Phoma stem canker disease on oilseed rape (*Brassica napus*) in China is caused by *Leptosphaeria biglobosa* 'brassicae'

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Abstract Phoma stem canker of oilseed rape (*Brassica napus*) is a globally important disease that is caused by the sibling ascomycete species *Leptosphaeria maculans* and *L. biglobosa***.** Sixty fungal isolates obtained from oilseed rape stems with phoma stem canker disease symptoms collected from four provinces in China in 1999, 2005 and 2006 were all identified as *Leptosphaeria biglobosa*, not *L. maculans*, by PCR diagnostics based on species-specific primers. There were no differences in cultural characteristics (e.g. pigmentation and *in vitro* growth) between these *L. biglobosa* isolates from China and those of 37 proven *L. biglobosa* isolates from Europe or Canada. In studies using amplified fragment length polymorphism (AFLP) markers, Chinese *L. biglobosa* populations were genetically more similar to European *L. biglobosa* populations than to the more diverse Canadian *L. biglobosa* populations. Sequencing of gene fragments of β-tubulin, actin and the internal transcribed spacer (ITS) region of rDNA from *L. biglobosa* isolates from China, Europe, Australia and Canada showed a closer taxonomic similarity of Chinese *L. biglobosa* to the European *L. biglobosa* 'brassicae' than to Canadian *L. biglobosa* 'canadensis' or to the

Australian *L. biglobosa* ‘occiaustralensis’ or ‘australensis’ subclades. These results suggest that the Chinese *L. biglobosa* population in this study is in the same subclade as European *L. biglobosa* ‘brassicae’ populations.

Keywords AFLP · Blackleg · *Brassica napus* · Genetic structure · ITS · *Leptosphaeria biglobosa* subclades · Phylogeny

Introduction

Phoma stem canker (blackleg) is a disease of worldwide importance on oilseed rape (*Brassica napus* L. var. *oleifera*), causing annual losses of more than £1000M globally at a price of £370 t^{-1} (Howlett 2004; Fitt et al. 2008). The disease is caused by two related fungal species, *Leptosphaeria maculans* (Desm.) Ces. & de Not. (anamorph = *Plenodomus lingam*) and *L. biglobosa* Shoemaker & Brun (anamorph = *P. biglobosus*) (Williams and Fitt 1999; Rouxel and Balesdent 2005). *L. maculans* is the more aggressive pathogen and usually causes severe epidemics and substantial yield losses (Fitt et al. 2006a; 2006b), associated with damaging stem base cankers (Zhou et al. 1999). However, even though the less damaging lesions caused by *L. biglobosa* usually occur higher up the stem (West et al. 2001), co-localization within the same niche sometimes occurs (West et al. 2002). Nevertheless, the two species occupy slightly different ecological niches, which enables them to coexist on oilseed rape crops in Europe (Fitt et al. 2006c), North America (Dilmaghani et al. 2009) and Australia (Van de Wouw et al. 2008; Vincenot et al. 2008). Both pathogens are spread by air-borne ascospores (Dawidziuk et al. 2012; Kaczmarek et al. 2012), from which they infect the leaves (Biddulph et al. 1999; Toscano-Underwood et al. 2001) and then spread along the leaf petioles to the stems (West et al. 2002). The global importance of phoma stem canker has increased over the last 20 years, with long-distance (e.g. inter-continental) spread of *L. maculans* on infected seed or debris and short-distance spread by air-borne ascospores (Fitt et al. 2008; Zhang et al. 2014).

China is a major oilseed rape producing country, providing *ca*. 30% of the total world yield. Since the late 1990s, the annual area of oilseed rape harvested in China has been greater than 8 million ha (http://www.fao.org/), with winter oilseed rape grown in the Yangtze River basin in central China (*ca*. 7M ha) and spring oilseed rape in northern China (*ca*. 1M ha). However, phoma stem canker has not caused serious economic losses in China (West et al. 2000; Fitt et al. 2006a; Li et al. 2013). In 1999, samples of plants with stem canker symptoms were collected in China from a few winter oilseed rape crops; only the less aggressive *L. biglobosa* (then known as B-group *L. maculans*) was isolated (West et al. 2000). When *L. maculans* has spread into areas where previously only *L. biglobosa* was present, such as Canada (1975-1998) and Poland (1994-2007) (Fitt et al. 2008; Zhang et al. 2014), the severity of phoma stem canker epidemics has increased greatly (Fitt et al. 2006a). There is thus good evidence that *L. maculans* should be considered as a global invasive species. Chinese oilseed rape cultivars are extremely susceptible to *L. maculans* (Li et al. 2008); if it becomes established in China, severe epidemics are likely to occur, with hardship for the subsistence farmers who grow the crop (Fitt et al. 2008). Therefore, it is important to determine the components of *Leptosphaeria* populations from a wider range of oilseed rape crops in China.

The global spread of *L. maculans* into areas where only *L. biglobosa* was previously present suggests that *L. maculans* may be ‘younger’ than *L. biglobosa,* in evolutionary terms. Further support for this conclusion is provided by genetic evidence, since *L. biglobosa* is genetically more diverse than *L. maculans* (Gall et al. 1995; Purwantara et al. 2000; Mendes-Pereira et al. 2003; Voigt et al. 2005). Mendes-Pereira et al. (2003) studied the phylogeny of the *L. maculans–L. biglobosa* species complex and other related *Leptosphaeria* species, using parsimony analysis of the sequences of the entire ribosomal internal transcribed spacer (ITS) region, including the 5.8S rDNA. *L. biglobosa* isolates were clustered into five subclades, i.e. *L. biglobosa* 'brassicae' (from various *Brassica* species, mostly in Europe), *L. biglobosa* 'canadensis' (mostly found in central Canada), *L. biglobosa* 'thlaspii' (from *Thlaspi arvense*), *L. biglobosa* 'erysimii' (from *Erysimum* sp.) and *L. biglobosa* 'australensis' (from Australia). In Australia, a further subclade, *L. biglobosa* 'occiaustralensis,' has now been identified (Vincenot et al. 2008) and *L. biglobosa* 'canadensis' has now been found (Van de Wouw et al. 2008). Whereas there were mixed populations of different *L. biglobosa* subclades in some countries (e.g. Australia), populations in Europe were exclusively *L. biglobosa* 'brassicae' and those in Canada were exclusively *L. biglobosa* 'canadensis'. From an evolutionary point of view, *L. biglobosa* may have evolved earlier than *L. maculans* from a common ancestor (Gudelj et al. 2004; Rouxel and Balesdent 2005).

Whilst two Chinese isolates from Guizhou province were classified as *L. biglobosa* 'brassicae' (Mendes-Pereira et al. 2003), it is not clear how representative they are of populations of *L. biglobosa* in China. Furthermore, it is unclear how genetically diverse Chinese populations are in comparison to *L. biglobosa* populations from Europe or Canada. Although Zhang et al. (2014) did a survey of *Leptosphaeria* species infecting Chinese *B. napus* and confirmed the presence of only *L. biglobosa*, neither the genetic structure nor subclade of this species was determined. This paper reports work to confirm whether phoma stem canker in China is caused solely by *L. biglobosa* and to use banding patterns of Amplified Fragment Length Polymorphism (AFLP) markers to study the genetic diversity of Chinese *L. biglobosa* isolates and their relatedness to *L. biglobosa* isolates from different parts of the world. While this method has been employed for evaluation of geographical differentiation of *Leptosphaeria* spp. within Australia (Barrins et al. 2004) and between Australian populations and those from Europe and North America (Pongram et al. 1999; Purwantara et al. 2000), our study presents the first use of AFLP for investigation of genetic diversity in *L. biglobosa* populations from China, Europe and Canada. Furthermore, this paper reports studies to identify the subclade of a sub-set of these Chinese phoma stem canker *L. biglobosa* isolates.

Materials and methods

Fungal sampling and isolation

Sixty isolates of *Leptosphaeria* species were obtained from four provinces of China. Oilseed rape stems with symptoms similar to those of phoma stem canker disease, generally on upper stems, were collected from three provinces in China in different years shortly before (or after) oilseed rape crops were harvested (samples were collected in May 2005 from winter oilseed rape crops in Wuhan, Hubei province, in September 2005 from spring oilseed rape crops in Hailar, Inner Mongolia and in May 2006 from winter oilseed rape crops in Hefei, Anhui province). Stem bases were always inspected for symptoms of canker but these were absent. Details of sampling procedures used in Chinese surveys to identify the pathogen causing phoma stem canker disease in oilseed rape crops are provided by Zhang et al. (2014). Such stems had previously been collected from Guiyang, Guizhou province and Hefei in 1999

(West et al. 2000) (Fig. 1). Diseased stems were first classified as affected by phoma stem canker disease by observation of the visible tissue discolouration and the presence of pycnidia. Pieces of the necrotic stem lesions (*ca*. 0.2 × 0.2 cm) were excised from these stems.

To isolate the causal pathogen(s), these pieces of stem were surface sterilised in 70% (v/v in water) ethyl alcohol for 2-3 seconds, and then immersed in 10% (v/v) sodium hypochlorite solution containing 8% available chlorine (Fisher Scientific, UK #S/5040/21) for 2 min, followed by a thorough rinse with sterile distilled water. Surface sterilised pieces were placed on water agar (WA) plates (9 cm diameter Petri dish, five samples per plate) and incubated at 15°C in darkness for 5-7 days to allow fungal colonies to grow from them. Then the hyphal tips were excised from these colonies and transferred to PDA$^+$ medium [potato dextrose agar (Oxoid Ltd., Basingstoke, Hampshire, UK) containing the antibiotics streptomycin (100 mg l^{-1}) and penicillin (50 mg l^{-1})] for 7 days at 15°C (five colonies per dish). The transfer was done using a sterilised Pasteur pipette (Fisherbrand, Fisher Scientific, UK) by cutting a plug (*ca*. 1 mm in diameter) containing only a few hyphal tips from the edge of each actively growing fungal colony. The colonies were then subcultured onto PDA medium and incubated for 3-6 days at 20°C (five colonies per dish). Finally, a pure culture of each isolate was subcultured onto PDA medium (one colony per dish) and incubated for 3-4 weeks at 15°C.

Preliminary identification of isolates was based on morphological characters of each colony on PDA medium (Williams and Fitt 1999). Colonies of *L. biglobosa* can be distinguished from those of *L. maculans* by size and pigment production (larger colonies, yellow to brown pigment, *L. biglobosa*; relatively smaller size, no brown/yellowish pigment production, *L. maculans*). No *Leptosphaeria* cultures were discarded.

A collection of 39 *L. biglobosa* ‘brassicae’ isolates from the UK (Rothamsted Research fungal culture collection), 35 isolates from Poland (provided by Malgorzata Jedryczka, Institute of Plant Genetics, Poznan, Poland), 34 isolates from France (provided by Hortense Brun, Institut National de la Recherche Agronimique, Le Rheu, France, and Marie-Hélène Balesdent, INRA, Thiverval-Grignon, France), 9 isolates from Austria (Rothamsted Research fungal culture collection) and 10 *L. biglobosa* ‘canadensis’ isolates from Canada (provided by Randy Kutcher, Crop Development Centre, Saskatoon, Canada) was also assembled for this work (Supplementary Table 1). All these isolates were confirmed as *L. biglobosa* by PCR using *L. biglobosa*-specific primers (Mahuku et al. 1996, specific to all *L. biglobosa sensu lato*; Liu et al. 2006, specific to *L. biglobosa* ‘brassicae’ *sensu stricto*) and maintained on PDA medium at 4ºC for short-term storage. All isolates, together with Chinese *L. biglobosa* isolates (confirmed as *L. biglobosa* by PCR with either or both pairs of diagnostic primers), were grown on PDA medium and the cultural characteristics of a subset were assessed.

DNA extraction

All the 60 isolates obtained from China and the 64 reference isolates (54 from Europe and 10 from Canada) were transferred to PDB (potato dextrose broth, Sigma-Aldrich® Inc., USA) liquid medium (six pieces of 2.5 × 2.5 mm PDA agar plugs with mycelia into 10 ml PDB medium) and maintained on an orbital shaker (23ºC, 180 rpm) for 10 days. Mycelia were harvested by centrifugation at 14,000 ***g*** for 5 min at 20ºC frozen and freeze-dried for at least 24 h. Genomic DNA from each isolate was extracted using a modified version of the method of Graham et al. (1994). Freeze-dried mycelial samples (150 µl fungal powder and 50 µl sterilised sand) were placed in 1.5 ml microfuge tubes. To each was added 600 µl CTAB (hexadecyltrimethyl ammonium bromide = cetyltriammonium bromide; Sigma, UK) lysis buffer with 2% β-mercapto-ethanol and the content was homogenised with a plastic pestle. The samples were vortexed and incubated at 70ºC for 30 min, followed by centrifugation (10 min at 15,000 ***g***) in a microcentrifuge. The supernatant was collected into a fresh tube and extracted against an equal volume of a chloroform : isoamyl alcohol (24 : 1) mixture by vortexing for 30 sec before centrifugation at 14,000 ***g*** for 10 min. The upper, aqueous phase was collected and a 0.1 volume of 3 M sodium acetate (pH 5.0) and 2 volumes of ice-cold absolute ethanol were added. Samples were mixed by gentle inversion and placed at –20ºC for 1 h to precipitate the genomic DNA. Pellets were collected by centrifuging the samples at 14,000 ***g*** for 10 min and discarding the supernatant. The precipitate was washed twice with ice-cold 70% ethanol. Pellets were dried at 37ºC and then dissolved in 200 µl 1 mM TE (tris-ethylenediamine tetra acetic acid, pH 7.5; Sigma, UK) buffer. DNA extract was stored at –20ºC.

PCR screening

Species identification was done with PCR using *L. maculans* and *L. biglobosa* specific primers (Mahuku et al. 1996; Liu et al. 2006; Table 1). The PCR reaction was done in a GeneAmp® 2700 PCR thermal cycler (Applied Biosystems Inc., Foster City, USA). Each 15 µl PCR reaction solution was made up of 7.5 µl RedTaq™ ReadyMix™ (2x concentrate) PCR reaction mix with $MgCl_2$ (Sigma®), 0.3 µl of each primer (10 pmol µl^{-1}), 5.9 µl sterile distilled water and 1 µl fungal genomic DNA. The uniplex PCR was programmed for initial denaturation at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 30 sec, annealing at 55-68°C, depending on the primer pair used, for 30 sec and elongation at 72°C for 1 min, followed by elongation at 72°C for 10 min and kept at 4°C. PCR products were electrophoresed on 1% agarose gel in 1 × TAE (1 M tris-ethylenediamine tetra-acetic acid plus ethylenediamine tetra-acetic, pH 7.2; Sigma, UK) buffer at 90 volts for 1 h and visualized under UV light. Only DNA from isolates confirmed by PCR as *L. biglobosa* was used in the population genetic diversity analysis.

Growth in culture of *L. biglobosa* from China, Europe and Canada

Sixty *L. biglobosa* isolates from China and, from the global collection, 32 isolates from the UK, 33 isolates from Poland, 33 isolates from France and 8 isolates from Canada were compared for cultural characteristics and growth. Each *L. biglobosa* isolate was sub-cultured on PDA medium by transferring a mycelial ‘plug’ from the colony edge with a Pasteur pipette and incubating at 15^{o}C in darkness for 10 days. Colony size (diameter of the colony) and pigmentation were assessed.

Pathogenicity assay

Three Chinese isolates that had been confirmed as *L. biglobosa* by PCR (CN60, CN22 and CN13) were tested for their pathogenicity to Chinese winter oilseed rape to satisfy Koch’s postulates in relation to phoma leaf spot lesions. In the first experiment in which isolate CN60 was used, seeds of Chinese *Brassica napus* cvs Deyou 829, Huiyou 50, Shifeng 1, Xingxuan 2 and Zhongshuang 10 were sown in a glasshouse at 20°C /16°C (16 h day/ 8 h night). There were five plants of each cultivar. To test for development of phoma leaf spot symptoms, seedlings with two to three true leaves were chosen for inoculation. A *L. biglobosa* conidial suspension was prepared by the method of Ansan-Melayah et al. (1995) and adjusted to 10^7 spores ml^{-1}. When plants were 4 weeks old, one leaf from each plant was wounded with a needle in six places and then a 10 μl drop of conidial suspension was placed on each wound. The inoculated plants were each covered with a polyethylene bag to maintain leaf wetness (*ca*. 100% relative humidity) for 48 h before removing it. Phoma leaf spot lesions were assessed 14 days post inoculation (dpi). In the second experiment in which all three isolates were used, Chinese winter oilseed rape cultivars Deyou 829 and Shifeng 1 were sown in a growth cabinet (20°C /16°C; 16 h day/ 8 h night). There were 10 plants of each cultivar. When plants were 4 weeks old, they were inoculated with *L. biglobosa* conidia at six wounded sites on one leaf from each plant (two inoculation points per isolate) with a 10 μl droplet of *L. biglobosa* conidial suspension (10^7 spores ml^{-1}). Leaf spot lesions were assessed 15 dpi. In both experiments, phoma leaf spot symptoms were photographed. In the second experiment, after assessment, lesions were excised from the leaves and the pathogen was re-isolated from them, and identified by cultural characteristics and PCR.

AFLP analysis

Genetic diversity of *L. biglobosa* populations was analysed by DNA fingerprinting, using amplified fragment length polymorphism (AFLP) markers, of a selection of 97 isolates from *L. biglobosa* populations in China, the UK, Poland, France, Austria and

Canada (Supplementary Table 1). These 97 isolates included Chinese isolates Gui2b2 and Gui2b2 (CN59 and CN60 in this study) and UK isolate BW70-11 (UK28) that had all been previously identified as *L. biglobosa* 'brassicae' (Mendes-Pereira et al. 2003; Liu et al. 2006). Samples of isolate DNA were used for the AFLP analysis after confirmation as *L. biglobosa* by PCR. DNA concentration was determined using a NanoDrop® spectrophotometer ND-1000 (Labtech International Ltd., East Sussex, UK). AFLP was done using an AFLP® Microorganism Primer Kit from Invitrogen™, following the instructions in the manual with slight modifications. Fungal genomic DNA (125 ng in 9 µl aqueous solution) was digested at 37°C with restriction enzymes MseI and EcoRI for 2 h and then complementary double stranded adaptors were ligated at 20-22°C to the digested fragment ends with T4 DNA ligase. This mixture was pre-amplified with MseI and EcoRI specific primers [0.5 µl each of primer E-0 and primer M-0 provided by the Kit (Table 1)]. Pre-amplification was done over 30 cycles of denaturation at 94°C for 30 sec, annealing at 46°C for 30 sec and elongation at 72°C for 1 min. PCR products of each reaction were diluted 10 times in TE buffer.

The selective AFLP amplification was done with 5 µl of the resulting diluted PCR samples in 20 µl (final volume) using primer E-AC and primer M-G in a touch-down PCR procedure. Cycling conditions were as follows. Firstly, one cycle was run at 94°C for 30 sec, 58°C for 30 sec and 72°C for 1 min. Secondly, 13 cycles were run, with the annealing temperature decreased by 0.7°C in each successive cycle. Thirdly, 28 additional cycles of amplification with an annealing temperature of 49°C were done. Finally, products were elongated at 72°C for 2 min and kept at 4°C. AFLP amplicons were denatured at 95°C for 5 min, and then placed on ice immediately.

The amplicons were electrophoresed on polyacrylamide gel. Sequi-Gen® GT Sequencing Cell plates (Bio-Rad Laboratories Ltd, Herts, UK) were assembled and the gel was poured and allowed to set horizontally for 1 h. After warming at 80 W for 1 h, samples (AFLP amplicons) were loaded and separated on the denaturing polyacrylamide gel in 1 × TBE buffer for 1.75 h at 50 W.

The gel was stained with silver nitrate before reading. At the end of each run, plates were separated and the gel which bound to the larger plate was fixed by shaking in 10% acetic acid for 20-30 min. The gel was then rinsed in cold ultra pure water (3 times) and shaken in staining solution (2 g $AgNO_3$ and 3 ml 33% formaldehyde in 2 l ultra pure water) for 30 min. At the end of the staining phase, the gel was rinsed (5-10 sec) in cold ultra pure water and developed in a solution of 60 g sodium carbonate in 2 l ultra pure water plus 400 µl sodium thiosulphate and 3 ml 33% formaldehyde until the bands became visible. The reaction was stopped by adding the fixing solution. Gels were rinsed three times with ultra pure water and left to dry at room temperature overnight. Gel images were scanned using an HP ScanJet 5470c scanner (Hewlett-Packard Development Company, L.P.).

Sequencing

Shake cultures of 15 isolates of *L. biglobosa*, including six from China (CN01, CN21, CN57, CN58; and CN59 and CN60, previously identified as *L. biglobosa* ‘brassicae’), four from Canada (CA02, CA03, CA07 and CA08), and five from Europe (Austrian AT01 & AT03; French FR08; British UK28 (previously identified as *L. biglobosa* ‘brassicae’) and Polish PL30) were grown in PDB (potato dextrose broth; 10 d at 180 rpm and 23^{o}C) medium. A culture of the Canadian *L. maculans* isolate (LEROY, IBCN80) was included in the study to serve as an outlier. DNA samples were subjected to polymerase chain reaction for the amplification of fragments from genomic regions corresponding to internal transcribed spacer (ITS) of rDNA (using primers PN3 & PN10, $T_a = 54^oC$, Table 1; Mendes-Pereira et al. 2003), actin (primers $T_a = 56^oC$, Table 1; Van de Wouw et al. 2008) or β-tubulin (primers $T_a = 58^oC$, Table 1; Vincenot et al. 2008). Amplicons from each of the isolates were excised from 1.5% agarose after electrophoresis in 1 x TAE buffer, purified and sent to Eurofin MWG Operon (Ebersberg, Germany) for bi-directional sequencing using these primers.

Data analyses

AFLP gels were scored manually for the presence or absence of bands to create a binary matrix. Data were collected for each isolate, band by band, with ‘presence’ or ‘absence’ recorded as 1 or 0, respectively. The binary data were analysed (Cluster Analysis) with a Multivariate Analysis using GenStat® (edition 9) software to draw a hierarchical tree illustrating the relatedness of the different groups of isolates (Payne et al. 2011). These band-based AFLP marker data were further analysed by principal coordinate analysis using a freely downloaded programme: PCO by Anderson (2003). The analysis was done on the basis of Bray-Curtis dissimilarities calculated on a binary matrix (i.e. 1 for the marker presence and 0 for the marker absence) of 97 isolates by 80 polymorphic bands. The first two principal coordinate axes were used to contrast and compare groups of isolates from different countries/ regions. Then the individual isolate estimate of the first principal coordinate axis was used to test for significant differences between isolates from China and those from other countries/regions. In addition, binary data from these AFLP gels were grouped on the basis of geographical origin and assessed with the POPGENE software (Yeh et al. 1997) for the evaluation of population differentiation and Nei’s genetic diversity (Nei

1972; 1987). The mean values for colony diameters measured on PDA plates were compared statistically using Analysis of Variance (ANOVA; GenStat® (edition 9) software; Payne et al. 2011). Nucleotide sequences of these gene fragments from the 16 *Leptosphaeria* isolates and those for three other isolates that had been published previously and obtained from the NCBI database (PHW 1268 (AJ550870.1, AY749001.1, AY748953.1), *L. biglobosa* 'australensis'; UWA A21-8 (AM410082.1, AM410084.1, AM410083.1), *L. biglobosa* 'occiaustralensis'; IBCN 82 (AJ550866.1, AY748958.1, AY749006.1), *L. biglobosa* 'canadensis') were aligned either separately or using a concatenated, multi-locus approach. Genetic distances were calculated using the Neighbor-Joining tree option (Saitou and Nei, 1987; Tamura et al. 2004) of the MEGA4 software (Tamura et al. 2007) and dendrogram stability was assessed using 100,000 bootstrap replications (Felsenstein, 1985). Nucleotide sequences of all gene fragments were lodged with the NCBI database and ascribed the following GenBank (Bankit) Accession numbers: for Actin sequences; Roth_LbCA02 (KJ574225), Roth_LbCA03 (KJ574229), Roth_LbCA07 (KJ574227), Roth_LbCA08 (KJ574228), Roth_LbCN 01 (KJ574238), Roth_LbCN21 (KJ574239), Roth_LbCN57 (KJ574234), Roth_LbCN58 (KJ574232), Roth_LbCN59 (KJ574230), Roth_LbCN60 (KJ574230), Roth_LbPL30 (KJ574224), Roth_LbUK28 (KJ574231), Roth_LbAT01 (KJ574235), Roth_LbAT03 (KL574237), Roth_LbFR08 (KJ574236), *L. biglobosa* PHW 1268 (KJ574224), *L. biglobosa* UWA A21-8 (KJ574226), *L. maculans* LEROY (KJ574242). For β–tubulin sequences; Roth_LbCA02 (KJ574253), Roth_LbCA03 (KJ574256), Roth_LbCA07 (KJ574257), Roth_LbCA08 (KJ574254), Roth_LbCN01 (KJ574243), Roth_LbCN21 (KJ574244), Roth_LbCN57 (KJ574255), Roth_LbCN58 (KJ574249), Roth_LbCN59 (KJ574248), Roth_LbCN60 (KJ574245), Roth_LbPL30 (KJ574251), Roth_LbUK28 (KJ574252), Roth_LbAT01 (KJ574246), Roth_LbAT03

(KJ574247), Roth_LbFR08 (KJ574250), *L. biglobosa* PHW 1268 (KJ574258), *L. biglobosa* UWA A21-8 (KJ574259), *L. maculans* LEROY (KJ574260). For ITS sequences; Roth_LbCA02 (KJ574220), Roth_LbCA03 (KJ574219), Roth_ LbCA07 (KJ574217), Roth_LbCA08 (KJ574218), Roth_LbCN01 (KJ574216), Roth_LbCN21 (KJ574208), Roth_LbCN57 (KJ574215), Roth_LbCN58 (KJ574209), Roth_LbCN59 (KJ574211), Roth_LbCN60 (KJ574213), Roth_LbPL30 (KJ574214), Roth_LbUK28 (KJ574210), Roth_LbAT01 (KJ574212), Roth_LbAT03 (KJ574206), Roth_LbFR08 (KJ574207), *L. biglobosa* PHW 1268 (KJ574222), *L. biglobosa* UWA A21-8 (KJ574221) and *L. maculans*, LEROY (KJ574223).

Results

Occurrence of only *L. biglobosa* on oilseed rape in China

Phoma stem canker symptoms were observed on both winter type (Anhui, Hubei, Guizhou provinces) and spring type (Inner Mongolia) oilseed rape (*B. napus*) crops in China in the period before or after harvest (May/June in the Yangtze River basin; September in north China). The canker symptoms on Chinese oilseed rape plants were mostly observed on parts of stems above ground level (Fig. 2a) rather than at the stem base where lesions are often observed in Europe (West et al. 2000; Fitt et al. 2006). Pycnidia were observed in the pale grey lesions. Discolouration of central stem pith tissues caused by the pathogen was also observed (Fig. 2b).

The phoma stem canker fungus was isolated from 113 diseased oilseed rape stems collected from three regions in China (namely Hailar in Inner Mongolia, Hefei in Anhui and Wuhan in Hubei). All cultures were morphologically similar to those of

L. biglobosa and no cultures similar to those of *L. maculans* were observed. These comprised 10 *L. biglobosa* isolates obtained from stem samples from Hefei (2006), 26 from Wuhan (2005) and 20 from Hailar (2005). In addition, two isolates from Hefei and two isolates from Guiyang, that had been collected in 1999 (West et al. 2000), were added. When cultures were tested by PCR using *L. maculans*- and *L. biglobosa*-specific primers, all of the 60 isolates were identified as *L. biglobosa* with both sets of primers. With the pair of primers of Mahuku et al. (1996), the *L. biglobosa* PCR amplicon was a single band at 230 bp while the *L. maculans* amplicon was a single 370 bp band. When the diagnostic primers of Liu et al. (2006) were used, however, the PCR amplicons were 444 bp (*L. biglobosa*) and 330 bp (*L. maculans*).

Growth in culture of *L. biglobosa* from China, Europe and Canada

L. biglobosa isolates collected from China demonstrated a wide range of pigmentation when cultured on PDA medium (Fig. 2d). Some Chinese *L. biglobosa* isolates produced a typical yellow-brown pigment on nutrient medium (e.g. CN53, CN57 and CN26), whilst others produced weak or even no pigmentation on PDA (e.g. CN49, CN55, CN52). A similar variation in pigment production was also observed in European *L. biglobosa* isolates, when a range of isolates was tested (Fig. 2e). Variation in pigmentation was also observed amongst Canadian *L. biglobosa* isolates; they were not as variable as Chinese or European *L. biglobosa* isolates (Fig. 2f), due perhaps, to the smaller sample size.

After incubating isolates on PDA medium at 15ºC in darkness for 10 days, the colony diameter of *L. biglobosa* isolates from China was 3.57 ± 0.55 cm (mean ± SD);

for the UK isolates it was 3.07 ± 0.42 cm, for the Polish isolates it was 3.36 ± 0.41 cm, for the French isolates it was 3.14 ± 0.49 cm and for Canadian isolates it was 3.25 ± 0.16 cm. Thus, there were no significant differences between Chinese *L. biglobosa* isolates and European or Canadian *L. biglobosa* isolates in *in vitro* colony diameter ($P > 0.05$).

Pathogenicity of the *L. biglobosa* isolates to Chinese oilseed rape cultivars

In the pathogenicity experiment, the Chinese *L. biglobosa* isolate (CN60) caused typical phoma leaf spot lesions on Chinese oilseed rape (cv. Deyou 829) seedling leaves (Fig. 2c). After 14 dpi, small, dark leaf spots without pycnidia surrounded by yellow margins were observed. These leaf symptoms were similar to those observed by Brun et al. (1997), Ansan-Melayah et al. (1997) and West et al. (2001) for European *L. biglobosa* ‘brassicae’ on European *B. napus* seedling leaves. In the second experiment, phoma leaf spot lesions were also observed after Chinese winter oilseed rape (cv. Deyou 829 or Shifeng 1) was inoculated with Chinese *L. biglobosa* isolates (CN60, CN13 or CN22). Both cultural and PCR identification confirmed that the isolates obtained from these leaf spots on inoculated Chinese winter oilseed rape were *L. biglobosa.*

Genetic relatedness of *L. biglobosa* isolates from China, Europe and Canada

In total, 97 *L. biglobosa* isolates from the collection were used for AFLP analysis. These comprised 33 isolates from China (12 from Hefei, 10 from Wuhan, 9 from Hailar and the 2 from Guiyang previously identified as *L. biglobosa* ‘brassicae’), 15 isolates from each of the UK, France and Poland, 9 isolates from Austria and 10 isolates from Canada (Supplementary Table 1). AFLP fingerprints of *L. biglobosa* isolates revealed that they were genetically diverse. A total of 86 amplified DNA fragments from the primer-combination were recorded. The size of the DNA fragments ranged from 200 bp to 5 kb. Amongst these amplified fragments, 80 showed polymorphism and were scored as discrete character data (as ‘1’ for presence and ‘0’ for absence). The combined character data matrix was analysed, assuming that co-migrating bands in an AFLP gel are homologous.

The 97 *L. biglobosa* isolates were clustered as two distinct groups, based on genetic relatedness from principal component analysis of the AFLP data (Fig. 3). One group comprised all the isolates from China and Europe and the other group consisted of all the Canadian isolates. The *L. biglobosa* isolates from China and Europe were separated from the Canadian isolates at a similarity level of *ca.* 25%. Except for one Canadian *L. biglobosa* isolate (CA08) with a similarity of 52%, the similarity between it and the other nine Canadian *L. biglobosa* isolates was more than 75%.

Chinese *L. biglobosa* isolates clustered together and similarities between them were over 90% (Fig. 3). No differences were apparent in AFLP data either within one region or between winter and spring oilseed rape producing regions of China. Some of the isolates showed 100% similarity to each other (e.g. CN08, CN10, CN12, CN14 and CN18), suggesting genotypic homology. This analysis suggested that Chinese isolates were closer to European *L. biglobosa* isolates than to Canadian *L. biglobosa* isolates. The similarities between Chinese isolates and most French, Austrian, UK and Polish isolates, for example, were at least 80%. Several isolates from Hefei (e.g. CN56 and CN53) clustered very closely with French isolates, with nearly 100% similarity. With only a few exceptions, *L. biglobosa* isolates from China and Europe showed considerable similarity (about 75%). For many European *L. biglobosa* isolates, isolates that were collected from each country grouped together regardless of when they were collected. Population differentiation of the AFLP data and POPGENE version 1.31 analyses of the 86 polymorphic bands generated a phenogram (Fig. 4) that confirmed the short genetic distance between Chinese *L. biglobosa* and *L. biglobosa* isolates from European countries. As shown in Fig. 4, there was a greater genetic distance between Chinese and Canadian isolates.

The principal coordinate analysis (PCA) showed that the first and second coordinate axes combined to account for 72.4% of the variation in the 97 isolates based on the 80 polymorphic AFLP markers (Fig. 5). The first principal coordinate axis explained 50.7% while the second coordinate axis accounted for 21.7% of the variation. It was again clear that isolates from Canada were distinctly different from isolates from both China and Europe (i.e. isolates combined from the UK, Poland, France and Austria). Since the first principal coordinate axis had the greatest discriminant power, an individual estimate of this axis was used to test differences between isolates from different countries/regions. As the isolate estimates of the first coordinate axis were not normally distributed across countries/regions, Kruskal-Wallis one-way analysis of variance on ranks was done to test the median variations between groups of isolates from different countries/regions. The results showed that the median (3.8) of first principal coordinate axis for isolates from China was significantly different from the median (-27.3) for isolates from Canada but was not different from the median (4.1) for isolates from Europe. The median values for isolates from European countries were also significantly different from the median for isolates from Canada.

Table 2 shows the genetic diversity indices from the 86 AFLP bands for isolates from China, Europe and Canada. Chinese isolates had genetic diversity and Shannon's Information Index values that were comparable to values for European (the UK, Poland, France and Austria) isolates; for these indices, Canadian *L. biglobosa* isolates had the greatest values (Table 2). Furthermore, Nm, the estimate of gene flow (Table 3) indicated both the infrequency and the unlikelihood of gene exchange between the Chinese *L. biglobosa* population and European *L. biglobosa* populations with which it shared the closest genetic similarity (Table 4) as judged by Nei's (1972) genetic identity and genetic distance.

Multilocus nucleotide sequencing and phylogeny of *L. biglobosa* to identify the subclade of Chinese *L. biglobosa* isolates

Phylogenetic analyses of the sequenced actin, β-tubulin and ITS gene fragments showed through clustering that *L. biglobosa* isolates from all four Chinese provinces were *L. biglobosa* 'brassicae' (Fig. 6). A comparison of the Clustal Omega (EMBL-EBI software) phylogram based on ITS sequences alone against a phylogram that was generated from a concatenation of the β-tubulin and actin sequences of the *L. biglobosa* isolates that were used in this study, confirmed the superiority of the 3-loci approach that was adopted. Chinese isolates Gui2b2 and Gui2b2 (CN59 and CN60 in this study) and UK isolate BW70-11 (UK28) had all been previously described as *L. biglobosa* 'brassicae' (Mendes-Pereira et al. 2003; Liu et al. 2006) and served as references for this infra-specific classification in this study. In addition, isolate IBCN 82 was also included (Mendes-Pereira et al, 2003) as a reference for the *L. biglobosa* 'canadensis' subclade. Similarity (and difference) matrices for the six isolates from the Hailar, Hefei, Wuhan and Guizhou regions of China that were sequenced showed >99.1% similarity across the entire 1451 bp gene fragments used in the combined analysis (Table 5)**.**

Discussion

These results suggest that phoma stem canker on oilseed rape in China is caused by the less aggressive *L. biglobosa* and that *L. maculans* is not currently present in China. In this study, only *L. biglobosa* was isolated from the 113 stems with phoma stem canker symptoms collected from Inner Mongolia, Anhui and Hubei provinces of China in 2005 and 2006 and no *L. maculans* was isolated from any of these provinces. Therefore, there is no evidence that the aggressive phoma stem canker pathogen *L. maculans* is present in different regions in China, including both winter (Anhui and Hubei) and spring (Inner Mongolia) oilseed rape producing regions. This is consistent with the isolation of only *L. biglobosa*, both from winter oilseed rape samples collected from Anhui and Guizhou provinces by West et al. (2000) and from samples taken from crops in 14 provinces in the period 2005-2012 (Zhang et al. 2014) and the observation that phoma stem canker does not generally cause serious yield losses in China (Li et al. 2013).

The AFLP results showed that the genetic diversity of *L. biglobosa* isolates collected in China for this study was comparable to that of isolates from European countries; Canadian isolates were the most genetically diverse (Table 2). The Chinese *L. biglobosa* isolates were collected from four different provinces separated in distance by more than 1000 km and from hosts comprising two different oilseed rape types, but were not less heterogeneous than those from the UK, France or Poland.

The low genetic diversity found in the Chinese *L. biglobosa* population from this study and close genetic relatedness to sub-populations in Europe conform to earlier suggestions that phoma stem canker may be a relatively new disease in China. *L. biglobosa* may originally have been introduced by a very small pathogen source, for example a few contaminated pieces of crop debris or seed (Chen et al. 2010), and then spread across China through transport of debris or seed from one region to another and by air-borne ascospores (Dawidziuk et al. 2012; Kaczmarek et al. 2012; Zhang et al. 2014) .

Analyses of the molecular data obtained reveal that Chinese *L. biglobosa* isolates had a genetic diversity that was comparable to those of the isolates from the UK, Poland and France. Isolates from Austria and, particularly, from Canada were genetically more diverse with the greatest scores for Nei's Genetic Diversity and Shannon's Information Index amongst the countries compared. It was equally instructive that the *L. biglobosa* isolates from Canada were the least phenotypically diverse in this study. The phylogenetic data from a concatenation of nucleotide sequences of the ITS, actin and β-tubulin gene fragments confirm that Chinese *L. biglobosa* isolates are more closely related to *L. biglobosa* isolates from Europe than to those from Canada or Australia. The multilocus approach to phylogeny that was used in this study has been adjudged (Crouch et al., 2006; Latunde-Dada and Lucas, 2007) to be more reliable for ascribing taxonomic similarity than dependence on one gene locus only. The current naming of clades in the phylogeny of *L. biglobosa* was initiated by the French group led by Thiery Rouxel (Mendes-Pereira et al. 2003) that established 'brassicae', 'canadensis', 'australensis', 'erysimii', 'thalspii' and later 'occiaustralensis' (Vincenot et al. 2008) as the six infraspecific taxa of this ascomycete fungal pathogen. The geographical delineations and specializations of isolates within these clades were clearly obvious and whilst ‘canadensis’, ‘australensis’ and ‘occiaustralensis’ are eponymous, the clade ‘brassicae’ comprised *L. biglobosa* isolates infecting *Brassica juncea*, *B. oleracea* and *B. napus* hosts from Europe. Our study confirms earlier reports (Mendes-Pereira et al. 2003; Vincenot et al. 2008) that placed two Chinese *L. biglobosa* isolates within the ‘brassicae’ subclade. Both the AFLP analysis and phylogeny results strongly indicate the close genetic similarity between *L. biglobosa* populations in China and Europe and suggest that isolates from both regions belong to the *L. biglobosa* ‘brassicae’ subclade instead of the ‘canadensis’ subclade (of Canadian and Australian isolates) or ‘australensis’ and ‘occiaustalensis’ subclades (of Australian isolates). It is possible that *L. biglobosa* may have been introduced into China from Europe; similar conclusions were made about the spread of *L. maculans* into North America (Pongam et al. 1999) and Mexico (Moreno-Rico et al. 2002). The conclusion that European *L. biglobosa* (‘brassicae’) is distinct from Canadian *L. biglobosa* (‘canadensis’) agrees with that of previous studies (Mendes-Pereira et al. 2003; Dilmaghani et al. 2009). While the small isolate population sizes used in the current study provide no evidence for a new, distinct or separate subclade for *L. biglobosa* from China, we propose the use of larger population sizes in future work.

The spread of the global invasive species *L. maculans* into Canada and Eastern Europe suggests that there is a risk that it may spread into China and cause severe phoma stem canker epidemics there. In Canada, before the 1970s only the less aggressive pathogen *L. biglobosa* was identified on oilseed rape crops and there were no severe stem canker epidemics (Gugel and Petrie 1992; Fitt et al. 2008). In 1975, *L. maculans* was first isolated from crops in the Saskatchewan province and by the early 1980s it had spread to Alberta and Manitoba provinces. Since then, *L. maculans* has spread and become endemic so that it causes serious yield losses in all the main oilseed rape producing regions in Canada (West et al. 2001). The social, natural and technical factors contributing to the rapid spread of *L. maculans* in Canada (Juska et al. 1997) also exist in China (large cropped area and social demand, high density of oilseed rape cropping in various geographic regions, inadequate knowledge about the disease amongst growers, etc.). There is therefore a serious risk that the pathogen *L. maculans* will spread into China and other Asian countries where only *L. biglobosa* is present, increasing the worldwide losses it causes. In the context of increasing severity of epidemics with climate change (Evans et al. 2008) and a world-wide shortage of vegetable oil for human consumption, there is therefore an urgent need for strategies to be developed to decrease the risk of *L. maculans* entry into China and to prevent the spread of the pathogen within China (Fitt et al. 2008; Zhang et al. 2014).

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Figure legends

Fig. 1 Sites in China from which oilseed rape stems with phoma stem canker were sampled to obtain isolates of *Leptosphaeria* species. Spring oilseed rape samples were from crops near Hailar, Inner Mongolia Province in 2005 (1). Winter oilseed rape samples were from crops near Hefei, Anhui in 2006 (2), near Wuhan, Hubei Province in 2005 (3) and near Guiyang, Guizhou Province in 1999 (4). Areas of winter oilseed rape (*ca*. 7M ha) production are indicated by solid diagonal lines; areas of spring oilseed rape (*ca*. 1M ha) production are indicated by dashed diagonal lines.

Fig. 2 Phoma stem canker caused by *Leptosphaeria biglobosa* in China. Diseased winter oilseed rape stems collected from Hefei, Anhui province in May 2005, showing phoma lesions at the site of leaf scars near the base of stems, with black pycnidia (P) observed in the lesions (a), and colonisation of the stem pith tissue (b). Phoma leaf spotting was observed on leaves of Chinese winter oilseed rape (cv. Deyou 829) point-inoculated with conidia of a Chinese *L. biglobosa* isolate (CN60) after wounding (14 days post inoculation at 20°C) (c). The pathogen responsible for symptoms was isolated from diseased stems from China (a, b) and diseased leaves (c) from controlled environment experiments and identified as *L. biglobosa* by pigment production in culture and by PCR. Range of pigmentation observed amongst *Leptosphaeria biglobosa* isolates grown on PDA (potato dextrose agar) medium, for Chinese isolates (d) (clockwise from top-left: CN53, CN57, CN26, CN52, CN55, CN49), European isolates (e) (clockwise, PL35, UK09, FR02, PL30) and Canadian isolates (f) (clockwise, CA16, CA19, CA01, CA12). All the isolates were incubated at 15°C on PDA -medium in darkness for 2 weeks.

Fig. 3 Genetic relatedness (0-1 scale) of *Leptosphaeria biglobosa* isolates obtained from China, the UK, France, Poland, Austria and Canada, assessed using AFLP markers and analysed using GenStat software. There were 33 *L. biglobosa* isolates from China (CN 🟥), 54 *L. biglobosa* isolates from Europe and 10 *L. biglobosa* isolates from Canada (CA 🟦) used for the AFLP analysis. In total, 80 polymorphic bands were compared between isolates. European isolates were from the UK (UK 🟦), Poland (PL 🟪), France (FR 🟩) and Austria (AT 🟫). These isolates are in the following order, starting from the top (CN01, 03, 06, 08, 10, 12, 14, 18, 16, 21, 25, 31, 27, 29, 33, 35, 39, 41, 45, 51; AT03, 07, 08, 06, 09; CN57, 58, 59, 60, 47, 48, 50, 54, 55, 49, 52, 53; FR26; CN56; FR14, 04, 12, 32, 24, 34, 35, 06, 08, 33, 10; UK01, 32; PL34, 24, 30, 29, 33; AT01; FR18, 30; UK08, 22, 21, 28, 31; PL19, 28, 35, 22, 23, 27; AT05; PL25, 26; UK03, 13, 36, 16, 15, 18, 10, 25; PL32; FR28; PL31; AT02, 04; CA01, 02, 10, 03, 09, 05, 04, 07, 06, 08). Details of the origins of these isolates are given in Supplementary Table 1.

Fig. 4 Differentiation of *L. biglobosa* isolates into geographical populations based on AFLP markers using the POPGEN32 software. The dendrogram (Neighbour-joining) is based on Nei's genetic distance to illustrate difference relationships within 86 AFLP bands from 97 isolates of *Leptosphaeria biglobosa* obtained from different countries.

Fig. 5 Results of the principal coordinate analyses based on the binary matrix of 97 *Leptosphaeria biglobosa* isolates tested by 80 polymorphic AFLP markers. Data represent means ±SD within each country/region. Of the 97 isolates, 33 were from China, 15 from the UK, 15 from Poland, 15 from France, 9 from Austria and 10 from Canada. Isolates from Austria, France, Poland and the UK were combined into a single group (Europe).

Fig. 6 Evolutionary relationships of 18 *Leptosphaeria biglobosa* isolates and *L. maculans* isolate LEROY (IBCN80) based a combined analysis of the nucleotide sequences of actin, β-tubulin and internal transcribed spacer (ITS) regions of the rDNA from mycelial cultures of these isolates. The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 100,000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% of bootstrap replicates were collapsed. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (100,000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 870 positions in the final dataset. Phylogenetic analyses were done in MEGA4.

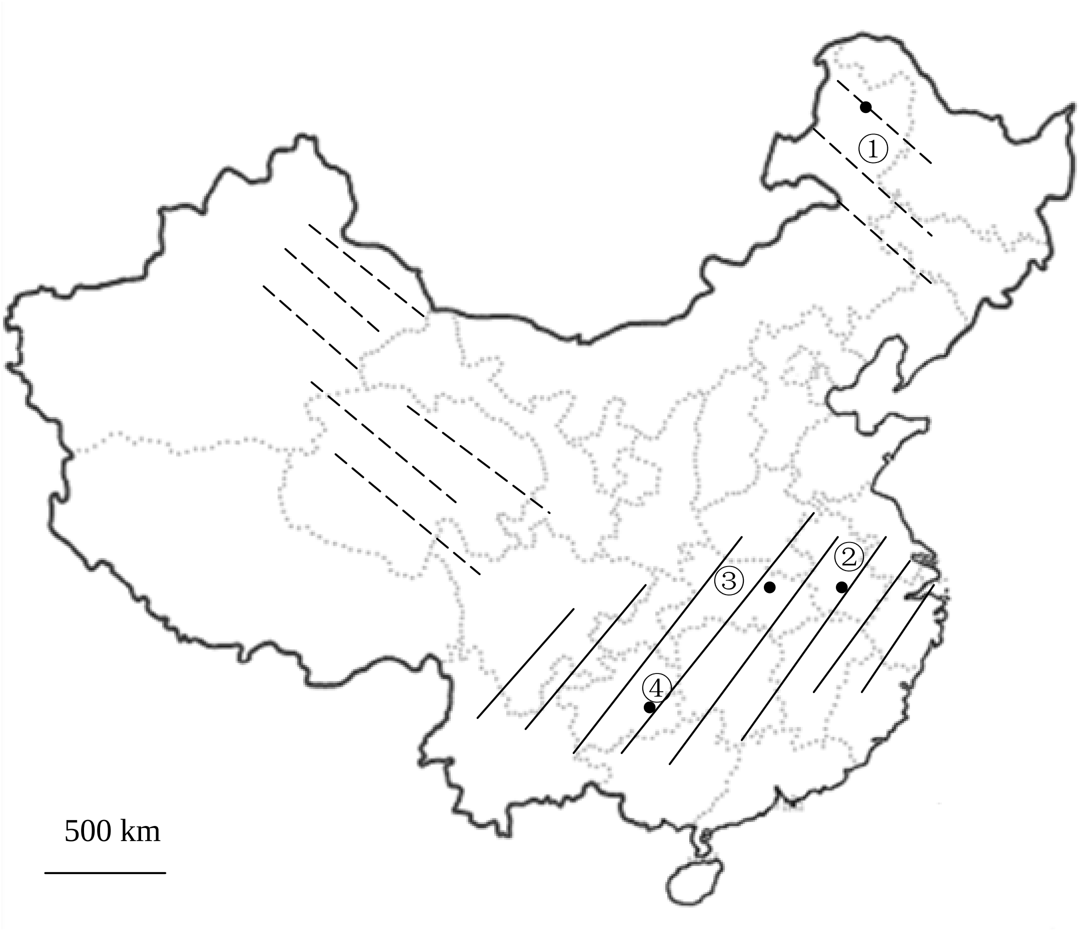

Fig. 1

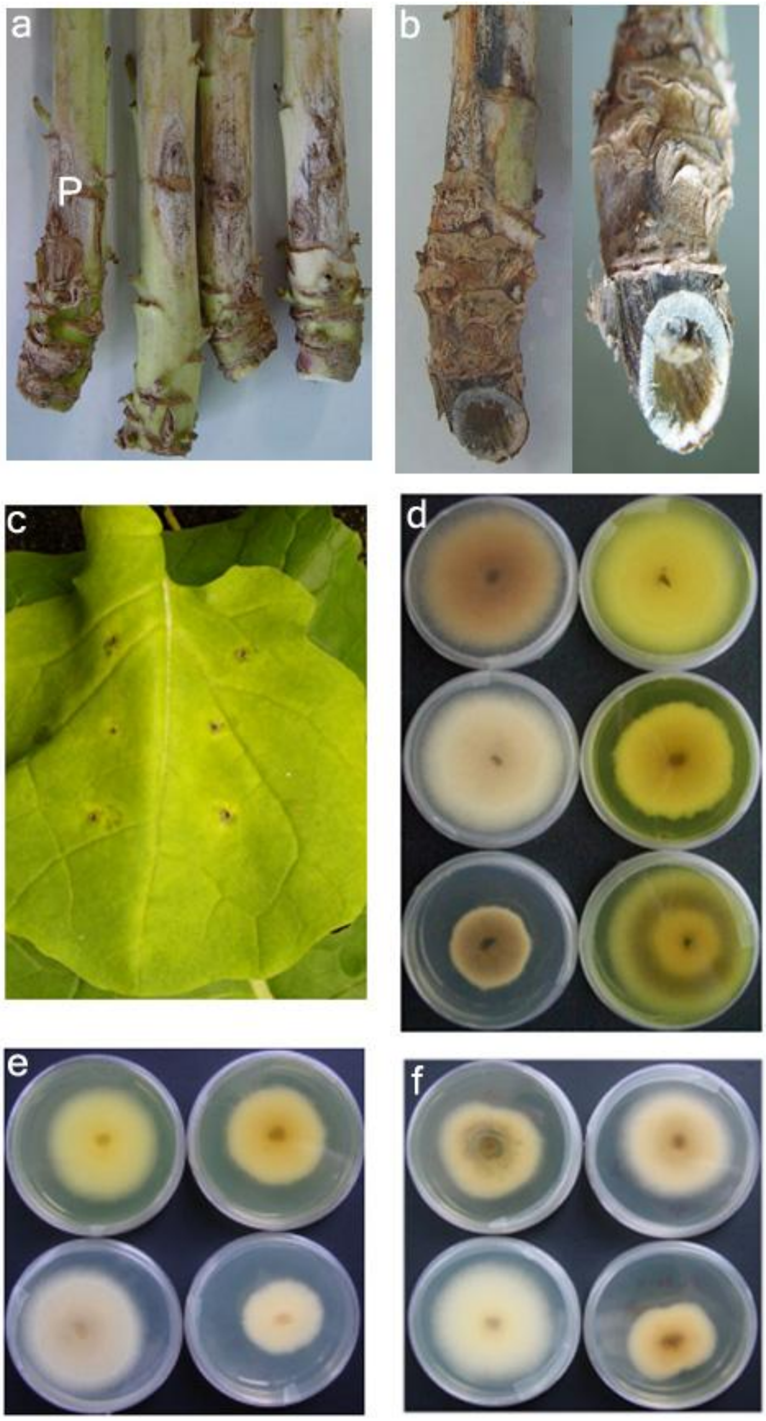

Fig. 2

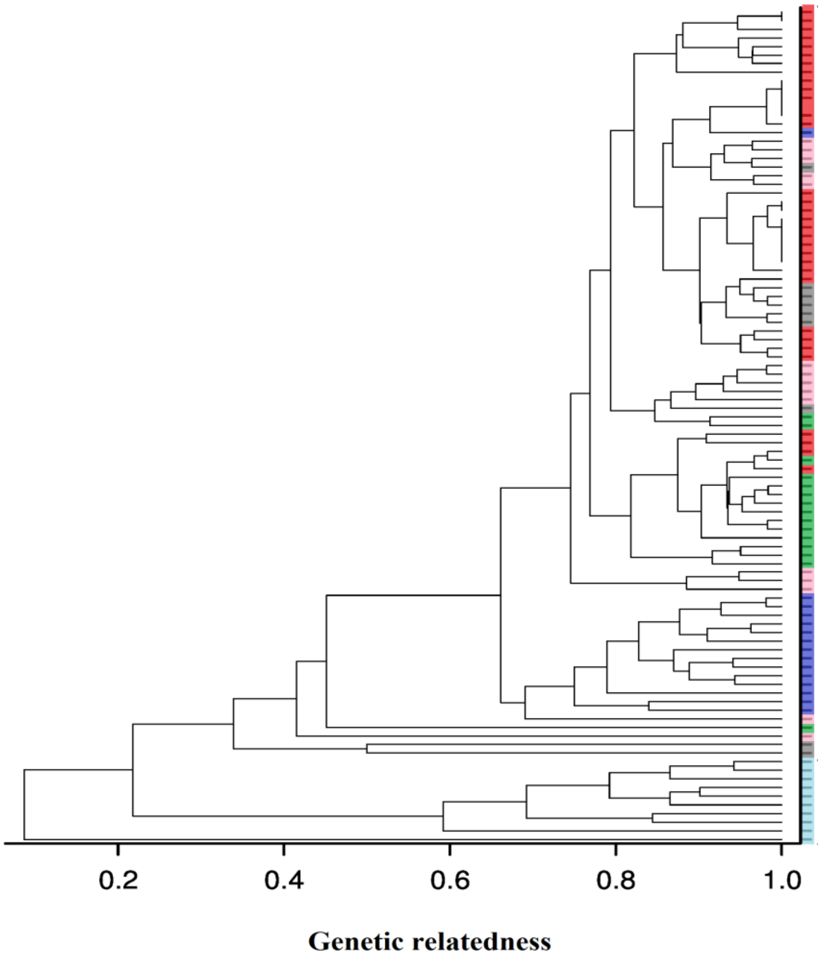

Fig. 3

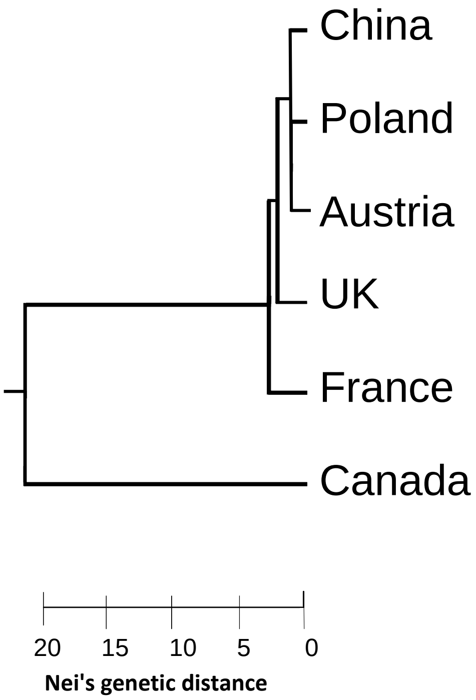
China
Poland
Austria
UK
France
Canada
20
15
10
5
0
Nei's genetic distance

Fig. 4

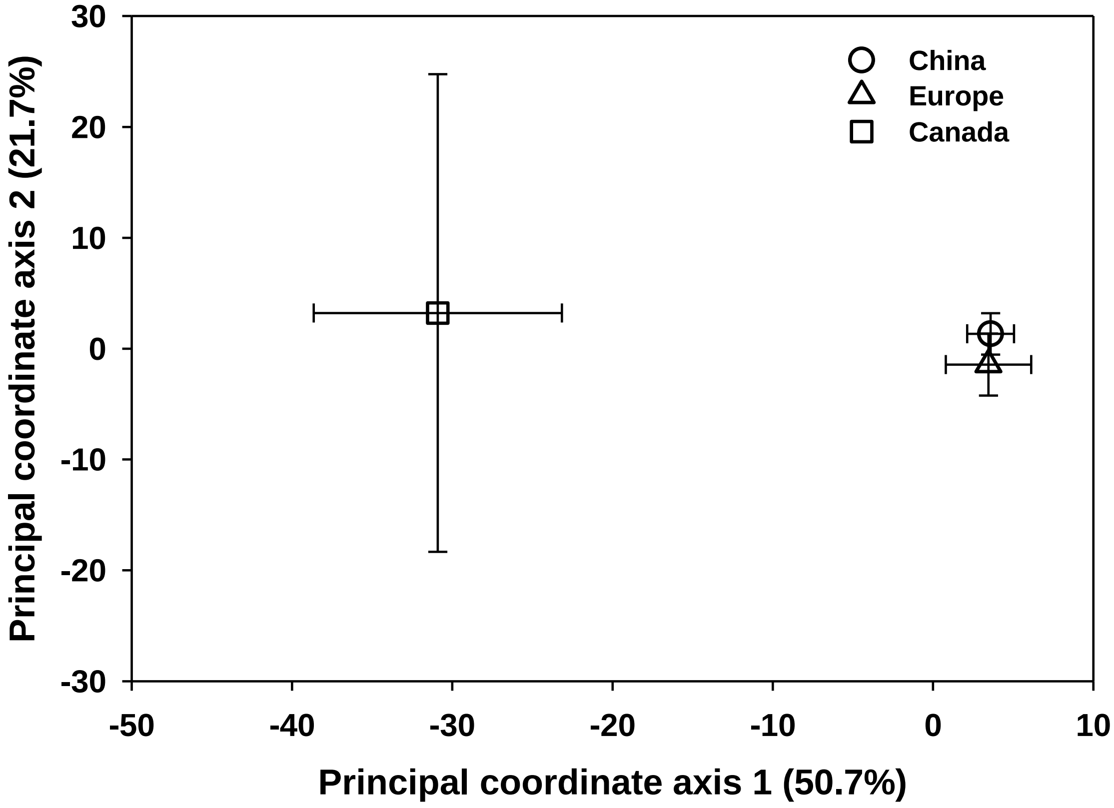
30
20
10
0
-10
-20
-30
-50
-40
-30
-20
-10
0
10
Principal coordinate axis 2 (21.7%)
Principal coordinate axis 1 (50.7%)
○ China
△ Europe
□ Canada

Fig. 5

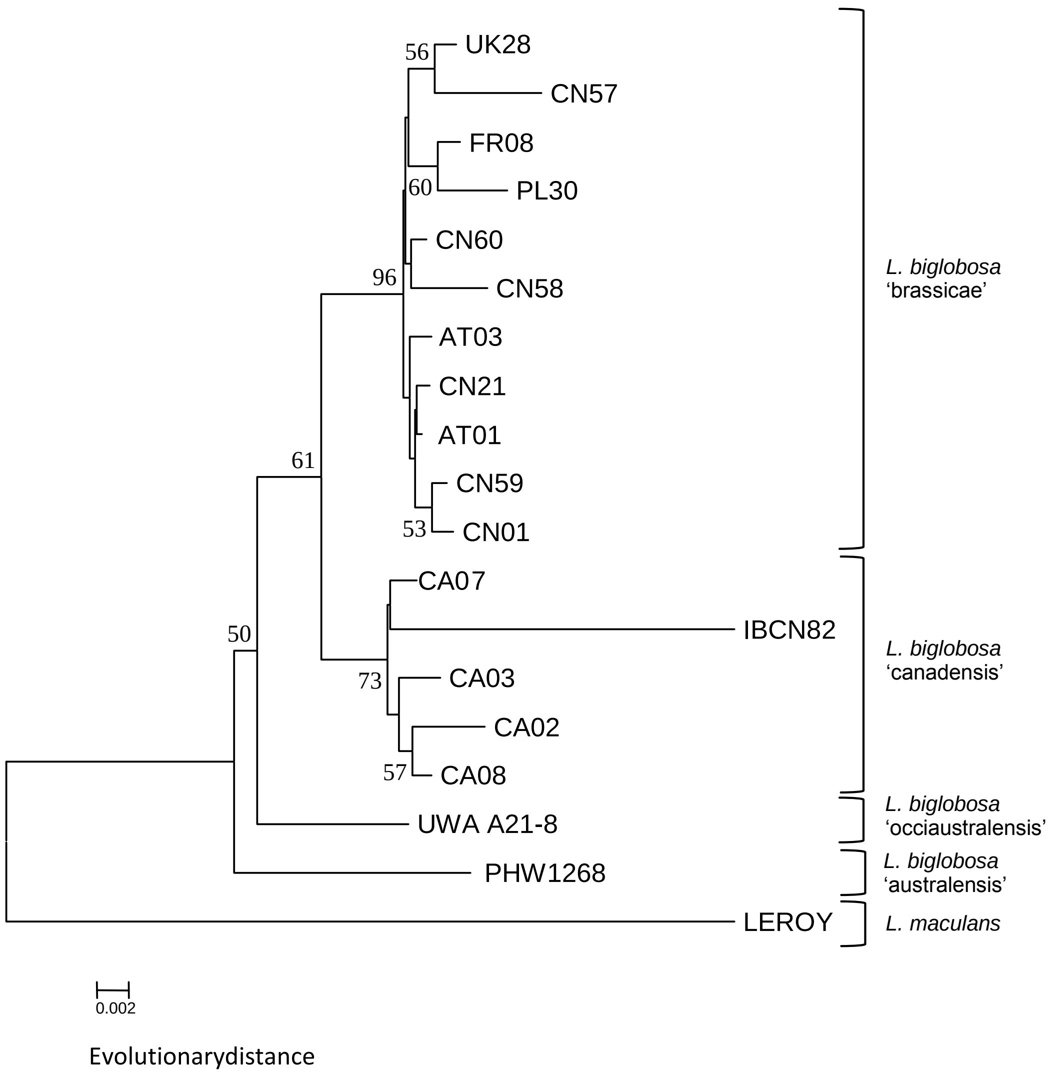
56
UK28
CN57
FR08
60
PL30
CN60
96
CN58
AT03
CN21
AT01
61
CN59
53
CN01
CA07
IBCN82
50
73
CA03
CA02
57
CA08
UWA A21-8
PHW1268
LEROY
L. biglobosa 'brassicae'
L. biglobosa 'canadensis'
L. biglobosa 'occiaustralensis'
L. biglobosa 'australensis'
L. maculans
0.002
Evolutionarydistance

Fig. 6

Table 1 Primers used for PCR assay to confirm the identity of *Leptosphaeria biglobosa* isolates (a), for AFLP assays to assess the genetic relatedness of *L. biglobosa* populations from different countries (b) and for phylogenetic analysis based on actin, β-tubulin and ribosomal internal transcribed spacer (ITS) nucleotide sequences (c)

Primer	Sequence	Source
(a) *Leptosphaeria maculans* (Lm/Lmac) and *L. biglobosa* (Lb/Lbig) primers		
LmF[a]	5’-GTG GCG GCA GTC TAC TTT GA -3’	Mahuku et al. (1996)
LmR	5’-GAG TCC CAA GTG GAA CAA ACA-3’	
LbF[b]	5’-CCT TCT ATC AGA GGA TTG GT-3’	
LbR[b]	5’-CGT TCT TCA TCG ATG CCA GA-3’	
LmacF	5’-CTT GCC CAC CAA TTG GAT CCC CTA-3’	
LmacR	5’-GCA AAA TGT GCT GCG CTC CAG G-3’	Liu et al. (2006)
LbigF[c]	5’-ATC AGG GGA TTG GTG TCA GCA GTT GA-3’	
LbigR[c]	5’-GCA AAA TGT GCT GCG CTC CAG G-3’	
(b) AFLP primers		
E-0	5’-GAC TGC GTA CCA ATT C-3’	AFLP® Microorganism Primer Kit (Invitrogen™,USA)
M-0	5’-GAT GAG TCC TGA GTA A-3’	
E-AC	5’-GAC TGC GTA CCA ATT CAC-3’	
M-G	5’-GAT GAG TCC TGA GTA AG-3’	
(c) Actin, β-tubulin and ITS primers		
ActinF	5’-GAG CAG GAG ATC CAG ACT GC-3’	Van de Wouw et al. (2008)
ActinR	5’-TTC GAG ATC CAC ATC TGC TG-3’	
β-tubulinF	5’-GTC GAG AAC TCC GAC GAG AC-3’	Vincenot et al. (2008)
β-tubulinR	5’-ATC TGG TCC TCG ACC TCC TT-3’	
PN3	5’-CCG TTG GTG AAC CAG CGG AGG GAT C-3’	Balesdent et al. (1998)
PN10	5’-TCC GCT TAT TGA TAT GCT TAA G-3’	

[a] F-forward; R-reverse

[b] Designed using *L. biglobosa* ‘canadensis’ isolates

[c] Designed using *L. biglobosa* ‘brassicae’ isolates

Table 2 Mean Genetic Diversity indices from POPGEN version 1.31 analyses of 86 polymorphic AFLP bands from 97 *Leptosphaeria biglobosa* isolates from China, the UK, Poland, Austria and Canada.

	Number of isolates	**na**	**ne**	**h**	**I**
China	33	1.605	1.132	0.089	0.155
UK	15	1.279	1.127	0.078	0.123
Poland	15	1.465	1.173	0.115	0.186
France	15	1.465	1.121	0.089	0.154
Austria	9	1.50	1.207	0.141	0.225
Canada	10	1.616	1.286	0.182	0.286
Summary	**97**	**1.98**	**1.265**	**0.185**	**0.312**

na= observed number of alleles; ne= effective number of alleles; h = Nei’s Genetic Diversity; I = Shannon’s Information Index.

Table 3 Nei's* analysis of gene diversity in subdivided *L. biglobosa* populations from China, the UK, Poland, France and Austria using the POPGENE version 1.31 software.

	Number of isolates	**Ht**	**Hs**	**Gst**	**Nm**
Mean	97	0.2214	0.1157	0.4776	0.5468

Ht= total heterozygosity; Hs= intraspecific heterozygosity; Gst= degree of genetic difference; Nm= estimate of gene flow

*Nei (1987).

Table 4 Similarity matrices based on Nei's* original measures of Genetic Identity and Genetic Distance among 97 *L. biglobosa* isolates from China, the UK, Poland, France, Austria and Canada, based on 86 polymorphic AFLP bands using the POPGENE version 1.31 software.

	CHINA	UK	POLAND	FRANCE	AUSTRIA	CANADA
CHINA	100	**94.75**	**97.18**	**95.46**	**97.27**	**64.39**
UK	94.61	100	**96.73**	**92.29**	**94.43**	**66.90**
POLAND	97.13	96.68	100	**94.68**	**97.12**	**66.30**
FRANCE	95.35	91.97	94.53	100	**95.08**	**61.72**
AUSTRIA	97.23	94.27	97.00	94.96	100	**66.65**
CANADA	55.97	59.80	58.91	51.76	59.42	100

Nei's Genetic Identity (in **bold type** above the diagonal) and Nei's Genetic Distance are expressed on a 1-100 scale.
*Nei (1972)

Table 5 Similarity matrix[a] of a combined analysis of the nuclotide sequences of gene fragments for actin, β-tubulin and ITS region of the rDNA from 18 isolates of *Leptosphaeria biglobosa* and one *L. maculans* isolate

	CA02	CA08	CA07	CA03	CN21	UK28	FR08	PL30	CN60	CN58	CN57	AT01	AT03	CN59	CN01	PHW1268	UWA A21-8	LEROY	IBCN82
[CA02]	100																		
[CA08]	99.4	100																	
[CA07]	99.1	99.5	100																
[CA03]	99.2	99.5	99.5	100															
[CN21]	98.1	98.7	98.7	98.7	100														
[UK28]	98.5	98.4	98.4	98.4	98.4	100													
[FR08]	98.1	98.7	98.5	98.3	99.4	99.3	100												
[PL30]	97.9	98.1	98.4	97.9	99.1	99.2	99.4	100											
[CN60]	98.1	98.7	98.7	98.5	99.8	99.4	99.7	99.1	100										
[CN58]	98.1	98.4	98.4	98.1	99.4	99.1	99.1	99.0	99.4	100									
[CN57]	98.1	98.3	97.8	98.0	99.1	99.2	99.0	98.4	99.1	98.7	100								
[AT01]	98.0	98.4	98.8	98.4	99.9	99.5	99.5	99.2	99.9	99.3	99.0	100							
[AT03]	98.1	98.5	97.3	98.7	99.7	99.5	99.5	99.2	99.7	99.1	99.0	99.8	100						
[CN59]	98.0	98.4	98.6	98.6	99.7	99.5	99.3	90.0	99.7	99.1	99.0	99.8	99.8	100					
[CN01]	98.0	98.4	98.7	98.4	99.7	99.3	99.3	99.1	99.7	99.2	98.7	99.8	99.5	99.8	100				
[PHW 1268]	96.4	96.7	96.9	97.7	97.4	97.3	97.2	97.1	97.4	97.1	96.5	97.5	97.4	97.3	97.3	100			
[UWA A21-8]	98.0	98.1	98.4	98.1	97.8	97.7	97.5	97.4	97.8	97.4	96.8	97.9	97.8	97.7	97.7	97.5	100		
[LEROY]	92.1	92.7	93.0	92.7	92.8	92.5	92.6	92.2	92.8	92.2	91.8	93.0	92.9	93.7	92.7	92.5	92.7	100	
[IBCN82]	97.3	97.4	97.7	97.5	96.6	96.6	96.3	96.2	96.6	96.5	95.7	96.7	96.7	96.6	96.5	95.4	96.8	90.6	100

[a] Similarity matrix of estimates of evolutionary divergence between nucleotide sequences of actin, β-tubulin and ITS region of the rDNA gene fragments from mycelia cultures of 18 isolates of *Leptosphaeria biglobosa* and one *L. maculans* isolate (LEROY, IBCN80) that were analysed in this study. The number of base substitutions per site from analysis between sequences is shown. All results are based on the pairwise analysis of 19 sequences and were obtained by a bootstrap procedure (10,000 replicates). Analyses were conducted using the Maximum Composite Likelihood method in MEGA4. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 870 positions in the final dataset.

Supplementary Table 1 Origins of 97 isolates of the phoma stem canker pathogen *Leptosphaeria biglobosa* collected from oilseed rape (*Brassica napus*) crops in China, the UK, France, Poland, Austria or Canada used for Amplified Fragment Length Polymorphism analysis

RRes Code	Original code	Location	Year	RRes Code	Original code	Location	Year
Roth_LbCN01	HL05-1[a]	Hailar, CN	2005[h]	Roth_LbPL19	PolB2-5[d]	Poznan, PL	2001[h]
Roth_LbCN03	HL05-3[a]	Hailar, CN	2005[h]	Roth_LbPL22	GR2-E 1-1[d]	Grabow, PL	2006
Roth_LbCN06	HL05-6[a]	Hailar, CN	2005[h]	Roth_LbPL23	GR2-E 1-2[d]	Grabow, PL	2006
Roth_LbCN08	HL05-8[a]	Hailar, CN	2005[h]	Roth_LbPL24	CER-EMX 1-3[d]	Cerekwica, PL	2006
Roth_LbCN10	HL05-10[a]	Hailar, CN	2005[h]	Roth_LbPL25	CER-DMX 5-2[d]	Cerekwica, PL	2006
Roth_LbCN12	HL05-12[a]	Hailar, CN	2005[h]	Roth_LbPL26	GR2-D 6-2[d]	Grabow, PL	2006
Roth_LbCN14	HL05-14[a]	Hailar, CN	2005[h]	Roth_LbPL27	GR1-E 1-1[d]	Grabow, PL	2006
Roth_LbCN16	HL05-16[a]	Hailar, CN	2005[h]	Roth_LbPL28	IGR-D 28-3[d]	Poznan, PL	2006
Roth_LbCN18	HL05-18[a]	Hailar, CN	2005[h]	Roth_LbPL29	IGR-E 6-3[d]	Poznan, PL	2006
Roth_LbCN21	WH05-1[a]	Wuhan, CN	2005[h]	Roth_LbPL30	GL-68 5Db[d]	Szpregawsk, PL	2002
Roth_LbCN25	WH05-5[a]	Wuhan, CN	2005[h]	Roth_LbPL31	GL-76 2Ga[d]	Trepnowy, PL	2002
Roth_LbCN27	WH05-7[a]	Wuhan, CN	2005[h]	Roth_LbPL32	GL-90 5Ga[d]	Malbork, PL	2002
Roth_LbCN29	WH05-9[a]	Wuhan, CN	2005[h]	Roth_LbPL33	SW-D 2-3[d]	Swadzim, FR	2006
Roth_LbCN31	WH05-11[a]	Wuhan, CN	2005[h]	Roth_LbPL34	ZL-D 6-2[d]	Zlotniki, FR	2006
Roth_LbCN33	WH05-13[a]	Wuhan, CN	2005[h]	Roth_LbPL35	SW-EMX 1-1[d]	Swadzim, FR	2006
Roth_LbCN35	WH05-15[a]	Wuhan, CN	2005[h]	Roth_LbFR04	CHR1b 30[e]	Chateauroux, FR	2006
Roth_LbCN39	WH05-19[a]	Wuhan, CN	2005[h]	Roth_LbFR06	SamRCE 7R[e]	Le Rheu , FR	1995
Roth_LbCN41	WH05-21[a]	Wuhan, CN	2005[h]	Roth_LbFR08	VERb 31[e]	Vergognes , FR	2006
Roth_LbCN45	WH05-25	Wuhan, CN	2005[h]	Roth_LbFR10	COG a 31[e]	Coglès , FR	2006
Roth_LbCN47	HF06-1[a]	Hefei, CN	2006[h]	Roth_LbFR12	MSMc 30[e]	Mt St Michel, FR	2006
Roth_LbCN48	HF06-2[a]	Hefei, CN	2006[h]	Roth_LbFR14	G06-413[f]	Grignon, FR	2006
Roth_LbCN49	HF06-3	Hefei, CN	2006[h]	Roth_LbFR18	G-49[f]	Bagneux, FR	2006
Roth_LbCN50	HF06-4[a]	Hefei, CN	2006[h]	Roth_LbFR24	L-41[f]	Leblanc, FR	2006
Roth_LbCN51	HF06-5[a]	Hefei, CN	2006[h]	Roth_LbFR26	L-48[f]	Leblanc, FR	2006
Roth_LbCN52	HF06-6[a]	Hefei, CN	2006[h]	Roth_LbFR28	Fr 6[f]	Richeville, FR	1996
Roth_LbCN53	HF06-7[a]	Hefei, CN	2006[h]	Roth_LbFR30	Fr 12[f]	Boissay, FR	1997
Roth_LbCN54	HF06-8[a]	Hefei, CN	2006[h]	Roth_LbFR32	Fr 16[f]	Avord, FR	1997
Roth_LbCN55	HF06-9[a]	Hefei, CN	2006[h]	Roth_LbFR33	IBCN41[f]	Le Rheu, FR	1990
Roth_LbCN56	HF06-10[a]	Hefei, CN	2006[h]	Roth_LbFR34	IBCN48[f]	Deuxville, FR	1990
Roth_LbCN57	Hef A4-1[b]	Hefei, CN	1999[h]	Roth_LbFR35	IBCN49[f]	Demanges aux eaux FR	1991
Roth_LbCN58	Hef B2[b]	Hefei, CN	1999[h]	Roth_LbCA01	RL1-41-1[c]	Manitoba, CAN	1989
Roth_LbCN59	Gui 2a 2[bk]	Guizhou, CN	1999[h]	Roth_LbCA02	RL96[c]	CAN	1989
Roth_LbCN60	Gui 2b 3[bk]	Guizhou, CN	1999[h]	Roth_LbCA03	RL41[c]	Alberta, CAN	1989
Roth_LbUK01	UK07-1[a]	Rothamsted, UK	2007[i]	Roth_LbCA04	99-27[g]	Saskatchewan, CAN	1999
Roth_LbUK03	UK07-3[a]	Rothamsted, UK	2007[i]	Roth_LbCA05	00-31[g]	Alberta, CAN	2000
Roth_LbUK08	UK07-8[a]	Rothamsted, UK	2007[j]	Roth_LbCA06	05-07[g]	Alberta, CAN	2005
Roth_LbUK10	UK07-10[a]	Rothamsted, UK	2007[j]	Roth_LbCA07	05-39[g]	Saskatchewan, CAN	2005
Roth_LbUK13	UK07-13[a]	Rothamsted, UK	2007[j]	Roth_LbCA08	05-70[g]	Saskatchewan, CAN	2005
Roth_LbUK15	UK07-15[a]	Rothamsted, UK	2007[j]	Roth_LbCA09	05-78[g]	Saskatchewan, CAN	2005
Roth_LbUK16	B2003-2-8[c]	Orston, UK	2003[h]	Roth_LbCA10	05-84[g]	Saskatchewan, CAN	2005
Roth_LbUK18	10.6[c]	Northant., UK	2002[h]	Roth_LbAT01	AUT 1[c]	St. Pölten, Austria	2002[j]
Roth_LbUK21	37.9[c]	North Yorkshire, UK	2002[h]	Roth_LbAT02	AUT 2[c]	St. Pölten, Austria	2002[j]
Roth_LbUK22	UK 4[c]	Canterb., UK	1996[h]	Roth_LbAT03	AUT 3[c]	St. Pölten, Austria	2002[j]
Roth_LbUK25	2002-35.8[c]	Baldesby, UK	2002[h]	Roth_LbAT04	AUT 4[c]	St. Pölten, Austria	2002[j]
Roth_LbUK28	BW70-11[ck]	Cambridgeshire, UK	2001[j]	Roth_LbAT05	AUT 5[c]	St. Pölten, Austria	2002[j]
Roth_LbUK31	RES S11[c]	Rothamsted, UK	2002[j]	Roth_LbAT06	AUT 6[c]	St. Pölten, Austria	2002[j]
Roth_LbUK32	TE6[c]	Terrington, UK	2001[j]	Roth_LbAT07	AUT 7[c]	St. Pölten, Austria	2002[j]
Roth_LbUK36	Gp 9-1[c]	Cambridgeshire, UK	2001[j]	Roth_LbAT08	AUT 8[c]	St. Pölten, Austria	2002[j]
				Roth_LbAT09	AUT 9[c]	St. Pölten, Austria	2002[j]

[a] isolated by Z Liu; [b] isolated by JS West; [c] isolated/supplied by M Eckert; [d] isolated by M Jedryczka; [e] isolated by R Travadon; [f] isolated by MH Balesdent; [g] isolated by R Kutcher; [h] isolated from plant stem lesions or cankers; [i] isolated from petiole; [j] isolated from plant leaf lesions; [k] ITS regions from these three isolates have been sequenced and they have been confirmed as *L. biglobosa* ‘brassicae’ (Mendes-Pereira et al. 2003; Liu et al. 2006).

Fig. 1

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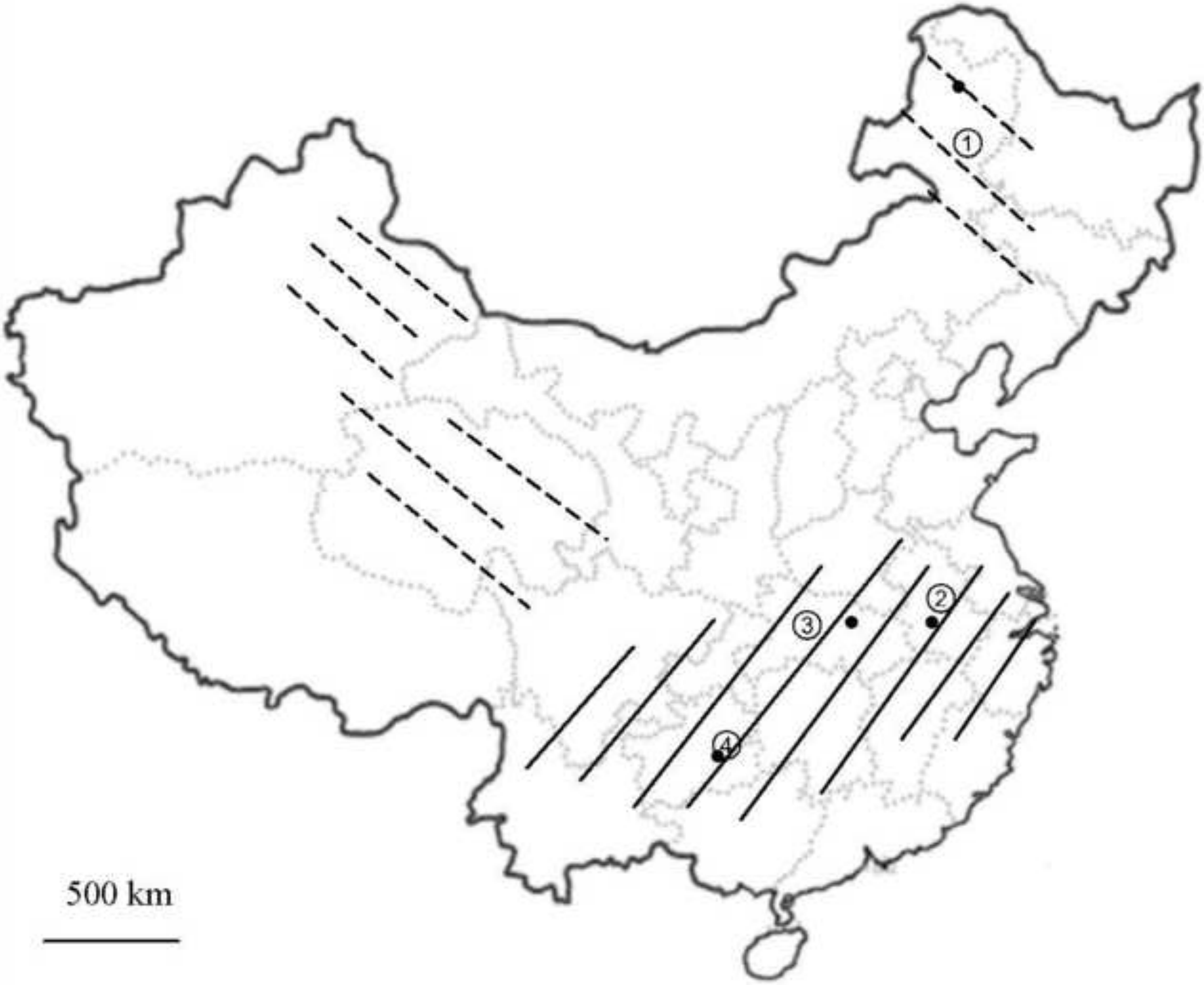

Fig. 2

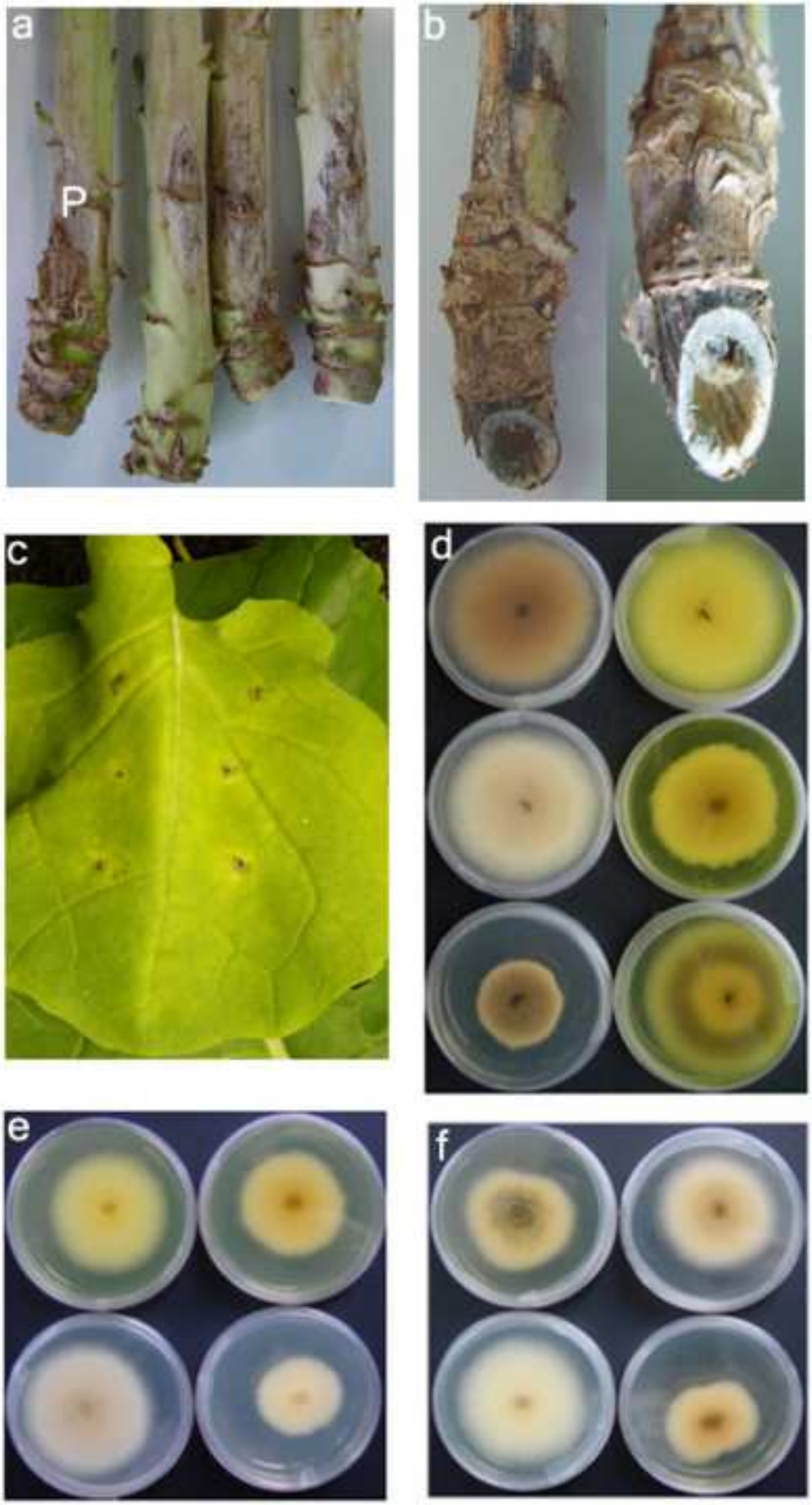

Fig. 3

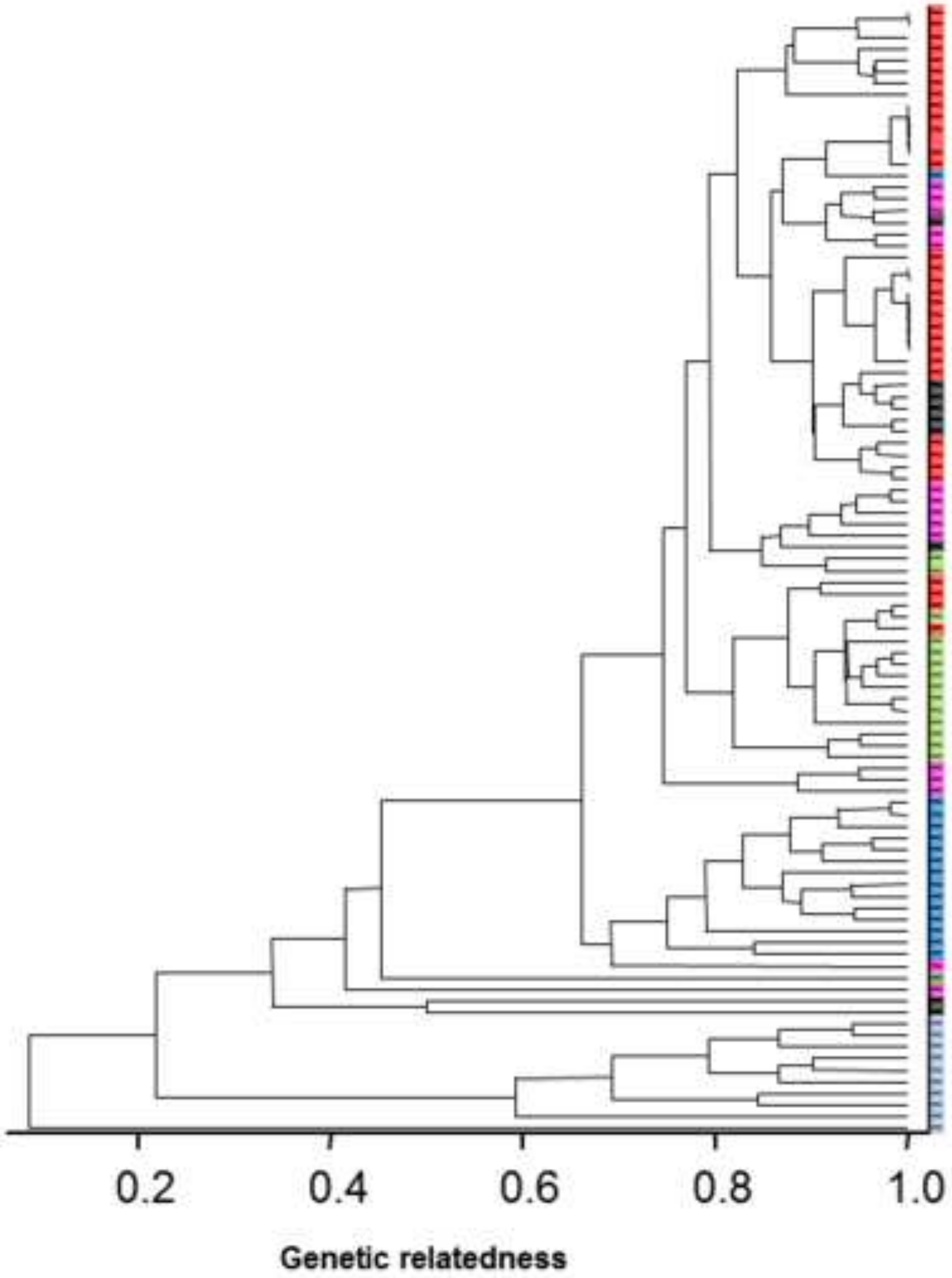

Fig. 4

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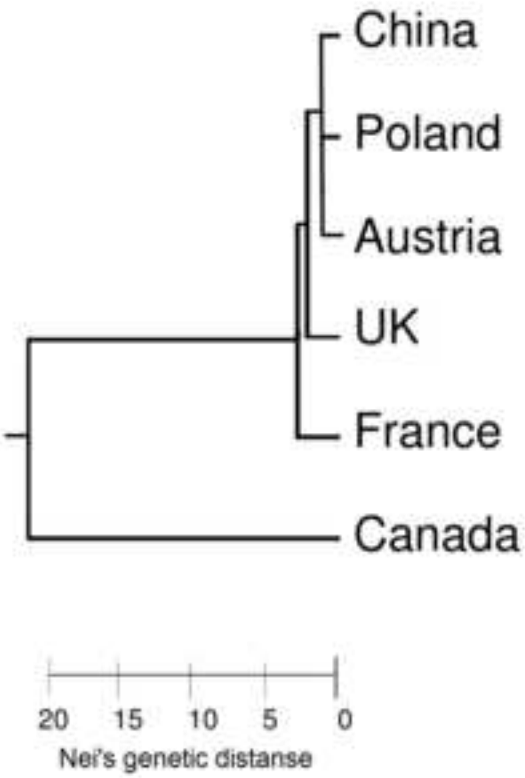

Fig. 5

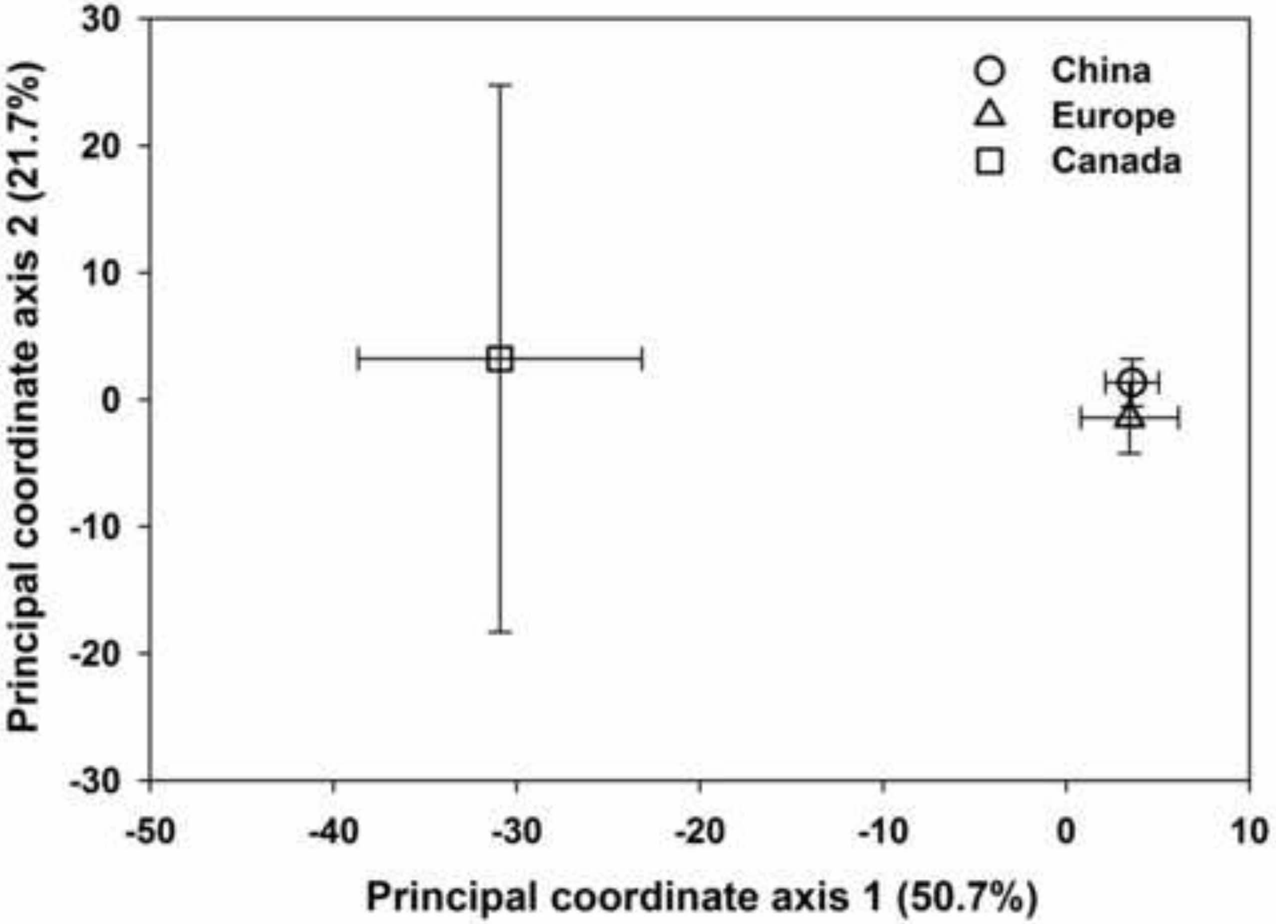
○ China
△ Europe
□ Canada
Principal coordinate axis 2 (21.7%)
30
20
10
0
-10
-20
-30
-50
-40
-30
-20
-10
0
10
Principal coordinate axis 1 (50.7%)

Fig. 6

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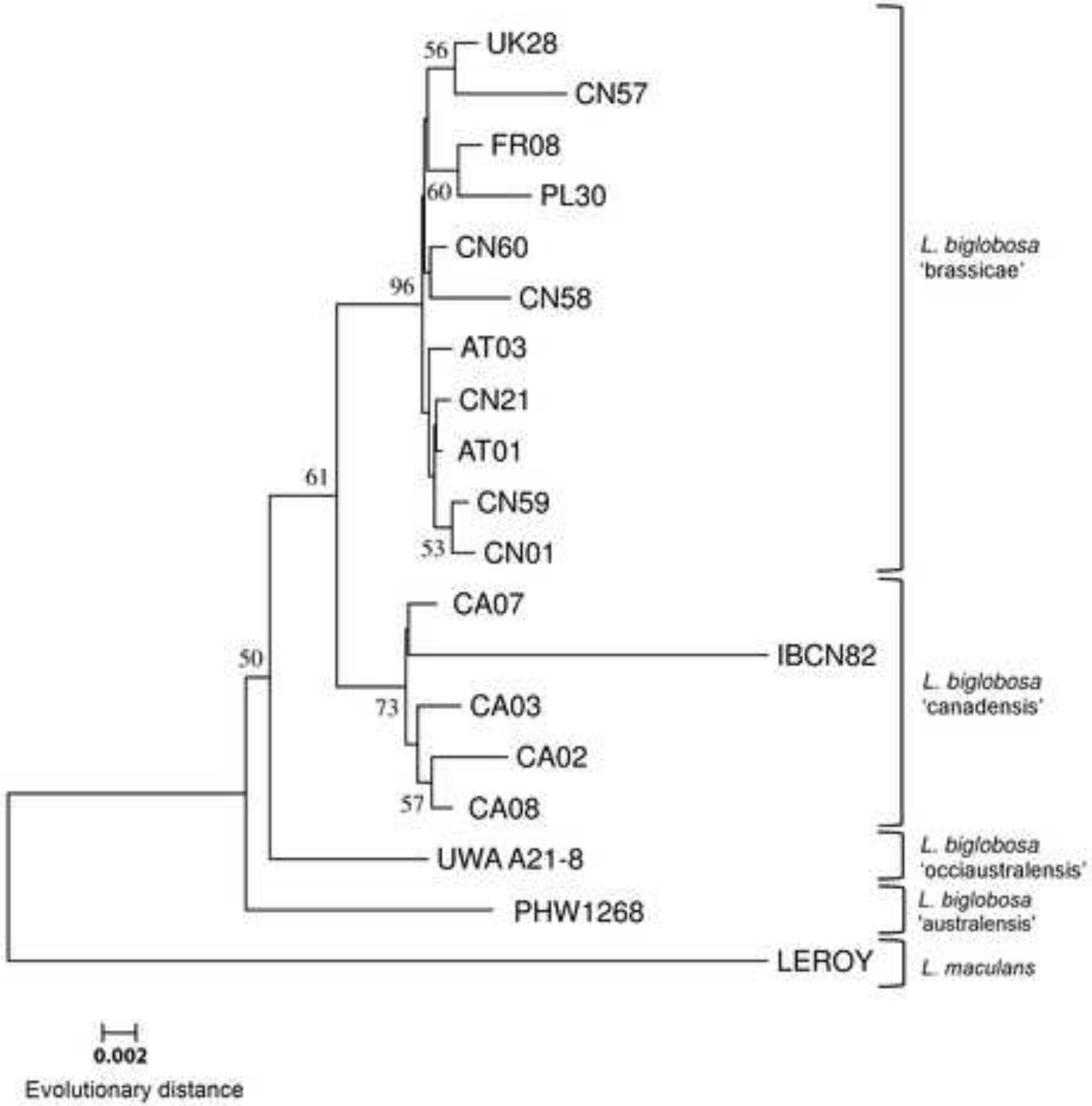

Table 1 Primers used for PCR assay to confirm the identity of *Leptosphaeria biglobosa* isolates (a), for AFLP assays to assess the genetic relatedness of *L. biglobosa* populations from different countries (b) and for phylogenetic analysis based on actin, β-tubulin and ribosomal internal transcribed spacer (ITS) nucleotide sequences (c)

Primer	Sequence	Source
(a) *Leptosphaeria maculans* (Lm/Lmac) and *L. biglobosa* (Lb/Lbig) primers		
LmF	5’-GTG GCG GCA GTC TAC TTT GA -3’	Mahuku et al. (1996)
LmR	5’-GAG TCC CAA GTG GAA CAA ACA-3’	
LbF	5’-CCT TCT ATC AGA GGA TTG GT-3’	
LbR	5’-CGT TCT TCA TCG ATG CCA GA-3’	
LmacF	5’-CTT GCC CAC CAA TTG GAT CCC CTA-3’	Liu et al. (2006)
LmacR	5’-GCA AAA TGT GCT GCG CTC CAG G-3’	
LbigF	5’-ATC AGG GGA TTG GTG TCA GCA GTT GA-3’	
LbigR	5’-GCA AAA TGT GCT GCG CTC CAG G-3’	
(b) AFLP primers		
E-0	5’-GAC TGC GTA CCA ATT C-3’	AFLP® Microorganism Primer Kit (Invitrogen™,USA)
M-0	5’-GAT GAG TCC TGA GTA A-3’	
E-AC	5’-GAC TGC GTA CCA ATT CAC-3’	
M-G	5’-GAT GAG TCC TGA GTA AG-3’	
(c) Actin, β-tubulin and ITS primers		
ActinF	5’-GAG CAG GAG ATC CAG ACT GC-3’	Van de Wouw et al. (2008)
ActinR	5’-TTC GAG ATC CAC ATC TGC TG-3’	
β-tubulinF	5’-GTC GAG AAC TCC GAC GAG AC-3’	Vincenot et al. (2008)
β-tubulinR	5’-ATC TGG TCC TCG ACC TCC TT-3’	
PN3	5’-CCG TTG GTG AAC CAG CGG AGG GAT C-3’	Balesdent et al. (1998)
PN10	5’-TCC GCT TAT TGA TAT GCT TAA G-3’	

[a] F-forward; R-reverse

Table 2 Standard deviations (SD) of the principal coordinate axis 1 and principal coordinate axis 2 for isolates within each country/region based on the binary matrix of 97 *Leptosphaeria biglobosa* isolates tested by 80 polymorphic AFLP markers

Country/region	SD of axis 1	SD of axis 2
China	1.02	1.64
Canada	7.74	21.58
Austria	3.63	4.25
France	1.75	2.34
Poland	3.07	2.32
UK	2.12	2.09
Europe[b]	2.68	2.78

[a] Details of isolates tested are given in Supplementary Table 1. Results of principal coordinate analyses are given in Figure 5.

[b] Europe refers to the combination of isolates from Austria, France, Poland and the UK.

Table 3 Similarity matrix[a] of a combined analysis of the nuclotide sequences of gene fragments for actin, β-tubulin and ITS region of the rDNA from 18 isolates of *Leptosphaeria biglobosa* and one *L. maculans* isolate

	CA02	**CA08**	**CA07**	**CA03**	**CN21**	**UK28**	**FR08**	**PL30**	**CN60**	**CN58**	**CN57**	**AT01**	**AT03**	**CN59**	**CN01**	**PHW1268**	**UWA A21-8**	**LEROY**	**IBCN82**
[CA02]	100																		
[CA08]	99.4	100																	
[CA07]	99.1	99.5	100																
[CA03]	99.2	99.5	99.5	100															
[CN21]	98.1	98.7	98.7	98.7	100														
[UK28]	98.5	98.4	98.4	98.4	98.4	100													
[FR08]	98.1	98.7	98.5	98.3	99.4	99.3	100												
[PL30]	97.9	98.1	98.4	97.9	99.1	99.2	99.4	100											
[CN60]	98.1	98.7	98.7	98.5	99.8	99.4	99.7	99.1	100										
[CN58]	98.1	98.4	98.4	98.1	99.4	99.1	99.1	99.0	99.4	100									
[CN57]	98.1	98.3	97.8	98.0	99.1	99.2	99.0	98.4	99.1	98.7	100								
[AT01]	98.0	98.4	98.8	98.4	99.9	99.5	99.5	99.2	99.9	99.3	99.0	100							
[AT03]	98.1	98.5	97.3	98.7	99.7	99.5	99.5	99.2	99.7	99.1	99.0	99.8	100						
[CN59]	98.0	98.4	98.6	98.6	99.7	99.5	99.3	90.0	99.7	99.1	99.0	99.8	99.8	100					
[CN01]	98.0	98.4	98.7	98.4	99.7	99.3	99.3	99.1	99.7	99.2	98.7	99.8	99.5	99.8	100				
[PHW 1268]	96.4	96.7	96.9	97.7	97.4	97.3	97.2	97.1	97.4	97.1	96.5	97.5	97.4	97.3	97.3	100			
[UWA A21-8]	98.0	98.1	98.4	98.1	97.8	97.7	97.5	97.4	97.8	97.4	96.8	97.9	97.8	97.7	97.7	97.5	100		
[LEROY]	92.1	92.7	93.0	92.7	92.8	92.5	92.6	92.2	92.8	92.2	91.8	93.0	92.9	93.7	92.7	92.5	92.7	100	
[IBCN82]	97.3	97.4	97.7	97.5	96.6	96.6	96.3	96.2	96.6	96.5	95.7	96.7	96.7	96.6	96.5	95.4	96.8	90.6	100

[a] Similarity matrix of estimates of evolutionary divergence between nucleotide sequences of actin, β-tubulin and ITS region of the rDNA gene fragments from mycelia cultures of 18 isolates of *Leptosphaeria biglobosa* and one *L. maculans* isolate (LEROY, IBCN80) that were analysed in this study. The number of base substitutions per site from analysis between sequences is shown. All results are based on the pairwise analysis of 19 sequences and were obtained by a bootstrap procedure (10,000 replicates). Analyses were conducted using the Maximum Composite Likelihood method in MEGA4. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 870 positions in the final dataset.

Supplementary Fig. 1

Supplementary Table 1