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Geostatistical analysis of the distribution of *Leptosphaeria* species causing phoma stem canker on winter oilseed rape (*Brassica napus*) in England

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In June/July 2001, 2002, 2003 and 2006, regional variation in distribution of the pathogens *Leptosphaeria maculans* and *L. biglobosa* that are causally associated with phoma stem canker was surveyed on winter oilseed rape crops in England. In 2001–2003, when isolates from basal cankers were visually identified as *L. maculans* or *L. biglobosa* based on cultural morphological characteristics, 70% were *L. maculans* and 30% *L. biglobosa*. In 2001, 2002, 2003 and 2006, when amounts of DNA of each species in basal cankers were determined by quantitative PCR, the abundance of *L. maculans* DNA was greater than that of *L. biglobosa* DNA in 77% of samples. When regional differences in amounts of *L. maculans* and *L. biglobosa* DNA were mapped geostatistically, quantities of *L. maculans* DNA were greater in cankers from southern England and those of *L. biglobosa* DNA were greater in northern England. A comparison with geostatistically mapped predictions made using a weather-based model describing stages in development of phoma stem canker epidemics suggested that these differences in *Leptosphaeria* populations may have been a consequence of differences in temperature after onset of leaf spotting between northern and southern England. Both PCR and morphological evidence suggested that the abundance of *L. maculans* in England has increased since the last surveys in the 1980s. Implications of these surveys for control of phoma stem canker are discussed.

Keywords: climate-disease interactions, invasive species, *Phoma lingam*, quantitative PCR, spatial statistics, weather-based disease forecasting

Introduction

The most damaging disease of winter oilseed rape in the UK is phoma stem canker, which is responsible for £70–140 M of losses each growing season at a price of £250 per tonne, despite expenditure of more than £20 M on fungicides to control it (Fitt *et al.*, 2006a). During the last decade winter oilseed rape has been grown on about 415 000 ha annually in England (http://tinyurl.com/nkh652); it is generally sown in late August/early September and harvested in mid-July the following year. Losses from phoma stem canker are generally associated with basal stem cankers, although less damaging upper stem lesions also occur. The disease is associated with infection of crop stems by two related pathogen species, *Leptosphaeria maculans* and *L. biglobosa,* but it is not clear how proportions of these species in cankers should affect strategies to control the disease.

In Europe, phoma stem canker epidemics are largely initiated in the autumn by ascospores released from pseudothecia on the stubble of the previous season's crop (West *et al.*, 2001). Germ-tubes produced by ascospores penetrate leaves through stomata and wounds (Huang *et al.*, 2003a). Leaf lesions caused by *L. maculans* are grey with many pycnidia while those caused by *L. biglobosa* are smaller, and have a dark margin with a light brown centre with few pycnidia (Toscano-Underwood *et al.*, 2001). From the leaf lamina, both *L. maculans* and *L. biglobosa* spread, without expressing symptoms, along the petiole to the stem, resulting in the formation of basal stem cankers or upper stem lesions in the spring/summer (West *et al.*, 2001). On the stem *L. maculans* is mainly associated with basal (crown) stem cankers and *L. biglobosa* mainly with upper stem lesions (West *et al.*, 2002; Fitt *et al.*, 2006b). After harvest, *L. maculans* and *L. biglobosa* are able to survive on crop debris (West *et al.*, 2002; Huang *et al.*, 2003b).

Effective control of phoma stem canker on winter oilseed rape can be achieved by foliar fungicide sprays in the autumn; however, timing of such applications is crucial (West *et al.*, 2001; Aubertot *et al.*, 2006; Gladders *et al.*, 2006). Disease control is also achieved through the deployment in commercial cultivars of qualitative race-specific genes for resistance to *L. maculans* (Delourme

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et al., 2006) that operate after penetration of the leaf (Huang *et al.*, 2006). However, this resistance is not effective against *L. biglobosa* (Somda *et al.*, 1998; Fitt *et al.*, 2006a). Quantitative resistance to *L. maculans,* involving quantitative trait loci (QTL), operating in *B. napus* petiole and stem tissues, is considered to provide more durable resistance (Delourme *et al.*, 2006; Huang *et al.*, 2009).

Both *L. maculans* and *L. biglobosa* have worldwide distributions but there is evidence that *L. maculans* has been invading areas previously colonized only by *L. biglobosa* (Fitt *et al.*, 2008). Both species coexist in many countries (Fitt *et al.*, 2006a). The invasion of *L. maculans* into areas where only *L. biglobosa* was previously present suggests that *L. biglobosa* predates *L. maculans* in evolutionary terms (Fitt *et al.*, 2006a,b). In China, the country which has the largest land area sown to brassicas worldwide, only *L. biglobosa* is currently present (Fitt *et al.*, 2008); however, there is great concern that considerable losses would occur if the more damaging *L. maculans* were to be introduced (Fitt *et al.* 2006a, 2008).

Whilst the two species are known to differ in their biology, it is unclear how regional variation in climate and agronomy influence the balance between them. In England, the regional distribution of *L. maculans* and *L. biglobosa* was investigated by isolation from infected brassica leaves and stems in 1982 and 1983 (Humpherson-Jones, 1986). The two species were distinguished by visual observation of characteristics of cultures on plates and by pathogenicity tests on cabbage (*B. oleracea*). Of the 1611 *Leptosphaeria* isolates collected, 41% were identified as *L. maculans* and 59% as *L. biglobosa*. Regional variation in the proportion of the two species was observed (Fig. 1); the proportion of *L. maculans* was greatest in areas with a longer history of cropping with oilseed rape (south-east and east England), which had been reintroduced during the 1970s. Nevertheless, there was no evidence of differences between the north and the south of England. There has been no detailed survey of the regional distribution of the two species since the 1980s.

The relationships between regional differences in the severity of stem canker epidemics, differences in climate and differences in the proportions of *L. maculans* and *L. biglobosa* in populations need to be investigated. In the UK, there is regional variation in the incidence of phoma stem canker, which may be related to differences in climate; disease incidence (http://www.cropmonitor.co.uk/wosr/surveys/phoma-RiskMap.cfm) and severity (Evans *et al.*, 2008) are greater in south-east England than in northern England. In Scotland, although phoma leaf spotting occurs, phoma stem cankers do not develop (Evans *et al.*, 2008). Temperature affects both the epidemiology of phoma stem canker and interactions between *L. maculans* and *L. biglobosa*. Pseudothecial maturation of each species is affected by temperature; maturation of *L. maculans* pseudothecia is faster than that of *L. biglobosa* at temperatures of <10°C whilst maturation of pseudothecia of both species is similar at 15–20°C (Toscano-Underwood *et al.*, 2003).

Controlled environment experiments have shown that with increasing temperature from 5 to 20°C the rate of ascospore germination and the efficiency of stomatal penetration by *L. maculans* and *L. biglobosa* increases (Huang *et al.*, 2003a), whilst incubation period (time from inoculation to appearance of leaf lesions) decreases (Toscano-Underwood *et al.*, 2001). In addition, the rate of endophytic growth of *L. maculans* within the petiole is affected by temperature (Sun *et al.*, 2001). The effect of climate change on phoma stem canker epidemiology has been demonstrated using a weather-based disease forecasting model combined with a model predicting climate

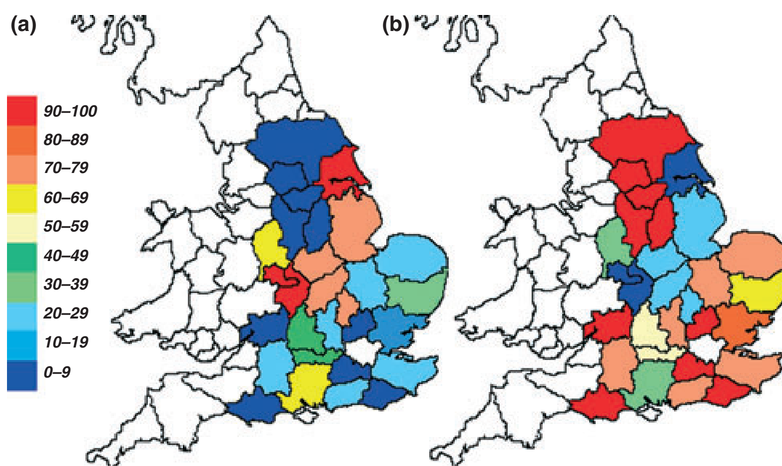

Figure 1 County maps of England showing the proportion (%) of isolates identified, by visual assessment of culture morphology, as *Leptosphaeria maculans* (a) or *L. biglobosa* (b) in each county surveyed in the early 1980s. Surveys were done in 1982 and 1983 by Humpherson-Jones (1986) and a total of 1611 isolates were obtained from affected brassica samples collected throughout England. Isolates were identified as *L. maculans* or *L. biglobosa* by visual identification in culture and by pathogenicity tests on cabbage (*Brassica oleracea*).

change under high- or low-CO_2 emissions for the 2020s and 2050s (Evans *et al.*, 2008). The model predicted that stem canker would increase in severity in England and spread northwards into Scotland by the 2020s.

Biological and epidemiological differences between *L. maculans* and *L. biglobosa* interact with the fluctuating biotic (crop leaves, stems, pods, residues) and abiotic (weather, agronomic practices) environment during the growing season and inter-crop period (Fitt *et al.*, 2006b). The surveys of Humpherson-Jones (1986) measured average proportions of stems affected by *L. maculans* and/or *L. biglobosa* within the counties of England by isolation. In contrast, PCR and geostatistical techniques (Webster & Oliver, 2001) were used in this study to fit models describing the variation in the amounts, based on DNA quantification, of *L. maculans* and *L. biglobosa* DNA in diseased stems. The relationship between these patterns of pathogen distribution and climate was explored by using the geostatistical techniques to map the severity of stem canker epidemics as predicted by a weather-based model. Where similarities between the patterns of *L. maculans* and *L. biglobosa* and the patterns of predicted epidemic severity occurred, the weather-based model was analysed to determine which weather variables accounted for the similarities. This paper describes work to determine the spatial and temporal variation in amounts of *L. maculans* and *L. biglobosa* across England on winter oilseed rape stems with phoma stem canker between 2001 and 2006.

Materials and methods

Sampling winter oilseed rape

A survey of *Leptosphaeria* populations in different parts of England was done by sampling winter oilseed rape plants with visible basal stem cankers 1–2 weeks before harvest (i.e. in late June or early July) in 2001, 2002, 2003 and 2006. Samples were collected by ADAS consultants (2001–2003) and The Arable Group (TAG) growers and farm consultants (2006) from commercial crops (fungicide treated; not all the same cultivar) in England and Wales. In 2001–2003, 25 plants were randomly selected from each site, stem canker severity was assessed (West *et al.*, 2002) and the 10 plants with the most severe basal stem cankers were selected. However, at a few sites <10 plants had visible basal stem cankers; therefore fewer diseased stems were available from these sites. In 2006, a minimum of 10 plants with visible basal stem cankers were selected from each of the different commercial crops sampled. The incidence of upper stem lesions on these plants was also assessed. The six figure Ordnance Survey map grid reference of each site was recorded. The selected plants were cut to give lengths of stem (30 cm), including the stem base and tap root. These samples of stem pieces from each site were sent to Rothamsted in padded envelopes or boxes with individual pieces wrapped in laboratory paper tissue. No samples were 'in transit' for more than 48 h. Samples were either processed on the day of arrival or placed in storage at −20°C. In total, 811 stems from 117 sites were processed in the 4 years (Table 1).

Table 1 Numbers of winter oilseed rape basal stem samples received in June/July in 2001, 2002, 2003 and 2006 to assess proportions of *Leptosphaeria maculans* and *L. biglobosa* present by cultural techniques or qPCR and mean severity of stem cankers on these stems at each site in each year

Year	Number of sampling sites[c]	Total number of stems examined		Mean basal canker severity per site[d]
		Cultural techniques	qPCR	
2001[a]	27	67	182	0·69
2002[a]	20	241	87	1·13
2003[a]	36	242	178	0·34
2006[b]	34	0	364	3·17

[a]Twenty-five plants from each site were assessed for disease severity; the 10 plants with the most severe symptoms were selected (at a minority of sites <10 stems had visible basal stem cankers). Selected stems were cut 30 cm above the base to give basal stem samples.
[b]A minimum of 10 plants with visible basal stem cankers were selected.
[c]The locations of sampling sites in England are shown in Fig. 3a-d.
[d]The internal severity of basal stem cankers was assessed on a 0–4 scale (0 = no symptoms, 4 = 100% stem cross-sectional area affected (Zhou *et al.*, 1999)). Mean canker severity per site was calculated from the mean canker severity of 25 (2001–2003) or 10 (2006) stems assessed at each site.

Assessment of stem canker severity

In the years 2002, 2003 and 2006, the internal stem canker severity, assessed by cutting a cross-section of basal stem cankers, was scored on a 0–4 scale according to the percentage of the internal stem cross-sectional area affected (0 = no symptoms visible, 1 = 0–25% affected, 2 = 26–50%, 3 = 51–75%, 4 = 76–100%) (Zhou *et al.*, 1999).

Cultural isolation

In the years 2001, 2002 and 2003, visual identification of *L. maculans* and *L. biglobosa* cultures was done. Pieces of basal stem canker lesions were excised from a sub-set of the diseased stems received (67 in 2001, 241 in 2002 and 242 in 2003). These pieces were surface sterilized in 70% (v/v in water) ethyl alcohol for <10 s, and then immersed in 10% (v/v) sodium hypochlorite solution containing 8% available chlorine (Fisher Scientific) for 2 min; following this, samples were rinsed with sterile distilled water. Surface sterilized samples were then placed onto 9 cm diameter water agar plates and incubated at 15°C in darkness for 5–7 days. Hyphal tips were then subsequently excised and transferred to 9 cm diameter potato dextrose agar (PDA; Oxoid Ltd) plates supplemented with streptomycin (100 mg L^{-1}) and penicillin (50 mg L^{-1}) and incubated at 15°C for 7 days. Isolates were identified by visual observation of plate cultures, growth rate and pigment production; larger colonies with yellow/brown pigment were recorded as *L. biglobosa* whilst

smaller colonies with no pigment production were recorded as *L. maculans* (Williams & Fitt, 1999).

DNA extraction

In all four years, stems were rinsed with sterile water, dipped in 70% ethanol and allowed to air dry at 20°C for 5 min. A piece, approximately 10 mm in diameter, was excised from a canker lesion on each stem and DNA extraction, based on the method of Graham *et al.* (1994), was done in 1·5 mL microfuge tubes. Where upper stem lesions were present, they were also sampled for DNA extraction. Samples were each macerated in 500 μL of CTAB extraction buffer (2% CTAB (hexadecyltrimethylammonium bromide), 100 mM Tris-HCl, 1·4 mM NaCl and 20 mM EDTA (ethylenediaminetetraacetic acid)) and 10 μL β-mercaptoethanol, vortexed, incubated for 60 min at 70°C and the homogenate centrifuged for 15 min at 16 000 ***g***. The supernatant was collected and extracted against an equal volume of a 24:1 chloroform:isoamyl alcohol mixture by vortexing. The mixture was centrifuged (16 000 ***g***) for 15 min and the upper, aqueous phase (450 μL) was collected. Sodium acetate (45 μL, 3 M, pH 5·2) and 900 μL ice-cold absolute ethanol were added and the samples were mixed by gentle inversion and placed at −20°C for 1 h to precipitate genomic DNA, followed by centrifugation at 16 000 ***g*** for 10 min to pellet the DNA. The supernatant was discarded. DNA pellets were washed with 1 mL ice-cold 70% ethanol, dried at 37°C for 15 min and dissolved in 100–200 μL sterile deionized water or 1 mM TE buffer (10 mM Tris HCl (pH 7·5), 1 mM EDTA). The amount of DNA was quantified using a NanoDrop-1000 spectrophotometer (Labtech International), diluted to obtain an optical density (OD) of no more than 2 and adjusted with sterile deionized water to give a concentration of 50 ng μL^{-1}.

Quantitative PCR

To determine the relative amounts of *L. maculans* and *L. biglobosa* DNA in samples, quantitative PCR (qPCR) was done using the Sigma SYBR Green qPCR kit (Sigma). Sterile deionized water and *B. napus* DNA (50 ng μL^{-1}) were used as no-template controls in all qPCR reactions. Species-specific primers for the identification of *L. maculans* and *L. biglobosa* (Mahuku *et al.*, 1996) were used. Preliminary work, in which the qPCR of one species was done with known DNA from the other species, confirmed that there is no cross detection between the *L. maculans* and *L. biglobosa* assays, as no amplification was observed. A standard 20 μL reaction volume consisted of 5 μL of DNA sample, 10 μL of SYBR Green Jump Start Ready Mix (Sigma), 0·08 μL of Internal Reference dye (R4626, Sigma), 0·4 μL (forward) and 0·6 μL (reverse) of 10 μM primers and 3·92 μL sterile deionized water. For this study, 10 μL assays were routinely done in duplicate (capped 96-well Thermo-Fast PCR plates, ABgene) in an ABI 7500 Real Time PCR system (Applied Biosystems) for 2 min at 95°C followed by 38 cycles of 15 s at 95°C, 30 s at 60°C and 45 s at 72°C. The increase in fluorescence from amplicons was recorded at 72°C during every cycle.

For each sample, the threshold cycle (C_t) for the SYBR-labelled amplicon was determined. Standard curves, with a minimum R^2 of 0·98, were generated by plotting known amounts of *L. maculans* or *L. biglobosa* DNA (between 0·0001 and 10 ng μL^{-1}) in a background of *B. napus* DNA (total DNA concentration 50 ng μL^{-1}) against C_t values. The resulting regression equations were used to quantify the amount of DNA in unknown samples. Results were expressed as amount (pg) of *L. maculans* or *L. biglobosa* DNA in 50 ng DNA from infected plant tissue. To ascertain the specificity of the procedure, a dissociation (melting) curve was done after the final amplification cycle by heating samples at 95°C for 15 s, cooling to 60°C for 1 min and then heating to 95°C for 15 s; fluorescence was measured continuously. To further confirm the specificity of the assays, PCR products were stained with 6× loading dye solution (10 mM Tris-HCl (pH 7·6), 0·03% bromophenol blue, 0·03% xylene cyanol FF, 60% glycerol, 60 mM EDTA) before separation by electrophoresis on 1% agarose, incorporated with ethidium bromide (0·5 μg mL^{-1}), in 1× TBE buffer (89 mM Tris base, 89 mM boric acid (pH 8·3), 2 mM Na_2EDTA) at 80 volts for 1 h and the presence of one amplicon was identified under ultraviolet light using a transilluminator (Gene Genius Bio Imaging System, Syngene, Synoptics Ltd).

Weather-based model predicting development of phoma stem canker epidemics

For each survey year, the severity of phoma stem canker epidemics was predicted at the sites of weather stations in England and Wales using the model of Evans *et al.* (2008). This is a weather-based model fitted to data from 40 oilseed rape field experiments done under different weather conditions in growing seasons between 1992–1993 and 2001–2002 at a wide range of sites in England. The model uses climatic variables to predict two key dates in the development of a phoma stem canker epidemic. These dates are *Dl*, the date in autumn when 10% of plants in a crop are affected by phoma leaf spotting and *Dc*, the date in the following spring when 10% of plants are affected by phoma stem canker. Having predicted these dates, the severity of a phoma stem canker epidemic at harvest can be predicted by summing the thermal time between *Dc* and the harvest date *Dh* (taken as 15 July). The equation to predict *Dl* is written

$$Dl = 216{\cdot}5 - 0{\cdot}24R_{\mathrm{sum}} - 4{\cdot}55T_{\mathrm{max}} \quad (1)$$

where R_{sum} and T_{max} are the total rainfall (mm) and mean maximum daily temperature (°C) between 15 July and 26 September and day zero is 15 July. Day *Dc* is the first day where the accumulated thermal time since *Dl* exceeds 1097°C-days for susceptible cultivars (resistance rating 1–5; http://tinyurl.com/l6ddkw, $A = 0$) or 1386°C-days for resistant cultivars (resistance rating 6–9; $A = 1$) i.e.

$$Dc = \min\left(x \in \{Dl, Dl+1, ...\} | \sum_{i=Dl}^{x} T_i \geq (1097 + 289A)\right) \quad (2)$$

where T_i is defined as the average of the minimum and maximum temperature in °C on day i. The equation to predict the canker severity on day Dh is

$$Sc = \sum_{i=Dc}^{Dh} T_i(0{\cdot}00135 - 0{\cdot}00035A) \quad (3)$$

where Sc is the mean stem canker severity of plants (0–4 scale; Zhou *et al.*, 1999).

The daily rainfall, daily minimum temperature and daily maximum temperature were sourced from the UK Meteorological Office database (Crown copyright 2007, the Meteorological Office) for 49 weather stations across England and Wales in each survey year. The date of start of leaf spotting, the data of start of stem canker and the severity of the canker at harvest was predicted at each of these weather stations for each year by Equations (1), (2) and (3) respectively. The parameter describing the resistance rating of the cultivar was set at A = 0·5 (average resistance) for all sites.

Mapping spatial variables by geostatistics

Each of the four study years had spatial observations of the abundance of *L. maculans* and *L. biglobosa* DNA and the outputs from the weather model of Evans *et al.* (2008). Geostatistical methods were used to map these variables across England and Wales. Classical geostatistical techniques (Webster & Oliver, 2001) are known to have a number of short-comings. In particular they require the user to select a number of arbitrary parameters which affect the outputs and it is not clear how multiple observations at the same site can be handled. Therefore model-based geostatistical techniques were used (Diggle & Ribeiro, 2007).

Model-based methods fit a model, in this case a linear mixed model (LMM), of spatial variation to the data and then use this model to interpolate the observed variable across the study region. Full details are given in the Appendix. The fitted model divides the variation between components that are purely random and components which are spatially correlated. It is these spatially correlated components that give the map patterns. The magnitude of the variations in each map will be indicative of the proportion of the variation that is spatially correlated rather than the actual variance of the variable. Therefore only the pattern of spatial variation is presented and not the magnitude, since this may be misleading.

Table 2 Visual identification by cultural characteristics as *Leptosphaeria maculans* or *L. biglobosa* of isolates obtained from stems of commercial winter oilseed rape crops with basal stem canker collected before harvest June/July in 2001, 2002 and 2003

Year[b]	Total no. isolates	Frequency (%) of isolates[a]	
		L. maculans	*L. biglobosa*
2001	67	61	39
2002	241	81	19
2003	242	62	38
	550	70	30

[a]Isolates were classified as *L. maculans* or *L. biglobosa* by growth rate and production of pigment on potato dextrose agar; *L. biglobosa* produces larger colonies with a yellow/brown pigment, *L. maculans* produces smaller colonies with no pigment.
[b]Numbers of stems examined each year are shown in Table 1.

Results

Visual identification of *L. maculans* and *L. biglobosa* isolates

In total, 550 isolates (from individual stems) were identified as *L. maculans* or *L. biglobosa* in years 2001–2003. In all years, a greater proportion of isolates was identified as *L. maculans* (mean 70% (387 isolates)) than as *L. biglobosa* (mean 30% (163)) (Table 2).

Phoma stem canker severity and amounts of *L. maculans* and *L. biglobosa* DNA

There were differences between seasons in basal stem canker severity in crops sampled; stem canker severity was greater in 2002 and 2006 than in 2001 and 2003 (Table 1). *Leptosphaeria maculans* DNA was identified in 100% of the 811 basal stem canker samples tested, at quantities ranging from 300 fg to 29·7 ng of DNA in 50 ng total DNA (Table 3). *Leptosphaeria biglobosa* DNA was identified in 96% (781) of the samples, at quantities ranging from 0·001 pg to 48 ng in 50 ng

Table 3 Exploratory statistics for amount (pg), determined by quantitative PCR, of *Leptosphaeria maculans* (*Lm*) DNA and $\log_{10}$-*L. maculans* DNA (log(*Lm*)) in basal phoma stem cankers on oilseed rape stems collected from England before harvest in June/July 2001, 2002, 2003 and 2006

	2001		2002		2003		2006	
	Lm	log(*Lm*)	*Lm*	log(*Lm*)	*Lm*	log(*Lm*)	*Lm*	log(*Lm*)
Mean	$3{\cdot}69 \times 10^3$	6·78	$1{\cdot}56 \times 10^3$	4·74	$1{\cdot}58 \times 10^3$	5·34	$1{\cdot}21 \times 10^3$	6·13
Maximum	$2{\cdot}48 \times 10^4$	10·01	$2{\cdot}14 \times 10^4$	9·97	$2{\cdot}97 \times 10^4$	10·03	$7{\cdot}33 \times 10^3$	8·90
Minimum	1·56	0·44	0·30	1·23	1·41	0·34	0·61	–0·50
Variance	$5{\cdot}07 \times 10^3$	2·24	$4{\cdot}18 \times 10^3$	2·68	$3{\cdot}42 \times 10^3$	2·34	$1{\cdot}27 \times 10^3$	1·92
Skew	2·20	–0·63	3·91	–0·03	4·66	0·01	1·39	–1·28

Table 4 Exploratory statistics for amount (pg), determined by quantitative PCR, of *Leptosphaeria biglobosa* (*Lb*) DNA and $\log_{10}$- (*L. biglobosa* DNA + 10^{-7}) (log(*Lb*)) in basal phoma stem cankers on oilseed rape stems collected from England before harvest in June/July 2001, 2002, 2003 and 2006

	2001		2002		2003		2006	
	Lb	log(*Lb*)	*Lb*	log(*Lb*)	*Lb*	log(*Lb*)	*Lb*	log(*Lb*)
Mean	2.37×10^3	4·07	1.16×10^3	3·45	5.22×10^2	0·72	9.34×10^2	4·57
Maximum	4.80×10^4	10·80	3.34×10^4	10·40	2.87×10^4	10·32	2.94×10^4	10·34
Minimum	0	−16·12	0	−16·12	0	−16·12	0·01	−10·38
Variance	6.92×10^3	3·95	4.30×10^3	2·81	2.97×10^3	6·33	2.91×10^3	2·42
Skew	4·61	−1·49	6·04	0·26	7·74	−1·61	6·34	0·13

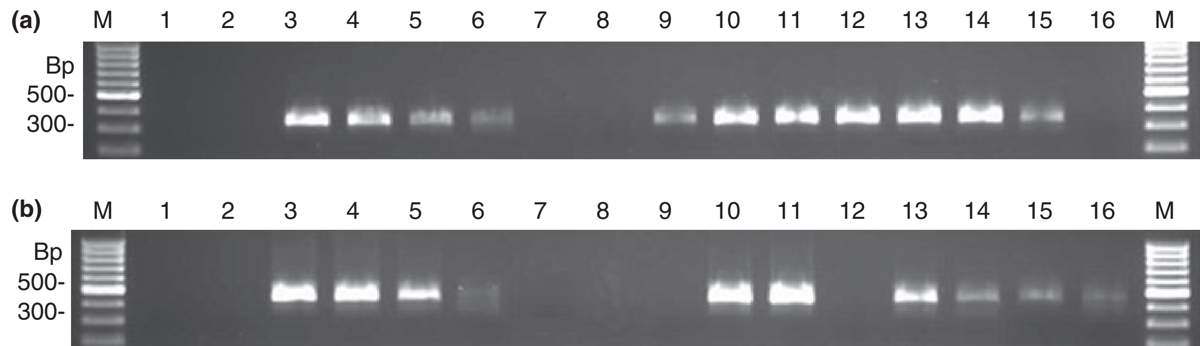

Figure 2 Visualization of *Leptosphaeria maculans* and *L. biglobosa* amplicons on agarose gels following qPCR. Amplicons from qPCR of (a) *L. maculans* and (b) *L. biglobosa* were separated by electrophoresis in 1 × TBE buffer on 1% agarose incorporated with ethidium bromide and viewed under uv light. Lanes 1 (sterile water) and 2 (50 ng *B. napus* DNA) contained negative controls. Lanes 3 to 8 contained the 10-fold dilution series. Lanes 9 to 16 contained DNA extracted from stem canker samples; for *L. maculans* (a) quantities of DNA were: lane 9 = 0·5 ng, 10 = 20·9 ng, 11 = 12·2 ng, 12 = 6·3 ng, 13 = 24·8 ng, 14 = 28·3 ng, 15 = 0·1 ng and 16 = 0·01 ng, and for *L. biglobosa* (b) they were: lane 9 = 0·002 ng, 10 = 2·3 ng, 11 = 11·2 ng, 12 = 0·001 ng, 13 = 1·1 ng, 14 = 0·1 ng, 15 = 0·4 ng and 16 = 0·03 ng. Marker (M) lanes contained 1 kb ladder (Invitrogen).

Table 5 Estimates of linear mixed model parameters for $\log_{10}$- (*Leptosphaeria maculans* DNA) (log(*Lm*)) and $\log_{10}$- (*L. biglobosa* DNA + 10^{-7}) (log(*Lb*)) in basal phoma stem cankers on oilseed rape stems collected from England before harvest, in June/July 2001, 2002, 2003 and 2006

	$\hat{c}_0$[a]		$\hat{c}_1$[b]		$\hat{r}$[c]		$\hat{\sigma}^2$[d]		$\hat{\beta}$[e]	
Year	log(*Lm*)	log(*Lb*)	log(*Lm*)	log(*Lb*)	log(*Lm*)	log(*Lb*)	log(*Lm*)	log(*Lb*)	log(*Lm*)	log(*Lb*)
2001	0·05	3·32	1·49	1·01	10·71	10·68	3·93	11·83	9·32	5·20
2002	0·07	1·67	0·12	0·08	3·58	6·50	7·06	6·05	6·44	3·65
2003	2·14	5·37	1·20	1·13	11·81	11·81	2·49	33·92	6·88	0·96
2006	0·32	1·10	2·87	0·58	9·70	8·65	2·17	4·23	7·45	5·17

[a]Nugget variance of spatially correlated variation.
[b]Sill variance of spatially correlated variation.
[c]Spatial parameter of spatially correlated variation (km).
[d]Variance of observations at same site.
[e]Fitted mean parameter.

total DNA (Table 4). For 77% (625) of samples, the amount of *L. maculans* DNA was greater than that of *L. biglobosa* DNA, in proportions ranging from 1 to 127 000 times; in 36 samples in which *L. maculans* DNA was detected, no *L. biglobosa* DNA was found. These qPCR determinations were authenticated by the diagnostic sizes of the amplicons of *L. maculans* and *L. biglobosa* after gel electrophoresis (Fig. 2).

In total, 151 upper stem lesions (60 in 2001, 12 in 2002, 61 in 2003 and 18 in 2006) were also examined. The results of a *t*-test show that the mean amount of *L. biglobosa* DNA in upper stem lesions was significantly greater ($P < 0.001$) than the mean amount in basal cankers. By contrast, there was no difference between basal cankers and upper stem lesions in the amount of *L. maculans* DNA. Since yield losses are associated with basal stem cankers and the data for upper stem lesions were less extensive than those for basal cankers, only the data for basal cankers are considered further.

In each year, the skewness values of data for quantities of DNA in basal cankers from both *L. maculans* and *L. biglobosa* were greater than one (Tables 3 & 4) and therefore a $\log_{10}$-transformation was applied before fitting the parameters of the LMM. The *L. biglobosa* data included some zero values and therefore 10^{-7} was added to each value to ensure that the log-transformation was possible.

Spatial distribution of amounts of *L. maculans* and *L. biglobosa* DNA

The fitted LMM parameters are shown in Table 5. These parameters are defined in the Appendix. For each of the fitted LMMs in years 2001–2003 the largest component of variation was between observations made at the same site. This is indicated by $\hat{\sigma}^2$ being greater than $\hat{c}_0$ and $\hat{c}_1$ (Table 5). The same source of variation was dominant in the 2006 *L. biglobosa* LMM but in 2006 the spatially correlated variation for *L. maculans* was larger than the independent variation (i.e. $\hat{c}_1 > \hat{\sigma}^2$). The map of *L. biglobosa* distribution for 2006 displayed no discernable spatial variation and was therefore omitted from Fig. 3. There were some persistent features of the maps of the spatial correlation portion of *L. maculans* variation (Fig. 3a-d). In each year, the smallest amounts of *L. maculans* DNA were observed in the north of England. Conversely, in 2001 and 2002 this region had the largest amounts of *L. biglobosa* DNA (Fig. 3e-f), although in 2003 there was more *L. biglobosa* DNA in the south than found in the north (Fig. 3g).

Spatial distribution of stem canker severity predicted by a weather-based model

The maps of stem canker severity produced by the weather-based model of Evans *et al.* (2008) predicted that the stem canker severity was less in the north than in the

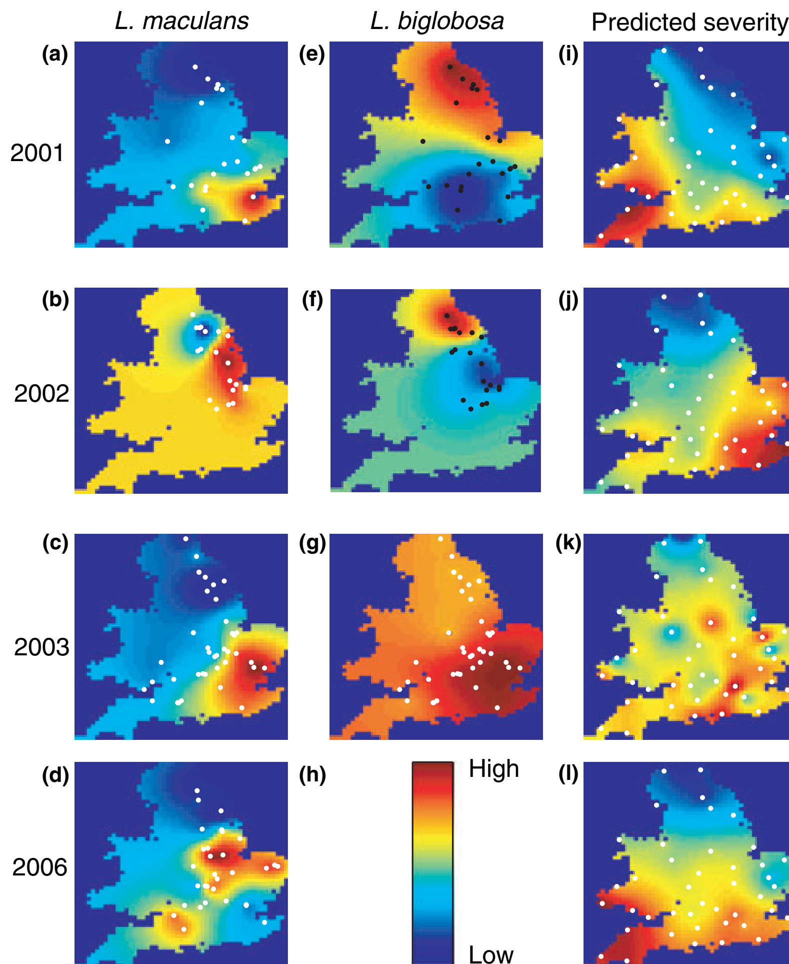

Figure 3 Maps of the pattern of the spatially correlated portion of variation for (i) $\log_{10}$-transformed amount of *Leptosphaeria maculans* DNA (a–d), (ii) $\log_{10}$-transformed amount of *L. biglobosa* DNA (e–g) in stems of winter oilseed rape crops sampled in England and (iii) phoma stem canker severity (0–4 scale, Zhou *et al.*, 1999) as predicted from weather data by the model of Evans *et al.* (2008) (i–l) at harvest, in 2001, 2002, 2003 and 2006. The locations of sample crop sites (a–g)/weather station sites (i–l) are marked. The map of *L. biglobosa* distribution for 2006 showed no discernable spatial variation and is omitted.

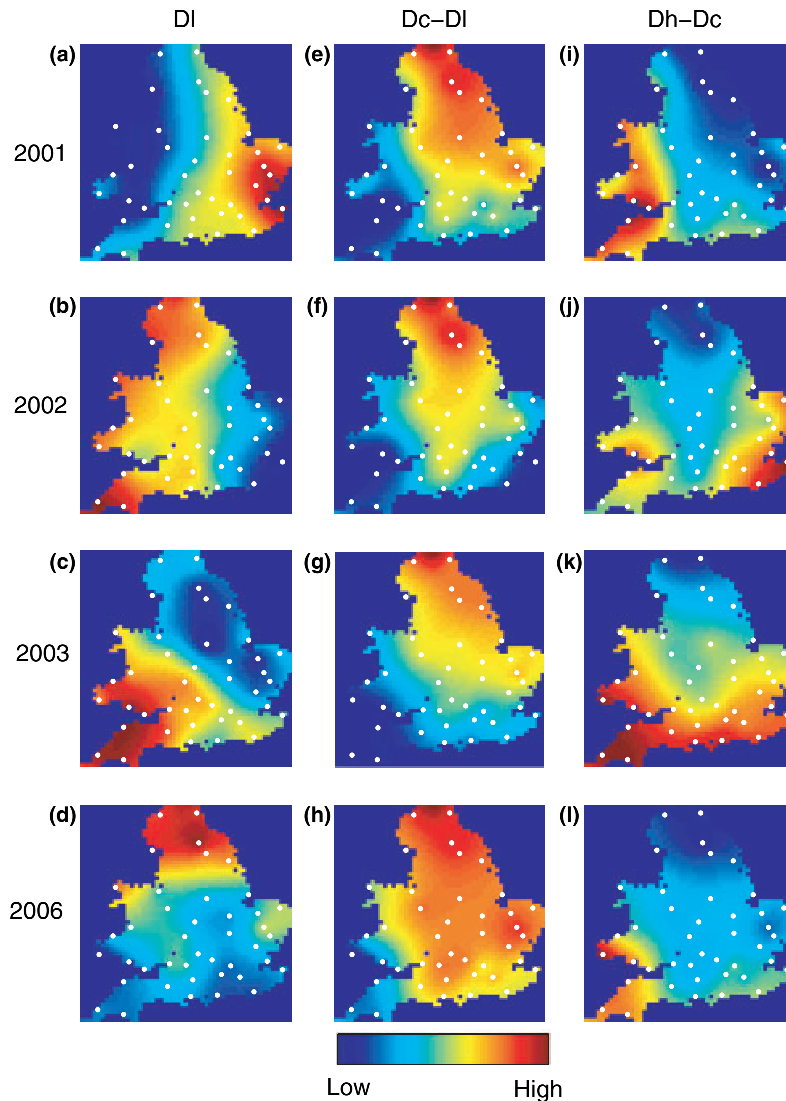

Figure 4 Maps of number of days between stages in development of phoma stem canker on winter oilseed rape as predicted by the model of Evans *et al.* (2008) from weather data for 2001, 2002, 2003 and 2006. These stages are *Dl* (the number of days after 15 July at which 10% of plants in crop are affected by phoma leaf spotting, a–d); *Dc–Dl* (the number of days after *Dl* when 10% of plants in crop are affected by phoma stem lesions, e–h) and *Dh–Dc* (the number of days between *Dc* and harvest, i–l). The locations of weather station sites are marked.

south of England in 2001, 2002, 2003 and 2006 (Fig. 3). The maps showing predicted values of key stages in the development of stem canker (Fig. 4) suggest which weather parameters had influence on this spatial pattern of variation. There was no obvious consistent spatial pattern in the predicted number of days after 15 July until the date of the appearance of leaf spotting in autumn (Fig. 4). However, the time taken between dates of onset of leaf spotting and onset of stem canker in spring was greater in the north than south of England in all four years (Fig. 4). Thus the time between onset of stem canker and harvest (15 July) was shortest in the north (Fig. 4), which explains why stem canker severity at harvest was predicted to be less in the north than in the south (Fig. 3i-l). These results suggest that the key weather parameter causing the north-south difference in predicted severity of stem canker was temperature; 1971–2000 average mean daily temperature is greater in southern (13·6°C) than in northern (12·1°C) England (UK Meteorological Office). This north-south difference in temperature influenced the rate at which thermal time (accumulated temperature in °C-days) was accumulated after onset of leaf spotting in autumn.

Discussion

This combination of geostatistical and molecular techniques provides evidence for the first time that there is regional variation in the proportions of *L. maculans* and *L. biglobosa* in basal stem cankers on winter oilseed rape

crops in England, with greater proportions of *L. maculans* in the south and *L. biglobosa* in the north in 2001–2006. Results suggest that this regional variation may have been associated with differences in temperature between the north and the south during autumn, winter and spring. The comparison with maps of stages in the development of phoma stem canker epidemics generated using the weather-based model of Evans *et al.* (2008) suggest that warmer temperatures in the south during the period between onset of leaf spotting in autumn and harvest in summer may favour *L. maculans,* with cooler temperatures in the north favouring *L. biglobosa*. Regional variation in the proportions of these *Leptosphaeria* species could also be affected by agronomical differences between the north and south. On winter oilseed rape in northern England, there is a greater incidence of light leaf spot (caused by *Pyrenopeziza brassicae*) than in southern England (http://www.cropmonitor.co.uk). Therefore, choice of fungicide application regime differs between the north and south; use of different azole fungicides might affect the proportion of *L. maculans* and *L. biglobosa* in populations (Eckert, 2005). In addition, there may be regional variation in cultivation methods and other agronomic practices that affect phoma stem canker epidemics and *Leptosphaeria* populations (Fitt *et al.*, 2006b; Schneider *et al.*, 2006).

The results, for both visual observation on characteristics of plate cultures and qPCR, suggest that the proportion of *Leptosphaeria* species on brassicas in England has changed over the last 20 years and support the hypothesis that *L. maculans* has been increasing in abundance since the reintroduction of oilseed rape in the 1970s. The current predominance of *L. maculans* in basal stem cankers on winter oilseed rape in England suggests that there has been a substantial increase in the abundance of *L. maculans* over the two decades since the surveys done by Humpherson-Jones (1986) in the 1980s, in which only 41% of isolates were identified as *L. maculans*. This increase suggests the gradual supplanting of *L. biglobosa* in England by the more invasive *L. maculans*. This explanation is supported by the evidence that *L. maculans* has been expanding into other areas previously occupied by *L. biglobosa,* such as in Canada and Poland (Karolewski *et al.*, 2002; Fitt *et al.*, 2008). Thus, these results provide further evidence that *L. maculans* should be considered as a global invasive species (http://www.issg.org/database), which is threatening production of oilseed rape in countries such as China (Fitt *et al.*, 2008).

The regional variation in the abundance of *L. maculans* and *L. biglobosa* in basal cankers may contribute to the regional variation in severity of phoma stem canker epidemics (http://www.cropmonitor.co.uk/wosr/surveys/lls-riskMap.cfm) since *L. maculans* is generally more damaging than *L. biglobosa* (West *et al.*, 2001; Fitt *et al.*, 2006a). As basal stem cankers derive from infections of lower leaves in the autumn and upper stem lesions derive from infection of leaves formed later, the greater amounts of *L. maculans* DNA in stem cankers collected in southern England suggest that *L. maculans* ascospores are released earlier in the season than those of *L. biglobosa*. This is consistent with the observation in southern England of a predominance of typical *L. maculans* leaf lesions in the autumn and characteristic *L. biglobosa* leaf lesions in the spring (Toscano-Underwood *et al.*, 2003). Since *L. maculans* and *L. biglobosa* differ in their sensitivity to different azole fungicides (Eckert, 2005), the regional variation in the proportions of the two species in England can be used to improve recommendations on use of fungicides to control phoma stem canker. Furthermore, since it is known that the resistance response of oilseed rape cultivars differs between *L. maculans* and *L. biglobosa* (Somda *et al.,* 1998; Fitt *et al.,* 2006a), these differences in populations between north and south should influence choice of winter oilseed rape cultivars grown in each region.

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Appendix

The abundance of *L. maculans* and *L. biglobosa* DNA in stem cankers and the outputs from the weather model of Evans *et al.* (2008) were mapped across England and Wales by model-based geostatistical methods (Diggle & Ribeiro, 2007). Such methods assume that the observations of the variable constitute a realization of a random function. The random function is represented by a parametric model such as a linear mixed model (LMM). The LMM divides the variation of the random function between three components. The first component is called the fixed effects and describes the expected value of the variable at each site. In general the fixed effects may vary according to auxiliary variables such as altitude or spatial coordinates. However, in this paper it is assumed that the fixed effects are constant across England and Wales. The second component describes the correlation between observations of the variable made at different sites and is referred to as the random effects. It is this component that causes pattern to be seen in the maps of the variable since there is a larger correlation between neighbouring observations than distant ones. The final component of variation accounts for the difference between observations made at the same site. This process is assumed to be both independent and random. The LMM may be expressed as

$$\mathbf{z} = \mathbf{M}\beta + \mathbf{Z}\mathbf{u} + \varepsilon \quad (4)$$

where $\mathbf{z}$ is the observed data vector of length n, $\mathbf{M}$ is an $n \times p$ matrix which contains the values of p auxiliary variables (or fixed effects) that correspond to each of the n observations, $\boldsymbol{\beta}$ is a vector of length p containing the coefficients of the linear relationship between $\mathbf{M}$ and $\mathbf{z}$, $\mathbf{u}$ is a length n_s vector containing the random effect for each

of the n_s sites and $\boldsymbol{\varepsilon}$ is a length n random process which accounts for variation between observations made at the same site. The matrix $\mathbf{Z}$ is an $n \times n_s$ design matrix which relates each of the n observations to one of the n_s sites. If observation i was made at site j then row i of $\mathbf{Z}$ contains a one in column j and zeros in the other columns. For the outputs from the weather model, since there is only one observation per site each year $\boldsymbol{\varepsilon}$ is removed from Equation (4) and $\mathbf{Z}$ is the identity matrix. Since it is assumed that the expectation of the random function is constant throughout England and Wales, then $p = 1$ and $\mathbf{M}$ consists of a length n column of ones.

It is assumed that $\boldsymbol{\varepsilon}$ is a realization of an independent Gaussian random process with mean zero and variance σ^2 whereas $\mathbf{u}$ is a realization of a spatially correlated Gaussian random with mean zero and covariance matrix $\mathbf{C}$. The covariance between two sites (i, j) decreases as the distance separating the sites (h_{ij}) increases. The decay in the covariance with increasing h_{ij} is described by an exponential function (Webster & Oliver, 2001) such that the covariance between sites i and j is written as

$$\mathbf{C}_{ij} = \begin{cases} c_0 + c_1 & \text{if } h_{ij} = 0 \\ c_1 \exp\left(\frac{-h_{ij}}{r}\right) & \text{if } h_{ij} > 0 \end{cases} \quad (5)$$

The parameters of this model are the nugget variance c_0, the partial sill variance c_1 and a distance parameter r. The nugget variance describes spatial variation of the variable over distances less than the shortest distance between observation sites. The partial sill variance is the portion of variation that is spatially correlated and the distance parameter describes how quickly the correlation between the variable at two sites decays with the distance separating these sites. This correlation decays by 95% over a distance of three times the spatial parameter.

Thus the LMM representation of the spatial variation of amounts of *L. maculans* and *L. biglobosa* has $4 + p$ parameters namely c_0, c_1, r, σ^2 and the p elements of $\boldsymbol{\beta}$ whereas the LMMs for the outputs of the weather model have $3 + p$ parameters, namely c_0, c_1, r and the p elements of $\boldsymbol{\beta}$. These parameters were fitted to the data by residual maximum likelihood (REML) (Pardo-Igũzquiza, 1997). This method uses a numerical optimization technique to find the values of the parameters which maximize a measure of fit known as the residual log-likelihood function. Once the parameters have been fitted, the LMM may be substituted into the best linear unbiased predictor (BLUP) to map the expected value of $\mathbf{z}$ at sites where it has not been observed (Lark *et al.*, 2006). Highly skewed data are not consistent with the Gaussian assumption within the LMM and therefore if the data have skew greater than one then they are log-transformed prior to analysis.

These techniques are used to produce maps of the abundance of *L. maculans* and *L. biglobosa* DNA, the predicted severity of the phoma stem canker epidemics, the date of leaf spotting, the number of days between leaf spotting and stem canker appearance and the number of days between stem canker appearance and harvest. The $\boldsymbol{\varepsilon}$ term does not affect the maps because it has an expectation of zero everywhere. Therefore the spatial variation within the maps is due wholly to the random effects. Thus the magnitude of the variations in each map is indicative of the proportion of variation that is spatially correlated rather than the total variation of the variable. Therefore, it is the pattern of variation that is critical in these maps rather than its magnitude, which is not displayed. When $c_1 = 0$, the LMM has no spatial correlation and the predicted map is constant across England and Wales.