3.44E-02	9.40E-04	0.06
70589_c2_seq5	---NA---	7.86E-03	2.77E-04	0.06
72155_c0_seq40	---NA---	8.15E-03	7.06E-06	0.06
67047_c0_seq2	tpa: cuticle protein	3.52E-02	7.68E-04	0.06
70283_c0_seq9	---NA---	2.53E-02	7.91E-04	0.06
63439_c0_seq10	---NA---	3.83E-02	6.90E-04	0.06
62027_c0_seq16	endonuclease-reverse	4.38E-03	8.43E-04	0.05
68281_c2_seq21	g protein-coupled receptor	2.41E-02	6.22E-04	0.05
71678_c0_seq4	low quality protein: protein	1.68E-06	7.39E-04	0.05
25655_c0_seq1	PREDICTED: uncharacterized	1.16E-04	1.48E-05	0.05
62906_c0_seq2	---NA---	2.48E-02	7.00E-05	0.05
67396_c0_seq6	PREDICTED: uncharacterized	1.47E-02	4.85E-04	0.05
69683_c2_seq2	---NA---	3.38E-02	3.58E-04	0.05
65233_c0_seq1	gram domain-containing protein	2.94E-02	3.58E-04	0.05
71086_c0_seq5	---NA---	2.39E-02	3.65E-04	0.05
58763_c0_seq2	hypothetical protein X777_02025	1.76E-02	3.59E-04	0.05
73022_c0_seq3	indole-3-acetaldehyde oxidase-	4.07E-02	1.84E-04	0.05
66419_c2_seq6	---NA---	3.05E-02	2.02E-04	0.05
71315_c0_seq14	---NA---	2.33E-02	2.28E-04	0.05
67725_c1_seq2	u11 u12 small nuclear	2.41E-02	2.52E-05	0.05
68128_c2_seq8	---NA---	1.70E-02	2.22E-04	0.05
65849_c0_seq4	methionine aminopeptidase	4.84E-02	1.18E-04	0.05
68125_c0_seq3	---NA---	3.10E-02	1.37E-04	0.05
72315_c0_seq4	coiled-coil domain-containing	2.16E-02	1.68E-04	0.05
71154_c0_seq38	---NA---	9.94E-03	5.87E-06	0.05
59845_c0_seq1	hypothetical protein KGM_18556	4.61E-02	9.85E-05	0.05
59365_c1_seq2	similar to CG11050	3.23E-02	1.22E-04	0.05
66626_c0_seq1	---NA---	1.99E-03	1.44E-10	0.05
65177_c0_seq7	exosc7 protein	1.63E-02	1.60E-04	0.05
69130_c0_seq3	adp ribosylation factor	2.64E-02	1.25E-04	0.05
68861_c0_seq3	zinc finger protein 28 homolog	3.99E-02	9.01E-05	0.05
70865_c0_seq1	cathepsin o2-like protease	1.16E-02	1.61E-04	0.05
67273_c0_seq3	---NA---	1.54E-02	1.42E-04	0.05
69197_c0_seq4	glycerol kinase 5- partial	1.69E-02	1.34E-04	0.05
70907_c0_seq10	-like protein 9	4.74E-02	6.52E-05	0.05
68625_c1_seq9	---NA---	2.46E-02	2.35E-05	0.05
65260_c2_seq4	cuticular protein hypothetical 5	2.20E-02	1.02E-04	0.04
60796_c1_seq3	---NA---	1.39E-02	9.45E-09	0.04
67660_c3_seq14	PREDICTED: uncharacterized	2.15E-04	1.07E-05	0.04
69165_c0_seq1	ubiquitin-associated domain-	6.06E-03	1.43E-04	0.04
69473_c0_seq1	---NA---	3.71E-02	6.14E-05	0.04
63022_c1_seq3	---NA---	1.30E-02	1.06E-04	0.04
72601_c0_seq23	synaptic vesicle glycoprotein 2c-	3.99E-02	5.02E-05	0.04
65319_c0_seq2	---NA---	2.40E-02	7.74E-05	0.04
67039_c1_seq6	---NA---	2.92E-02	5.99E-05	0.04
58821_c0_seq3	---NA---	3.63E-02	4.40E-05	0.04
69484_c1_seq10	hypothetical protein KGM_14717	2.66E-02	5.71E-05	0.04
62836_c0_seq5	probable very-long-chain enoyl-	2.30E-04	1.09E-05	0.04
69789_c0_seq15	---NA---	4.84E-02	2.56E-05	0.04
62984_c0_seq7	---NA---	4.96E-02	2.40E-05	0.04
67884_c0_seq1	carbonic anhydrase-related	2.36E-02	4.86E-05	0.04
69325_c0_seq5	---NA---	2.78E-04	8.11E-06	0.04
57398_c0_seq7	hypothetical protein	9.27E-03	6.46E-05	0.04
73202_c0_seq2	dna topoisomerase 2	1.82E-02	4.58E-05	0.04

70283_c0_seq12	---NA---	2.78E-03	8.33E-05	0.04
66136_c3_seq6	---NA---	2.07E-02	5.87E-06	0.04
61119_c0_seq4	---NA---	4.56E-02	1.58E-05	0.04
66951_c0_seq22	hypothetical protein YYC_03233	3.15E-03	6.53E-05	0.04
62522_c1_seq3	PREDICTED: uncharacterized	2.04E-02	2.82E-05	0.04
67554_c0_seq9	PREDICTED: uncharacterized	3.05E-02	1.02E-06	0.04
72012_c1_seq3	---NA---	6.81E-03	4.24E-05	0.04
63752_c0_seq5	rna-directed dna polymerase from	2.64E-02	1.62E-05	0.04
64405_c0_seq10	---NA---	4.82E-03	1.48E-05	0.04
62457_c0_seq2	gem-associated protein 5-like	7.21E-03	3.57E-05	0.04
61497_c1_seq1	---NA---	1.55E-02	2.33E-05	0.04
71662_c4_seq1	---NA---	1.62E-02	3.91E-07	0.04
66044_c1_seq12	thap domain-containing protein 9	4.34E-03	3.76E-05	0.04
68023_c2_seq18	---NA---	2.06E-02	1.64E-05	0.04
59224_c1_seq6	---NA---	2.33E-02	1.44E-05	0.04
71755_c0_seq3	regulator of g-protein signaling	2.33E-02	1.44E-05	0.04
62579_c0_seq1	centromere kinetochore protein	1.55E-02	1.91E-05	0.04
68632_c1_seq8	---NA---	2.95E-02	9.66E-06	0.04
69325_c0_seq8	---NA---	1.46E-05	1.87E-06	0.04
71059_c0_seq15	cytoplasmic dynein 1	2.24E-02	1.27E-05	0.04
72710_c0_seq17	---NA---	1.11E-04	5.47E-05	0.04
62555_c0_seq2	---NA---	2.98E-02	7.94E-06	0.04
68281_c2_seq20	g protein-coupled receptor	2.10E-02	1.15E-05	0.04
64343_c0_seq3	endonuclease-reverse	1.93E-02	1.22E-05	0.04
72046_c0_seq6	zinc finger c2hc domain-	2.46E-02	9.21E-06	0.04
73254_c0_seq10	low quality protein: dynein heavy	2.65E-02	8.23E-06	0.04
73194_c0_seq2	rho guanine nucleotide exchange	3.68E-05	3.37E-06	0.04
64578_c0_seq18	---NA---	1.62E-02	1.27E-05	0.04
60447_c0_seq4	---NA---	2.77E-02	6.58E-06	0.04
72601_c0_seq11	synaptic vesicle glycoprotein 2c-	1.43E-02	1.23E-05	0.04
67838_c0_seq2	hypothetical protein KGM_11873	2.49E-02	7.01E-06	0.04
66951_c0_seq11	---NA---	2.25E-02	7.90E-06	0.04
63814_c0_seq2	---NA---	1.82E-02	9.60E-06	0.04
67972_c0_seq7	unknown similar to MacoNPV-B	1.14E-03	2.39E-05	0.04
68322_c2_seq6	trna-specific adenosine	6.75E-03	1.22E-05	0.04
61839_c0_seq12	---NA---	1.47E-02	7.25E-06	0.04
67705_c0_seq43	calponin homology domain-	2.77E-02	3.11E-06	0.04
59343_c0_seq23	---NA---	8.39E-04	1.97E-05	0.04
66647_c0_seq5	leucine carboxyl	1.95E-02	2.28E-07	0.04
59211_c0_seq3	---NA---	2.07E-02	4.04E-06	0.03
72371_c0_seq3	---NA---	8.56E-03	8.47E-06	0.03
67330_c0_seq2	peroxisome assembly protein 12	7.88E-03	8.71E-06	0.03
67615_c0_seq4	inorganic phosphate	1.32E-03	1.60E-05	0.03
64509_c0_seq2	---NA---	2.14E-03	1.40E-05	0.03
71302_c0_seq6	pre-mrna 3 end processing	1.21E-02	3.89E-07	0.03
66304_c0_seq11	PREDICTED: uncharacterized	9.02E-04	1.45E-05	0.03
51027_c0_seq1	---NA---	2.07E-02	2.84E-06	0.03
63465_c0_seq5	glutathione s-transferase epsilon	3.87E-03	8.24E-06	0.03
61803_c0_seq4	---NA---	1.80E-02	2.58E-06	0.03
67354_c1_seq20	hypothetical protein	1.75E-02	2.55E-06	0.03
51706_c0_seq2	serf-like protein	3.60E-03	7.01E-06	0.03
68088_c0_seq2	hypothetical protein KGM_02762	3.59E-03	6.89E-06	0.03
63191_c1_seq4	---NA---	9.94E-03	3.42E-06	0.03
68124_c0_seq10	dna-binding protein ikaros-like	1.49E-03	8.00E-06	0.03
60185_c1_seq8	---NA---	9.94E-03	3.15E-06	0.03
71042_c0_seq2	---NA---	2.08E-02	1.36E-06	0.03
62543_c0_seq2	single-stranded dna-binding	1.01E-02	6.02E-09	0.03
68310_c2_seq8	vacuolar atpase subunit a	2.84E-03	5.51E-06	0.03
68731_c0_seq2	testis-specific serine threonine-	9.23E-03	2.53E-06	0.03
63096_c0_seq6	hypothetical protein	3.87E-03	3.66E-06	0.03
67606_c0_seq10	hypothetical protein KGM_11742	1.57E-02	1.05E-06	0.03

69327_c0_seq9	---NA---	5.64E-03	2.69E-06	0.03
70151_c0_seq2	---NA---	9.21E-04	5.51E-06	0.03
64181_c0_seq2	e3 ubiquitin-protein ligase amfr-	4.34E-03	2.86E-06	0.03
71591_c0_seq11	---NA---	1.82E-02	6.83E-07	0.03
65924_c0_seq1	---NA---	1.11E-02	1.08E-06	0.03
70915_c0_seq2	hypothetical protein KGM_04852	1.16E-02	9.69E-07	0.03
73024_c0_seq4	hypothetical protein KGM_14284	8.83E-03	1.20E-06	0.03
71307_c0_seq11	organic cation transporter	3.15E-05	2.68E-07	0.03
68731_c0_seq6	testis-specific serine threonine-	1.21E-02	4.21E-07	0.03
68960_c0_seq4	---NA---	1.17E-02	3.84E-07	0.03
65319_c0_seq6	---NA---	2.60E-03	1.31E-06	0.03
63865_c0_seq2	hemolin-interacting protein	5.11E-04	2.68E-06	0.03
65319_c0_seq4	---NA---	4.62E-03	8.09E-07	0.03
64688_c1_seq11	---NA---	6.22E-05	3.56E-06	0.03
64470_c0_seq22	---NA---	7.10E-03	5.19E-07	0.03
62095_c0_seq12	hypothetical protein	2.37E-04	7.58E-11	0.03
71059_c0_seq4	cytoplasmic dynein 1	6.75E-03	3.58E-07	0.03
63095_c1_seq2	limb and neural patterns protein	1.10E-02	1.92E-07	0.03
73206_c1_seq1	hypothetical protein KGM_11072	1.14E-03	1.08E-08	0.03
72461_c0_seq1	hypothetical protein KGM_13383	1.63E-03	5.19E-07	0.03
63165_c0_seq8	PREDICTED: uncharacterized	2.70E-05	1.83E-06	0.03
65405_c0_seq1	PREDICTED: uncharacterized	3.59E-03	2.68E-07	0.03
72253_c0_seq14	myelin expression factor 2	4.05E-03	1.88E-07	0.03
67273_c0_seq1	---NA---	2.07E-03	2.83E-07	0.03
67838_c0_seq15	hypothetical protein KGM_11873	1.72E-03	1.24E-08	0.03
72343_c0_seq10	---NA---	1.48E-03	2.83E-07	0.03
72255_c0_seq6	---NA---	7.83E-05	8.09E-07	0.03
72464_c2_seq3	juvenile hormone epoxide	1.25E-04	6.83E-07	0.03
46708_c0_seq1	rna-directed dna polymerase from	3.97E-03	1.03E-07	0.03
50693_c0_seq1	prolyl 4-hydroxylase subunit	2.17E-03	1.46E-07	0.03
64181_c0_seq1	e3 ubiquitin-protein ligase amfr	2.33E-04	3.93E-07	0.02
60796_c1_seq21	---NA---	2.86E-03	7.89E-08	0.02
67705_c0_seq13	calponin homology domain-	1.68E-04	3.64E-07	0.02
67519_c1_seq30	---NA---	3.40E-03	5.72E-08	0.02
70681_c0_seq14	williams-beuren syndrome	5.11E-06	7.30E-07	0.02
72507_c0_seq5	rna-directed dna polymerase from	4.95E-03	3.74E-08	0.02
65177_c0_seq6	exosc7 protein	4.45E-05	1.17E-07	0.02
67838_c0_seq6	hypothetical protein KGM_11873	2.44E-03	9.59E-10	0.02
71250_c1_seq3	phospholipid-transporting atpase	3.80E-04	1.93E-07	0.02
66298_c2_seq1	---NA---	2.60E-03	4.02E-10	0.02
61362_c3_seq1	---NA---	2.70E-05	9.72E-08	0.02
67838_c0_seq11	hypothetical protein KGM_11873	1.49E-03	5.22E-08	0.02
63950_c0_seq2	---NA---	2.44E-03	2.94E-08	0.02
70078_c0_seq5	hypothetical protein KGM_04783	3.42E-03	1.10E-08	0.02
68088_c0_seq7	hypothetical protein KGM_02762	3.06E-03	1.17E-08	0.02
55438_c0_seq3	---NA---	1.15E-03	2.32E-08	0.02
68889_c2_seq5	---NA---	3.61E-04	5.20E-08	0.02
70681_c0_seq6	williams-beuren syndrome	3.03E-04	1.37E-11	0.02
59343_c0_seq18	---NA---	8.41E-04	1.04E-08	0.02
72345_c2_seq3	---NA---	5.36E-04	1.40E-08	0.02
72337_c0_seq7	protein kinase c and casein kinase	9.02E-04	7.01E-09	0.02
66285_c0_seq1	alpha-tocopherol transfer	7.70E-05	3.16E-08	0.02
66951_c0_seq26	hypothetical protein YYC_03233	8.41E-04	3.84E-09	0.02
68371_c0_seq2	hypothetical protein KGM_02090	1.59E-03	1.59E-09	0.02
54716_c0_seq3	reverse transcriptase	4.13E-04	4.18E-09	0.02
71578_c0_seq7	cytochrome b5-like isoform x2	3.72E-05	1.40E-08	0.02
66806_c2_seq29	multidrug resistance protein	1.10E-05	2.12E-08	0.02
66948_c0_seq1	protein shq1-like protein	5.07E-06	2.60E-08	0.02
62589_c1_seq5	---NA---	6.81E-04	1.76E-09	0.02
67838_c0_seq13	hypothetical protein KGM_11873	9.41E-04	9.78E-10	0.02
68437_c0_seq1	---NA---	5.37E-04	1.81E-09	0.02

68437_c0_seq8	---NA---	1.14E-03	8.36E-10	0.02
70470_c0_seq3	---NA---	5.64E-04	1.50E-09	0.02
69745_c3_seq6	PREDICTED: uncharacterized	2.60E-06	2.18E-08	0.02
66073_c0_seq2	wd repeat domain	9.99E-09	5.44E-10	0.02
72316_c0_seq4	protein suppressor of white	1.72E-04	3.32E-09	0.02
68349_c1_seq3	---NA---	3.69E-08	4.21E-08	0.02
64416_c0_seq7	glutathione s transferase s1	2.00E-05	9.45E-09	0.02
51706_c0_seq6	serf-like protein	3.22E-08	3.94E-08	0.02
65177_c0_seq2	exosc7 protein	4.10E-08	3.66E-08	0.02
72847_c0_seq8	e3 ubiquitin-protein ligase	5.57E-04	9.59E-10	0.02
55342_c0_seq2	cdc2-related-kinase	3.61E-04	1.22E-09	0.02
72337_c0_seq1	protein kinase c and casein kinase	7.69E-05	3.00E-09	0.02
70325_c0_seq2	---NA---	1.27E-12	7.55E-23	0.02
66502_c0_seq1	hypothetical protein	2.01E-05	4.15E-12	0.02
63705_c1_seq8	#NAME?	3.72E-05	3.68E-09	0.02
56442_c0_seq7	hypothetical protein KGM_19177	6.22E-05	2.34E-09	0.02
63705_c1_seq2	#NAME?	1.04E-05	4.81E-09	0.02
69965_c0_seq2	bromodomain containing 3	1.22E-06	9.43E-09	0.02
73215_c0_seq1	protein lin-10	5.09E-06	4.73E-09	0.02
67887_c0_seq3	PREDICTED: uncharacterized	4.82E-05	1.59E-09	0.02
64988_c0_seq2	agap000179-pa-like protein	6.76E-09	1.50E-09	0.02
67030_c0_seq17	vacuolar atp synthase subunit d	1.67E-06	4.13E-09	0.02
66021_c0_seq3	---NA---	1.42E-06	4.13E-09	0.02
72914_c0_seq4	c-myc promoter-binding	2.44E-04	1.61E-10	0.02
72390_c0_seq2	hypothetical protein KGM_02108	4.62E-06	2.00E-09	0.02
63292_c2_seq4	---NA---	8.72E-06	1.66E-13	0.02
70480_c0_seq4	---NA---	5.89E-05	4.41E-10	0.02
64416_c0_seq5	glutathione s transferase s1	2.61E-07	6.00E-10	0.02
71687_c0_seq18	zinc transporter 2-like isoform x2	1.68E-05	5.12E-10	0.02
72773_c0_seq13	bm8 interacting protein	1.40E-04	6.47E-11	0.02
64969_c2_seq1	hypothetical protein KGM_00604	3.72E-05	1.77E-10	0.02
73261_c0_seq3	---NA---	2.54E-07	8.38E-10	0.01
66671_c0_seq2	cysteine-rich with egf-like	8.25E-05	2.38E-11	0.01
73357_c0_seq2	vacuolar protein sorting-	3.44E-07	4.79E-10	0.01
73048_c0_seq3	PREDICTED: uncharacterized	2.11E-06	5.07E-11	0.01
72847_c0_seq5	e3 ubiquitin-protein ligase	9.99E-08	1.61E-10	0.01
36924_c0_seq1	---NA---	8.79E-08	2.25E-12	0.01
70156_c0_seq19	---NA---	7.60E-06	3.49E-12	0.01
68231_c0_seq5	hypothetical protein KGM_17477	3.81E-06	2.97E-12	0.01
62749_c0_seq3	---NA---	1.98E-09	9.70E-15	0.01
66260_c0_seq6	PREDICTED: twinfilin-like	1.05E-05	8.65E-13	0.01
55740_c0_seq2	hypothetical protein KGM_19088	1.35E-07	9.30E-12	0.01
63664_c0_seq1	estradiol 17-beta-dehydrogenase	1.41E-07	2.00E-14	0.01
69623_c1_seq7	hypothetical protein KGM_01730	4.03E-10	5.29E-16	0.01
68088_c0_seq5	hypothetical protein KGM_02762	1.89E-10	3.80E-11	0.01
70856_c0_seq2	hypothetical protein KGM_19783	3.29E-06	9.56E-14	0.01
67169_c0_seq29	hypothetical protein KGM_07275	2.06E-08	1.26E-14	0.01
64416_c0_seq11	glutathione s transferase s1	1.86E-09	1.39E-13	0.01
71653_c0_seq2	hypothetical protein KGM_12768	7.14E-07	1.26E-13	0.01
73194_c0_seq1	rho guanine nucleotide exchange	9.99E-09	2.41E-14	0.01
72272_c0_seq3	similar to CG2519	3.29E-06	1.41E-14	0.01
69325_c0_seq1	---NA---	1.62E-07	1.39E-13	0.01
66671_c0_seq5	cysteine-rich with egf-like	1.30E-06	9.70E-15	0.01
72380_c1_seq15	branched-chain-amino-acid	1.02E-06	9.70E-15	0.01
67353_c0_seq4	hypothetical protein KGM_20861	3.61E-11	2.25E-16	0.01
71551_c0_seq3	---NA---	1.41E-06	4.48E-15	0.01
67329_c0_seq9	---NA---	1.61E-09	1.57E-13	0.01
67466_c0_seq16	hypothetical protein KGM_06296	5.00E-14	1.39E-13	0.01
69623_c1_seq6	hypothetical protein KGM_01730	7.59E-13	2.06E-21	0.01
73357_c0_seq1	vacuolar protein sorting-	1.62E-07	2.36E-16	0.01
68420_c0_seq14	calcium-activated potassium	2.87E-10	1.69E-14	0.01

62027_c0_seq19	endonuclease-reverse	1.46E-08	1.11E-15	0.01
67401_c0_seq11	---NA---	1.45E-11	8.92E-14	0.01
62486_c0_seq5	recombination repair protein 1-	2.65E-09	7.10E-16	0.01
68137_c0_seq2	transcription factor ets	1.45E-11	4.48E-15	0.01
66370_c0_seq19	ornithine decarboxylase antizyme	4.01E-11	7.10E-16	0.01
69913_c0_seq3	c1a cysteine protease precursor	1.54E-15	1.55E-13	0.01
70929_c1_seq3	PREDICTED: mitoferrin-like	1.01E-09	2.21E-17	0.01
51706_c0_seq5	serf-like protein	1.01E-09	1.83E-17	0.01
64849_c1_seq41	mitochondrial intermembrane	3.90E-11	2.21E-17	0.01
62486_c0_seq3	---NA---	1.66E-09	6.14E-19	0.01
72050_c0_seq7	hypothetical protein KGM_16968	2.25E-10	6.14E-19	0.01
68385_c1_seq17	---NA---	1.45E-11	6.14E-21	0.01
68420_c0_seq17	calcium-activated potassium	4.05E-13	2.80E-20	0.01
51706_c0_seq1	serf-like protein	3.31E-17	6.07E-21	0.004
66528_c0_seq6	SWP26	4.18E-15	6.87E-26	0.004
72380_c1_seq16	branched-chain-amino-acid	7.64E-17	1.87E-23	0.003
67030_c0_seq11	vatd_manse ame: full=v-type	3.42E-20	2.31E-29	0.002
64988_c0_seq3	agap000179-pa-like protein	1.61E-23	2.20E-28	0.002
69235_c0_seq3	hypothetical protein KGM_04418	1.98E-22	2.05E-35	0.002
68495_c0_seq7	hypothetical protein KGM_17464	1.90E-24	4.11E-31	0.001
72380_c1_seq11	branched-chain-amino-acid	2.28E-24	1.12E-34	0.001

Appendix 6B. Differentially expressed transcripts in Assembly 6. FDR = False discovery rate; FC = Fold change estimated by DEseq2. List filtered by FDR <0.05 by both EdgeR and DEseq2 analyses.

Contig ID	NCBI top hit	EdgeR FDR	DEseq2 FDR	FC
157678_c1_seq14	---NA---	1.39E-11	7.16E-22	200.87
150161_c0_seq9	activating signal cointegrator 1	4.02E-13	9.30E-19	182.60
155006_c0_seq13	28 kda heat- and acid-stable	1.96E-10	2.10E-18	158.78
156118_c1_seq4	---NA---	4.48E-10	4.40E-14	116.39
158509_c0_seq1	hypothetical protein KGM_03812	6.74E-08	1.79E-14	108.49
147582_c0_seq3	---NA---	6.73E-07	8.04E-16	80.71
152325_c0_seq8	nuclear pore complex protein	1.48E-06	8.34E-12	80.19
152051_c0_seq1	hypothetical protein KGM_04798	2.10E-06	1.03E-11	78.80
146107_c2_seq3	---NA---	5.31E-05	2.71E-12	75.69
148856_c2_seq3	hypothetical protein SINV_07136	6.07E-06	2.35E-11	73.99
158855_c0_seq3	PREDICTED: uncharacterized	1.11E-06	2.68E-10	69.01
150959_c0_seq1	signal peptide peptidase-like 3-	5.17E-05	4.83E-11	67.34
144096_c0_seq18	---NA---	2.76E-04	1.30E-11	66.98
158316_c1_seq3	maguk p55 subfamily member 5-	2.32E-04	2.37E-11	65.85
158370_c0_seq2	---NA---	1.89E-04	3.14E-11	65.70
151914_c0_seq1	neuronal membrane glycoprotein	1.29E-06	1.03E-09	64.18
153483_c0_seq6	hermansky-pudlak syndrome 5	5.06E-05	3.35E-10	62.06
157577_c0_seq6	---NA---	1.60E-04	2.58E-10	60.68
152063_c1_seq40	trna pseudouridine synthase	1.25E-05	1.82E-09	59.20
158448_c0_seq3	---NA---	3.41E-04	3.94E-10	57.66
146086_c1_seq11	protein canopy homolog 1-like	4.58E-07	1.01E-08	57.03
151985_c0_seq2	PREDICTED: uncharacterized	4.37E-04	6.98E-10	55.76
157100_c0_seq23	unknown	1.14E-03	3.15E-10	55.14
151761_c0_seq10	---NA---	3.35E-04	1.57E-09	54.37
153102_c1_seq1	chaperonin containing t-complex	5.03E-04	1.81E-09	53.00
148856_c2_seq30	hypothetical protein SINV_07136	1.40E-03	2.87E-09	49.62
158653_c0_seq26	hypothetical protein KGM_09317	1.67E-03	4.86E-09	48.00
149704_c1_seq4	---NA---	7.48E-06	3.16E-08	46.29
147196_c1_seq1	---NA---	1.35E-03	1.44E-08	45.98

158290_c0_seq95	dna mismatch repair protein	3.68E-04	4.35E-08	45.48
155984_c1_seq13	---NA---	2.19E-03	1.44E-10	45.07
157892_c0_seq2	polycomb protein l g0020-like	1.94E-03	4.10E-08	42.82
154821_c0_seq2	hypothetical protein KGM_02279	2.24E-03	4.10E-08	42.53
152114_c2_seq4	rna-directed dna polymerase from	1.20E-03	7.34E-08	42.24
146086_c1_seq10	protein canopy homolog 1-like	1.02E-03	2.82E-07	39.23
145601_c1_seq15	transcription-associated zinc	1.52E-03	2.05E-08	38.70
158636_c0_seq5	c-myc promoter-binding	2.96E-03	2.26E-07	38.14
154355_c0_seq10	hypothetical protein KGM_11873	3.82E-04	7.31E-07	37.79
156554_c1_seq5	---NA---	1.20E-02	1.68E-07	36.22
156673_c1_seq3	hormone-sensitive lipase	1.43E-02	3.07E-07	34.73
147141_c1_seq1	PREDICTED: uncharacterized	1.51E-02	3.05E-07	34.59
145454_c0_seq16	putative T21D12.3	1.89E-03	4.50E-07	33.39
158379_c0_seq8	---NA---	2.45E-03	2.88E-06	32.47
145245_c0_seq12	udp-n-acetylhexosamine	1.19E-02	1.36E-06	32.12
141797_c0_seq4	DnaJ	8.30E-04	4.82E-07	32.01
154629_c0_seq3	itg-containing peptide	6.97E-03	4.06E-06	30.51
157427_c0_seq26	Cullin-2	7.25E-03	5.34E-06	29.88
157100_c0_seq8	unknown	7.61E-03	5.77E-06	29.63
156155_c1_seq7	kv channel-interacting protein 1-	2.62E-02	2.65E-06	29.21
154658_c0_seq2	probable 3 -cyclic	1.33E-02	5.67E-06	29.06
157002_c0_seq1	zinc transporter 2-like isoform x1	6.72E-03	1.06E-05	28.40
145895_c0_seq4	transmembrane protein 93	3.24E-02	3.43E-06	28.34
154748_c0_seq3	---NA---	2.14E-02	5.72E-06	28.23
150461_c0_seq7	---NA---	3.58E-02	4.17E-06	27.78
149366_c0_seq7	low quality protein: transcription	1.09E-02	1.33E-05	27.46
152998_c0_seq8	---NA---	1.53E-02	1.12E-05	27.43
153847_c0_seq3	stromal interaction molecule 1	6.09E-03	1.76E-05	27.34
157210_c0_seq6	hypothetical protein KGM_13115	3.94E-02	7.23E-06	26.59
159378_c0_seq1	---NA---	3.80E-02	5.16E-07	26.41
143751_c2_seq19	---NA---	4.62E-02	7.71E-06	26.11
142873_c1_seq3	---NA---	3.94E-02	1.01E-05	26.02
156500_c1_seq4	---NA---	3.80E-02	1.06E-05	25.95
145343_c0_seq3	hypothetical protein KGM_22419	4.56E-02	9.41E-06	25.82
144486_c2_seq3	PREDICTED: uncharacterized	3.56E-03	3.94E-05	25.81
150567_c3_seq9	---NA---	4.48E-02	1.02E-05	25.71
147141_c1_seq6	PREDICTED: uncharacterized	4.88E-02	1.29E-05	25.09
139526_c0_seq5	protein fam32a-like	1.19E-02	3.75E-05	25.06
158718_c2_seq3	aldehyde oxidase 1	4.19E-02	1.63E-05	24.99
153484_c1_seq7	S08405 hypothetical protein 2 -	2.26E-03	5.41E-06	24.71
156389_c2_seq31	GK15001	2.08E-02	1.29E-05	23.95
153603_c0_seq2	hypothetical protein	3.03E-02	5.00E-05	23.47
158548_c0_seq3	dual oxidase-like precursor	1.77E-02	7.17E-05	23.39
149311_c0_seq5	---NA---	3.40E-02	4.80E-05	23.37
147419_c0_seq4	---NA---	4.00E-02	4.48E-05	23.26
151992_c0_seq7	unknown	3.20E-02	5.58E-05	23.15
158191_c0_seq7	6-phosphofructokinase	1.38E-04	2.82E-07	22.96
152570_c3_seq7	---NA---	3.76E-02	6.23E-05	22.71
158969_c2_seq2	PREDICTED: uncharacterized	4.52E-02	5.59E-05	22.63
153311_c0_seq8	Sequestosome-1	2.22E-02	9.72E-05	22.51
155222_c1_seq9	---NA---	4.91E-02	5.53E-05	22.47
152426_c1_seq10	---NA---	4.52E-02	1.43E-05	22.15
157781_c0_seq2	---NA---	1.37E-02	1.55E-04	21.85
154841_c0_seq2	hypothetical protein	4.95E-02	1.37E-04	20.90
146828_c0_seq8	---NA---	4.61E-02	1.71E-04	20.60
149720_c2_seq1	PREDICTED: uncharacterized	3.24E-02	2.28E-04	20.41
143031_c0_seq7	---NA---	1.94E-02	3.92E-04	19.64
148790_c0_seq3	---NA---	2.31E-02	2.20E-05	19.38
157286_c0_seq20	lim and sh3 domain protein lasp	4.13E-02	7.85E-04	17.77
155108_c1_seq10	glutathione s-transferase zeta 1	4.60E-02	9.27E-04	17.38
156714_c0_seq24	---NA---	2.68E-02	7.64E-06	16.92

143503_c0_seq1	PREDICTED: uncharacterized	4.60E-02	1.43E-03	16.51
149889_c0_seq7	---NA---	2.30E-04	6.04E-03	12.90
155984_c1_seq24	---NA---	4.27E-02	3.94E-03	12.30
154756_c2_seq4	hypothetical protein KGM_03768	4.22E-02	3.89E-04	12.13
149386_c0_seq1	hypothetical protein KGM_10094	2.46E-02	3.84E-04	11.77
156078_c0_seq1	nad-dependent	2.40E-04	1.96E-05	11.25
151587_c0_seq8	---NA---	2.66E-02	8.72E-05	9.72
147243_c1_seq1	---NA---	2.80E-02	8.65E-03	9.30
156714_c0_seq36	---NA---	4.95E-02	3.38E-03	9.01
157712_c0_seq9	PREDICTED: uncharacterized	2.93E-02	3.90E-02	8.60
157058_c1_seq11	s-adenosylmethionine synthetase	5.01E-04	1.71E-05	8.30
150743_c0_seq2	all2_thapi ame: full=allergen tha	4.90E-04	4.90E-05	7.69
152307_c1_seq11	copper-zinc superoxide dismutase	2.41E-03	4.60E-03	7.66
153069_c0_seq6	serine protease	5.14E-03	1.20E-02	6.38
158112_c0_seq3	---NA---	4.98E-02	2.76E-02	4.93
158819_c0_seq1	PREDICTED: gelsolin,	3.76E-02	4.64E-02	4.36
142494_c0_seq1	zinc transporter zip1-like	2.44E-02	2.61E-05	0.21
153527_c0_seq1	PREDICTED: uncharacterized	4.11E-02	4.22E-04	0.19
138445_c0_seq1	---NA---	2.38E-02	4.82E-03	0.17
153146_c0_seq3	MG7	2.58E-03	3.20E-05	0.16
157921_c0_seq2	PREDICTED: uncharacterized	5.06E-03	5.19E-14	0.16
150151_c0_seq2	retrovirus-related pol polyprotein	1.69E-02	1.61E-03	0.15
158867_c0_seq1	hypothetical protein KGM_01703	4.36E-03	5.78E-05	0.14
153267_c0_seq2	c-type lectin 17	4.11E-02	8.21E-05	0.14
139867_c1_seq1	---NA---	3.74E-02	4.93E-02	0.12
146065_c0_seq3	probable leucine--trna	3.51E-03	3.36E-02	0.12
154097_c0_seq27	perilipin-4-like isoform x1	9.08E-03	1.54E-02	0.12
147641_c1_seq9	hypothetical protein	1.55E-02	1.53E-03	0.11
149484_c0_seq3	endonuclease-reverse	8.32E-03	7.00E-03	0.11
150866_c1_seq2	---NA---	3.54E-02	1.41E-03	0.10
143060_c2_seq2	---NA---	1.09E-02	2.48E-02	0.10
152785_c0_seq1	hypothetical protein	3.24E-02	1.99E-02	0.10
155133_c0_seq4	---NA---	2.94E-02	2.25E-02	0.10
151621_c1_seq1	rna-directed dna polymerase from	9.52E-03	2.21E-04	0.10
147143_c0_seq2	cuticular protein hypothetical 4	4.10E-03	3.00E-03	0.10
156521_c0_seq6	sulfate transporter	5.17E-04	7.97E-07	0.09
145252_c0_seq7	hypothetical protein	4.66E-02	9.31E-03	0.09
157678_c1_seq16	sodium-bile acid cotransporter	1.18E-03	6.67E-03	0.09
157678_c1_seq23	sodium-bile acid cotransporter	1.34E-04	1.22E-02	0.09
154028_c0_seq8	PREDICTED: uncharacterized	8.61E-06	1.33E-02	0.09
145252_c0_seq6	hypothetical protein	2.78E-03	8.85E-03	0.08
75815_c0_seq1	---NA---	2.08E-02	1.23E-02	0.08
157261_c0_seq2	low quality protein: protein	3.18E-06	8.95E-03	0.08
143227_c1_seq1	---NA---	6.31E-03	3.62E-03	0.08
156124_c0_seq1	n-acetyllactosaminide beta- -n-	1.10E-02	2.60E-03	0.08
154696_c3_seq2	---NA---	2.80E-02	8.08E-04	0.07
140668_c0_seq2	interferon-induced very large	2.08E-02	3.13E-03	0.07
153808_c0_seq15	transmembrane protein 184b-like	4.68E-02	4.49E-03	0.07
159029_c0_seq3	vacuolar protein sorting-	1.59E-06	3.39E-03	0.07
150365_c0_seq2	hypothetical protein KGM_03594	4.82E-02	2.42E-03	0.07
153931_c0_seq1	---NA---	8.83E-03	3.60E-07	0.06
152996_c2_seq3	cytochrome c-like isoform x1	3.72E-02	1.77E-03	0.06
145080_c0_seq4	aldose reductase-like isoform x1	1.36E-12	2.23E-03	0.06
147143_c0_seq5	cuticular protein hypothetical 4	1.38E-04	1.64E-05	0.06
155571_c0_seq12	cathepsin d	1.51E-04	1.98E-04	0.06
157763_c0_seq2	hypothetical protein KGM_13955	2.61E-02	7.53E-04	0.06
158707_c1_seq2	hypothetical protein KGM_06367	2.25E-03	1.97E-04	0.06
150431_c3_seq1	PREDICTED: uncharacterized	1.62E-04	2.02E-05	0.06
152909_c1_seq11	---NA---	1.53E-02	7.85E-04	0.06
143031_c0_seq4	---NA---	2.23E-02	6.43E-04	0.05
156753_c0_seq6	hypothetical protein KGM_18620	2.84E-02	5.93E-04	0.05

158977_c0_seq2	PREDICTED: cadherin-23-like	4.30E-02	4.96E-04	0.05
154870_c0_seq12	dnaj homolog subfamily c	4.65E-02	3.75E-04	0.05
153103_c3_seq10	---NA---	3.24E-02	5.43E-05	0.05
143946_c0_seq3	hypothetical protein X777_02025	2.24E-02	4.65E-04	0.05
152771_c1_seq3	uridine 5 -monophosphate	2.61E-02	4.16E-04	0.05
150865_c0_seq7	---NA---	3.50E-02	3.57E-04	0.05
145245_c0_seq6	udp-n-acetylhexosamine	4.00E-02	2.65E-04	0.05
146925_c1_seq1	PREDICTED: uncharacterized	3.54E-02	2.65E-04	0.05
153960_c0_seq13	---NA---	3.61E-02	2.46E-04	0.05
153051_c3_seq20	---NA---	1.49E-02	3.22E-04	0.05
146019_c0_seq2	hypothetical protein	1.77E-03	3.20E-12	0.05
151259_c0_seq1	ubiquitin-associated and sh3	2.15E-02	2.42E-04	0.05
152542_c0_seq2	limb and neural patterns protein	4.34E-02	1.52E-04	0.05
142486_c0_seq8	rna-binding protein 1	4.56E-02	1.44E-04	0.05
150190_c0_seq2	protein suppressor of white	4.95E-02	1.17E-04	0.05
145245_c0_seq17	udp-n-acetylhexosamine	2.55E-02	1.78E-04	0.05
158823_c0_seq2	hypothetical protein KGM_21981	4.68E-02	1.22E-04	0.05
149983_c0_seq13	ras-related protein ral-a-like	4.51E-02	1.27E-04	0.05
157237_c0_seq7	---NA---	2.14E-02	1.79E-04	0.05
150657_c0_seq2	cell growth-regulating nucleolar	4.66E-02	1.12E-04	0.05
158381_c0_seq15	orthopedia	4.14E-02	1.22E-04	0.05
149412_c0_seq5	methionine aminopeptidase	3.61E-02	1.24E-04	0.05
147101_c0_seq2	phd finger protein 22	3.80E-02	2.38E-05	0.05
157967_c0_seq16	odorant binding protein 11	4.68E-02	8.30E-05	0.05
146058_c1_seq2	PREDICTED: uncharacterized	3.93E-02	7.72E-05	0.05
156800_c0_seq8	PREDICTED: uncharacterized	4.74E-02	5.53E-05	0.04
152610_c2_seq1	protein peanut-like	4.42E-02	5.52E-05	0.04
158598_c0_seq1	hypothetical protein KGM_13459	3.03E-02	6.55E-05	0.04
158633_c0_seq30	atp-binding cassette sub-family b	3.34E-02	5.53E-05	0.04
158615_c0_seq2	acetylcholinesterase	4.13E-02	3.94E-05	0.04
151625_c0_seq1	PREDICTED: uncharacterized	4.71E-02	2.40E-05	0.04
137409_c0_seq6	hypothetical protein	1.13E-02	5.74E-05	0.04
158627_c0_seq4	hypothetical protein KGM_00262	4.13E-02	2.36E-05	0.04
154466_c0_seq19	hypothetical protein	2.61E-02	3.38E-05	0.04
143344_c0_seq4	---NA---	1.81E-02	4.34E-05	0.04
155363_c1_seq7	PREDICTED: uncharacterized	4.98E-02	1.58E-05	0.04
145817_c2_seq2	---NA---	3.07E-02	2.52E-05	0.04
144230_c0_seq2	ca2+-channel-protein-beta-	2.34E-02	3.21E-05	0.04
157678_c1_seq25	sodium-bile acid cotransporter	5.43E-06	2.47E-05	0.04
144230_c0_seq8	voltage-dependent l-type calcium	4.34E-02	1.64E-05	0.04
157451_c1_seq7	AGAP010235-PA	1.14E-02	4.00E-05	0.04
155418_c0_seq5	bromodomain containing 3	9.07E-03	4.22E-05	0.04
158149_c0_seq14	calcium-activated potassium	2.29E-03	5.53E-05	0.04
150572_c2_seq2	acidic repeat-containing	2.84E-02	2.09E-05	0.04
138969_c1_seq1	hypothetical protein	3.35E-04	7.31E-06	0.04
147474_c3_seq7	---NA---	4.70E-02	1.02E-05	0.04
153072_c3_seq2	similar to CG17680	2.68E-02	1.80E-05	0.04
157349_c0_seq11	PREDICTED: uncharacterized	2.90E-02	1.64E-05	0.04
156074_c0_seq5	PREDICTED: uncharacterized	2.08E-02	2.09E-05	0.04
158777_c0_seq15	---NA---	4.34E-02	9.68E-06	0.04
154618_c0_seq3	ubiquitin-associated domain-	6.60E-03	3.17E-05	0.04
151618_c0_seq6	---NA---	1.58E-02	2.16E-05	0.04
147570_c0_seq7	---NA---	1.39E-02	2.12E-05	0.04
154961_c0_seq1	carbonic anhydrase-related	2.20E-02	1.60E-05	0.04
157794_c0_seq10	---NA---	3.50E-02	1.02E-05	0.04
145746_c0_seq1	histone deacetylase 3	2.44E-02	1.26E-05	0.04
149718_c3_seq2	---NA---	2.31E-02	1.29E-05	0.04
159125_c0_seq1	---NA---	1.33E-02	1.78E-05	0.04
154234_c2_seq7	---NA---	4.05E-02	6.26E-06	0.04
157376_c1_seq11	PREDICTED: uncharacterized	2.84E-02	7.53E-06	0.04
146110_c4_seq4	---NA---	1.08E-02	1.52E-05	0.04

144445_c3_seq6	---NA---	8.19E-03	3.60E-07	0.04
146311_c0_seq5	e3 ubiquitin-protein ligase hakai-	1.91E-02	6.68E-06	0.04
156529_c0_seq4	protein isoform a-like	3.03E-02	3.79E-06	0.04
157947_c1_seq8	PREDICTED: uncharacterized	1.53E-02	7.63E-06	0.04
156003_c1_seq2	PREDICTED: uncharacterized	2.61E-02	3.95E-06	0.04
156701_c0_seq42	---NA---	9.03E-03	8.89E-06	0.04
153901_c0_seq4	---NA---	2.25E-02	4.06E-06	0.04
157733_c1_seq7	integrator complex subunit 4-like	9.08E-03	6.55E-06	0.03
159023_c0_seq12	vacuolar protein sorting-	2.93E-02	2.14E-06	0.03
155836_c0_seq4	unnamed protein product	2.93E-02	1.93E-06	0.03
152305_c0_seq3	---NA---	2.46E-02	2.30E-06	0.03
157292_c0_seq62	hypothetical protein	1.60E-04	1.63E-05	0.03
149015_c2_seq4	---NA---	2.72E-02	1.72E-06	0.03
150909_c1_seq1	---NA---	4.27E-04	1.33E-05	0.03
156815_c0_seq4	---NA---	2.90E-03	7.64E-06	0.03
156939_c0_seq3	protein cornichon homolog 4-like	2.23E-02	1.84E-06	0.03
146483_c1_seq12	---NA---	1.86E-02	2.24E-06	0.03
147634_c4_seq14	---NA---	6.41E-04	1.04E-05	0.03
155165_c0_seq6	mothers against decapentaplegic	9.08E-03	3.80E-06	0.03
155977_c0_seq10	PREDICTED: clavesin-1-like	1.67E-02	1.16E-07	0.03
147119_c0_seq1	zinc finger protein 836	1.17E-02	2.96E-06	0.03
155592_c3_seq4	transport and golgi organization	2.24E-02	1.47E-06	0.03
149951_c1_seq3	hypothetical protein KGM_08587	1.72E-02	1.93E-06	0.03
150825_c0_seq1	inorganic phosphate	5.03E-04	5.67E-06	0.03
148856_c2_seq22	hypothetical protein SINV_07136	6.73E-04	4.30E-06	0.03
157868_c0_seq16	hypothetical protein KGM_21130	6.25E-03	1.53E-06	0.03
158346_c0_seq3	hypothetical protein KGM_02056	1.91E-03	2.75E-06	0.03
152735_c1_seq1	prolyl 4-hydroxylase subunit	7.61E-03	9.25E-07	0.03
150967_c0_seq3	---NA---	1.78E-02	3.71E-07	0.03
152534_c1_seq10	rna-directed dna polymerase from	8.42E-03	7.41E-07	0.03
154790_c0_seq15	---NA---	3.75E-03	1.13E-06	0.03
154520_c0_seq36	location of vulva defective family	7.68E-04	2.30E-06	0.03
152227_c2_seq11	PREDICTED: uncharacterized	4.34E-03	7.97E-07	0.03
155062_c3_seq7	hypothetical protein KGM_11922	3.73E-04	2.12E-06	0.03
156104_c0_seq4	---NA---	1.92E-03	1.16E-06	0.03
144021_c4_seq10	PREDICTED: uncharacterized	6.26E-03	5.16E-07	0.03
158277_c0_seq8	retrovirus-related pol polyprotein	2.64E-03	8.76E-07	0.03
157129_c0_seq3	---NA---	6.76E-03	3.59E-07	0.03
158777_c0_seq4	---NA---	2.98E-03	1.57E-09	0.03
147196_c1_seq4	---NA---	1.01E-02	2.09E-07	0.03
147570_c0_seq9	hypothetical protein	5.06E-05	7.10E-08	0.03
140550_c1_seq3	---NA---	9.65E-06	1.47E-06	0.03
148823_c0_seq5	---NA---	6.87E-03	2.54E-07	0.03
157968_c0_seq1	hypothetical protein KGM_04783	4.07E-03	3.11E-07	0.03
146688_c2_seq8	probable prefoldin subunit 4-like	8.70E-05	1.45E-06	0.03
155797_c1_seq1	unc93a protein	2.29E-03	3.87E-07	0.03
155687_c1_seq17	---NA---	2.40E-04	9.38E-07	0.03
146888_c0_seq10	srb7prna polymerase ii	6.15E-03	1.45E-07	0.03
158441_c0_seq7	aldehyde oxidase aox2	1.72E-03	3.71E-07	0.03
147494_c2_seq4	---NA---	6.07E-03	1.16E-07	0.03
152168_c2_seq9	---NA---	6.07E-04	5.15E-07	0.03
157451_c1_seq20	tbc1 domain family member 24-	4.90E-04	5.27E-07	0.03
138562_c0_seq1	---NA---	7.36E-03	5.64E-08	0.03
150589_c0_seq3	protein shq1-like protein	3.21E-04	3.73E-07	0.03
150098_c0_seq2	---NA---	3.75E-03	6.94E-08	0.03
157451_c1_seq5	AGAP010235-PA	2.03E-03	1.18E-07	0.03
154285_c0_seq4	glucose dehydrogenase	1.49E-03	1.31E-07	0.02
154512_c0_seq3	tom1-like protein 2-like	3.83E-04	2.65E-07	0.02
150567_c3_seq2	---NA---	2.36E-03	7.90E-08	0.02
148777_c1_seq4	---NA---	9.38E-04	1.47E-07	0.02
154355_c0_seq26	hypothetical protein KGM_11873	1.03E-03	1.40E-07	0.02

158736_c0_seq1	e3 ubiquitin-protein ligase	7.79E-04	1.61E-07	0.02
147623_c1_seq4	---NA---	2.64E-03	6.16E-08	0.02
155179_c0_seq5	hypothetical protein KGM_04089	3.07E-03	4.87E-08	0.02
155102_c0_seq4	DnaJ-25	5.54E-03	2.50E-08	0.02
145601_c1_seq13	transcription-associated zinc	1.73E-04	2.15E-07	0.02
146107_c2_seq2	---NA---	1.32E-03	7.20E-08	0.02
150313_c2_seq5	e3 ubiquitin-protein ligase amfr	2.30E-04	1.43E-07	0.02
158662_c0_seq3	hypothetical protein KGM_20007	1.45E-03	4.07E-08	0.02
153416_c1_seq1	---NA---	2.95E-03	1.69E-08	0.02
158300_c0_seq7	hypothetical protein KGM_06279	3.07E-03	1.58E-08	0.02
156254_c0_seq24	---NA---	1.80E-03	2.69E-08	0.02
153596_c0_seq34	---NA---	2.73E-07	6.34E-10	0.02
145323_c5_seq2	cuticle protein	1.23E-03	2.90E-08	0.02
158346_c0_seq4	hypothetical protein KGM_02056	1.67E-03	2.17E-08	0.02
154646_c0_seq3	hypothetical protein KGM_10843	2.86E-05	1.56E-07	0.02
145598_c0_seq3	myofilin variant a	1.06E-06	2.94E-08	0.02
152534_c1_seq17	hypothetical protein	3.56E-03	8.63E-09	0.02
154520_c0_seq8	location of vulva defective family	3.04E-04	6.11E-08	0.02
150822_c0_seq3	PREDICTED: uncharacterized	2.65E-03	1.07E-08	0.02
146659_c0_seq3	hypothetical protein KGM_14452	1.96E-03	1.33E-08	0.02
157002_c0_seq11	zinc transporter 2-like isoform x2	1.60E-04	5.87E-08	0.02
142090_c1_seq3	---NA---	4.81E-04	2.94E-08	0.02
154048_c1_seq2	---NA---	1.02E-03	1.26E-08	0.02
156078_c0_seq11	nad-dependent	8.40E-06	1.17E-13	0.02
154203_c1_seq1	acidic nucleoplasmic dna-binding	1.20E-03	7.26E-09	0.02
142486_c0_seq10	rna-binding protein	1.55E-03	4.25E-09	0.02
156529_c0_seq6	protein isoform a-like	3.19E-04	1.58E-08	0.02
146387_c0_seq1	PREDICTED: uncharacterized	9.06E-04	6.41E-11	0.02
158639_c0_seq1	atp-binding cassette sub-family a	6.82E-04	4.50E-09	0.02
152802_c0_seq2	angiotensin-converting enzyme-	6.79E-04	4.50E-09	0.02
156027_c0_seq3	TRAF3	1.21E-04	1.24E-08	0.02
152482_c0_seq9	---NA---	1.34E-03	1.57E-09	0.02
156059_c2_seq8	---NA---	1.36E-11	5.30E-16	0.02
158977_c0_seq3	hypothetical protein KGM_22119	2.82E-05	1.47E-08	0.02
148823_c0_seq12	hypothetical protein KGM_21879	1.60E-04	6.52E-09	0.02
141394_c1_seq2	---NA---	2.77E-05	1.24E-08	0.02
149704_c1_seq7	---NA---	5.09E-04	1.71E-09	0.02
158332_c0_seq8	---NA---	4.47E-05	7.56E-09	0.02
147582_c0_seq1	---NA---	1.02E-03	7.18E-10	0.02
148241_c0_seq12	hemicentin 1	2.78E-04	1.78E-09	0.02
156311_c0_seq47	potassium voltage-gated channel	1.74E-04	2.44E-09	0.02
153594_c0_seq14	glucose-6-phosphate 1-	4.41E-08	2.86E-08	0.02
157892_c0_seq4	polycomb protein l g0020-like	3.07E-04	1.25E-09	0.02
157047_c0_seq9	---NA---	9.20E-04	2.69E-10	0.02
153847_c0_seq11	stromal interaction molecule 1	1.89E-04	1.18E-09	0.02
147491_c0_seq2	elongation of very long chain	2.69E-06	7.22E-09	0.02
151785_c0_seq1	tetratricopeptide repeat protein	3.28E-04	2.36E-10	0.02
157678_c1_seq32	sodium-bile acid cotransporter	5.21E-07	8.84E-12	0.02
157543_c1_seq2	hypothetical protein KGM_04788	1.57E-05	4.83E-11	0.02
128989_c0_seq1	---NA---	3.03E-08	8.65E-13	0.02
157589_c1_seq11	multidrug resistance protein	3.52E-05	2.37E-10	0.02
78949_c0_seq1	---NA---	7.17E-05	1.85E-13	0.02
157584_c0_seq3	plekhh1	2.29E-04	2.26E-11	0.02
157053_c0_seq4	fatty acid transport protein 4	9.78E-07	3.35E-10	0.01
147389_c0_seq2	---NA---	1.75E-07	3.72E-10	0.01
158149_c0_seq13	calcium-activated potassium	1.03E-09	1.11E-09	0.01
152909_c1_seq28	---NA---	1.83E-06	6.73E-11	0.01
157868_c0_seq20	PREDICTED: hemicentin-1-like	4.41E-06	4.04E-11	0.01
157868_c0_seq21	hypothetical protein KGM_21130	2.00E-05	1.31E-11	0.01
154355_c0_seq16	hypothetical protein KGM_11873	3.83E-06	3.46E-11	0.01
158823_c0_seq6	hypothetical protein KGM_21981	5.20E-06	1.64E-11	0.01

158882_c0_seq5	PREDICTED: uncharacterized	4.02E-06	1.79E-11	0.01
156175_c0_seq39	---NA---	1.20E-05	6.05E-12	0.01
158627_c0_seq14	hypothetical protein KGM_00262	1.41E-05	3.84E-12	0.01
149575_c0_seq3	alcohol partial	1.71E-05	1.36E-12	0.01
157230_c0_seq5	---NA---	2.25E-07	8.09E-12	0.01
158186_c0_seq18	heterotrimeric guanine	4.38E-10	4.83E-11	0.01
157155_c0_seq1	zinc transporter 8-like	2.25E-07	4.79E-12	0.01
154939_c0_seq13	PREDICTED: uncharacterized	5.18E-06	2.26E-13	0.01
152500_c6_seq5	apolipoprotein d-like	7.45E-08	3.27E-12	0.01
154881_c0_seq9	vatd_manse ame: full=v-type	4.71E-08	2.90E-12	0.01
150161_c0_seq12	activating signal cointegrator 1	1.19E-08	4.24E-12	0.01
151286_c0_seq5	transcription factor ets	1.39E-09	1.13E-12	0.01
151901_c0_seq3	uncharacterized protein	2.95E-07	2.78E-14	0.01
154520_c0_seq73	location of vulva defective family	2.24E-07	2.22E-14	0.01
154881_c0_seq12	---NA---	4.84E-07	3.65E-15	0.01
155796_c0_seq5	prefoldin subunit 2-like	1.53E-09	7.15E-14	0.01
157349_c0_seq8	PREDICTED: uncharacterized	6.74E-08	1.38E-16	0.01
144509_c0_seq2	---NA---	1.27E-09	8.71E-16	0.01
153751_c0_seq9	hypothetical protein KGM_06296	2.68E-15	2.87E-16	0.01
145823_c0_seq2	cdc2-related-kinase	7.02E-10	9.30E-19	0.01
152535_c0_seq3	hypothetical protein KGM_16968	1.96E-10	2.10E-18	0.01
157868_c0_seq13	PREDICTED: hemicentin-1-like	1.77E-11	2.65E-18	0.01
155969_c0_seq5	hypothetical protein KGM_04418	6.86E-10	1.73E-19	0.01
155987_c2_seq1	---NA---	1.39E-13	1.81E-17	0.01
145080_c0_seq1	aldo-keto reductase	1.39E-13	1.48E-17	0.01
157589_c1_seq5	multidrug resistance protein	1.48E-13	5.43E-19	0.01
154833_c1_seq1	protein disulfide-isomerase like	1.37E-12	1.04E-22	0.005
158826_c0_seq1	rho guanine nucleotide exchange	2.97E-18	1.11E-28	0.003
156998_c0_seq1	branched-chain-amino-acid	1.87E-22	3.04E-30	0.002
141495_c0_seq1	signal sequence receptor beta	9.79E-25	3.04E-30	0.002
157058_c1_seq12	s-adenosylmethionine synthetase	1.48E-28	3.80E-37	0.001
157058_c1_seq15	s-adenosylmethionine synthetase	1.19E-30	7.34E-43	0.001