Molecular Plant Pathology

MOLECULAR PLANT PATHOLOGY DOI: 10.1111/J.1364-3703.2011.00751.X

Analysis of the mating-type loci of co-occurring and phylogenetically related species of *Ascochyta* and *Phoma*

JOYCE H. C. WOUDENBERG[1,*], JOHANNES DE GRUYTER[1,2], PEDRO W. CROUS[1,3,4] AND LUTE-HARM ZWIERS[1]

[1]*CBS-KNAW Fungal Biodiversity Centre, Uppsalalaan 8, 3584 CT Utrecht, the Netherlands*
[2]*Plant Protection Service, Ministry of Economic Affairs, Agriculture and Innovation, Geertjesweg 15, 6706 EA Wageningen, the Netherlands*
[3]*Utrecht University, Department of Biology, Microbiology, Padualaan 8, 3584 CH Utrecht, the Netherlands*
[4]*Wageningen University and Research Centre (WUR), Laboratory of Phytopathology, Droevendaalsesteeg 1, 6708 PB Wageningen, the Netherlands*

SUMMARY

Ascochyta and *Phoma* are fungal genera containing several important plant pathogenic species. These genera are morphologically similar, and recent molecular studies performed to unravel their phylogeny have resulted in the establishment of several new genera within the newly erected Didymellaceae family. An analysis of the structure of fungal mating-type genes can contribute to a better understanding of the taxonomic relationships of these plant pathogens, and may shed some light on their evolution and on differences in sexual strategy and pathogenicity. We analysed the mating-type loci of phylogenetically closely related *Ascochyta* and *Phoma* species (*Phoma clematidina*, *Didymella vitalbina*, *Didymella clematidis*, *Peyronellaea pinodes* and *Peyronellaea pinodella*) that co-occur on the same hosts, either on *Clematis* or *Pisum*. The results confirm that the mating-type genes provide the information to distinguish between the homothallic *Pey. pinodes* (formerly *Ascochyta pinodes*) and the heterothallic *Pey. pinodella* (formerly *Phoma pinodella*), and indicate the close phylogenetic relationship between these two species that are part of the disease complex responsible for Ascochyta blight on pea. Furthermore, our analysis of the mating-type genes of the fungal species responsible for causing wilt of *Clematis* sp. revealed that the heterothallic *D. vitalbina* (*Phoma* anamorph) is more closely related to the homothallic *D. clematidis* (*Ascochyta* anamorph) than to the heterothallic *P. clematidina*. Finally, our results indicate that homothallism in *D. clematidis* resulted from a single crossover between *MAT1-1* and *MAT1-2* sequences of heterothallic ancestors, whereas a single crossover event followed by an inversion of a fused *MAT1/2* locus resulted in homothallism in *Pey. pinodes*.

INTRODUCTION

Ascochyta and *Phoma* are important fungal genera with a worldwide distribution. The genus *Ascochyta* harbours pathogens occurring on a broad range of plants, and is responsible for significant economic losses of peas, beans and forage legumes. The genus *Phoma* includes multiple plant pathogens, saprobes and even human pathogens (Aveskamp *et al*., 2008). These two genera are morphologically very similar (Boerema, 1997; Boerema and Bollen, 1975; Brewer and Boerema, 1965; Wollenweber and Hochapfel, 1936), and the criteria for their delimitation are based on conidiogenesis and the percentage of septate conidia produced (Boerema and Bollen, 1975). However, the distinctive features of conidiogenesis can only be observed using scanning electron microscopy, and conidiogenesis of the genus *Ascochyta* has been disputed (Buchanan, 1987; Punithalingam, 1979). As *Phoma* species produce predominantly aseptate conidia and *Ascochyta* species mainly septate conidia, the ratio of aseptate to septate conidia produced on artificial media is used as a practical criterion for identification. However, this trait is highly variable for *Phoma*, and therefore this criterion is also of limited value for accurate identification (Onfroy *et al*., 1999). As a result of these problems in correct identification, multiple synonyms of *Phoma* and *Ascochyta* species are known, e.g. *A. clematidina*, *P. clematidina*, *A. pinodella*, *P. pinodella*, *A. argillacea*, *P. argillacea*, *A. caricae-papayae* and *P. caricae-papayae* (Boerema *et al*., 2004).

Recent molecular studies of *Phoma* spp. (Aveskamp *et al*., 2008, 2009a, b, 2010; de Gruyter *et al*., 2009, 2010), aimed at unravelling the phylogeny of these genera, have resulted in the establishment of several new genera within the newly erected Didymellaceae family, including both *Ascochyta* and *Phoma* species (de Gruyter *et al*., 2009). In 2010, *Phoma pinodella* and *Didymella pinodes* (*Ascochyta* anamorph) were described in the newly erected genus *Peyronellaea* as *Pey. pinodella* and *Pey. pinodes* (Aveskamp *et al*., 2010). These fungi, together with *Ascochyta pisi* and the recently described *Phoma koolunga* (Davidson *et al*., 2009), constitute the disease complex causing Ascochyta blight of pea, one of the most important diseases affecting field peas in the world. Although the results of multiple studies have confirmed that there are differences between the species, these studies have also shown that the relationship between *Pey. pinodes* and *Pey. pinodella* is much closer than that between either of these species and *A. pisi* (Barve *et al*., 2003; Chilvers *et al*., 2009;

**Correspondence*: Email: j.woudenberg@cbs.knaw.nl

Faris-Mokaiesh *et al*., 1996; Fatehi *et al*., 2003; Peever, 2007) or *P. koolunga* (Davidson *et al*., 2009). The same situation was observed within a recently characterized disease complex occurring on *Clematis*. A multigene phylogeny performed on fungal strains isolated from wilting *Clematis* revealed three distinct clades among strains formerly identified as *Phoma clematidina*. The sexual fungi *Didymella vitalbina* (*Phoma* anamorph) and *Didymella clematidis* (*Ascochyta* anamorph) were recognized as being the cause of wilt of different wild *Clematis* spp., next to the previously recognized asexual *P. clematidina*, which seems to occur on *Clematis* hybrids (Woudenberg *et al*., 2009). Here, the relationship between *D. vitalbina* (*Phoma* anamorph) and *D. clematidis* (*Ascochyta* anamorph) is much closer than that between either of these species and *P. clematidina*. These results again indicate the need for taxonomic clarification and correct identification.

Although the internal transcribed spacer (ITS) is the region of the genome most commonly used as a genetic marker in identification and phylogeny studies to date (Begerow *et al*., 2010; Seifert, 2009), it has been shown that mating-type loci, as well as other rapidly evolving loci, may provide much better resolution among closely related taxa (Barve *et al*., 2003; Peever *et al*., 2007). Mating-type genes evolve more quickly than other regions of the genome, but are highly conserved within species, making them useful for the phylogenetic analysis of closely related species.

In all heterothallic filamentous ascomycetes studied to date, sexual reproduction is controlled by a single regulatory locus (the mating-type or *MAT* locus). In heterothallic (outbreeding) species, the mating-type locus contains one of two forms of dissimilar sequence. These sequences are named idiomorphs as the alternative versions of the mating-type locus are completely dissimilar, but are located at the same chromosomal location within the genome (Metzenberg and Glass, 1990). By convention, mating-type idiomorphs of complementary isolates are termed *MAT1-1* and *MAT1-2* (Turgeon and Yoder, 2000). In contrast, homothallic fungi that do not require the presence of a complementary isolate to complete a sexual cycle contain both mating-type genes (either physically linked or unlinked) in a single genome. Characteristic for *MAT1-1* isolates is the presence of a gene (*MAT1-1-1*) within the *MAT1-1* mating-type locus that encodes a protein with an α-domain and, for *MAT1-2* isolates, the presence of a gene (*MAT1-2-1*) within the *MAT1-2* mating-type locus that encodes a protein containing a high-mobility group (HMG) domain (Coppin *et al*., 1997).

In 1953, the homothallic nature of *Pey. pinodes* was confirmed by mono-ascospore cultures (Baumann, 1953). The sexual state of *Pey. pinodella* has also been described in the past (Bowen *et al*., 1997) and, as single ascospore-derived cultures failed to produce pseudothecia, it was assumed to be heterothallic. On the basis of the presence in pure cultures of both the teleomorph and anamorph state, *D. clematidis* was predicted to be a homothallic fungus, whereas no information was available about the sexual state of *P. clematidina* and *D. vitalbina* (Woudenberg *et al*., 2009). For none of these fungi molecular data on the underlying genetic basis of these mating systems were available. We aimed to use molecular data to confirm the sexual state of *Pey. pinodes*, *Pey. pinodella* and *D. clematidis* and to determine the sexual state of *P. clematidina* and *D. vitalbina*. Furthermore, using a phylogenetic analysis based on parts of the mating-type loci, we aimed to confirm the taxonomy and species boundaries. Finally, by genomic comparison and a study of the structural organization of the mating-type loci, we sought to obtain a better understanding of the evolution of homo- and heterothallism in these closely related and co-occurring *Phoma* and *Ascochyta* species. In this article, we describe the cloning, characterization and genomic comparison of the complete mating-type loci of *P. clematidina*, *D. vitalbina*, *D. clematidis*, *Pey. pinodes*, *Pey. pinodella* and *P. herbarum* (the type species of *Phoma*).

RESULTS

Cloning of mating-type loci

Full-length mating-type loci of the examined species were cloned by a combination of methods. Initially, an attempt was made to amplify conserved parts of the *MAT1-1-1* and *MAT1-2-1* mating-type genes using primers previously employed for the amplification of the α-box (*MAT1-1-1*) of *Leptosphaeria maculans* and the HMG-box (*MAT1-2-1*) of *Ascochyta rabiei* (Barve *et al*., 2003; Cozijnsen and Howlett, 2003). On the cloned partial *MAT* sequences, several subsequent chromosome walking steps in both the upstream and downstream directions were performed to obtain the whole mating-type loci. Only the entire *MAT1-2* of *P. herbarum* and *P. clematidina* could thus be generated. On the basis of these *MAT1-2* loci and the sequences of five reference *MAT* loci, new primers directed against DNA sequences flanking the idiomorphs were designed (Tables 1 and 2). The use of these 'idiomorph primers' resulted in the amplification of the entire *MAT1-1* of *P. clematidina* and *D. vitalbina*, and both *MAT1-1* and *MAT1-2* of *Pey. pinodella*. The sequences of all newly obtained

Table 1 Primers designed for *MAT* idiomorph polymerase chain reaction (PCR) and *Phoma*-specific high-mobility group (HMG) motif and partial *MAT1* PCR.

Name	Nucleotide sequence (5′–3′)	Used for
MATidio_Fd	GGRAGRATIGCIGAYTGGAARGG	MAT idiomorph
MATidio_Rv	TGGIGITGYGGIACKTTYATYTGG	
HMGF_Phoma	CGYCCRATGAAYTGCTGGAT	HMG motif
HMGR_Phoma	CRGGCTTRCGAGGRSWRTACTT	
MAT1F2_Phoma	CTGGAATIGCWGRCATGGC	Partial MAT1
MAT1R2_Phoma	TGTCGCTTYGYICGTCGC	

Table 2 GenBank numbers from control strains.

Name	*MAT1-1-1*	*MAT1-2-1*		ITS	
Didymella lentis	DQ341314	DQ341315	Chérif *et al*. (2006)	DQ383953	Peever *et al*. (2007)
Didymella rabiei	DQ341313	DQ341312	Barve *et al*. (2003)	DQ383949	Peever *et al*. (2007)
Pyrenophora graminae	DQ823079	DQ823080	Rau *et al*. (2007)	–	
Pyrenophora teres	AY950585	AY950586	Rau *et al*. (2005)	–	
Leptosphaeria maculans	AY174048	AY174049	Cozijnsen and Howlett (2003)	–	

mating-type loci were used to design *Phoma*-specific *MAT1-1* and *MAT1-2* primers (Table 1). For those species still lacking sequence information about the full-length mating-type locus (*D. clematidis*, *Pey. pinodes* and *D. vitalbina*), the fragments obtained after polymerase chain reaction (PCR) with the *Phoma*-specific primers were used as a starting point for a new chromosome walking procedure. Finally, the *Phoma*-specific primers were used on all isolates under investigation (Table 3). According to the *Phoma*-specific PCRs, seven *P. clematidina* strains were *MAT1-1* positive and three were *MAT1-2* positive, of the 12 tested *Pey. pinodella* strains five contained the *MAT1-1* and seven the *MAT1-2* idiomorph, and of the seven tested *D. vitalbina* strains four were *MAT1-1* and two were *MAT1-2*; the amplification of *MAT* sequences from one *D. vitalbina* strain remained unsuccessful. These results indicate the heterothallic nature of *P. clematidina*, *Pey. pinodella* and *D. vitalbina*. However, all *Pey. pinodes* strains and the *D. clematidis* strain gave positive results with both *Phoma*-specific *MAT1-1* and *MAT1-2* primers, thus indicating the homothallic nature of these species. Finally, no *P. herbarum* strains gave positive results with *MAT1-1* primers, but six of the 15 strains tested were *MAT1-2* positive.

Structural organization of heterothallic mating-type loci

The *P. clematidina MAT1-1* and *MAT1-2* sequences obtained resulted in the assembly of 3.6 and 3.5 kb of sequence, respectively. BLAST2 analysis of these sequences indicated that, in the *MAT1-1* and *MAT1-2* isolates, 1.8 kb of *MAT1-1* and 2.1 kb of *MAT1-2* were dissimilar and thus belonged to the idiomorphs (Metzenberg and Glass, 1990). Similar analysis of the *D. vitalbina MAT1-1* and *MAT1-2* sequences (3 and 3.1 kb, respectively) obtained identified a *MAT1-1* idiomorph of 1.9 kb and a *MAT1-2* idiomorph of 2.2 kb. The sequenced mating-type loci of the heterothallic *Pey. pinodella* (2.6 kb of *MAT1-1* and 3.1 kb of *MAT1-2*) revealed a *MAT1-1* idiomorph of 2.4 kb and a *MAT1-2* idiomorph of 2.8 kb. As indicated above, the amplification of mating-type sequences from *P. herbarum* was only successful for 40% of the isolates tested and only *MAT1-2* sequences were obtained. Chromosome walking resulted in the generation of 4.3 kb of *P. herbarum MAT1-2* sequences. BLAST2 analyses of the *P. herbarum MAT1-2* sequences against the *MAT1-1* sequences of *P. clematidina*, *D. vitalbina* and *Pey. pinodella* suggested that the *P. herbarum MAT1-2* idiomorph had a length of 3.2 kb (Table 4).

Upstream of the idiomorphs of all of these species, (part of) an additional open reading frame (ORF) was identified with highest similarity to *ORF1*, an ORF found near the idiomorph of other loculoascomycetes e.g. *Ascochyta lentis*, *A. rabiei* (Chérif *et al*., 2006), *Pyrenophora teres* and *Pyrenophora graminea* (Rau *et al*., 2007). Furthermore, part of an ORF similar to a DNA lyase was found downstream of the idiomorphs (Fig. 1a). DNA lyases are found more often near the *MAT* loci of other fungi (Arzanlou *et al*., 2010; Waalwijk *et al*., 2002).

Structural organization of homothallic mating-type loci

Chromosome walking along the genomic DNA of the homothallic species *Pey. pinodes* and *D. clematidis* resulted in the generation of 5.3 and 5.4 kb of sequence, respectively. The *D. clematidis MAT1/2* mating-type locus contained a *MAT1-1-1* located downstream on the same strand as the *MAT1-2-1* gene. Similar to the situation in the heterothallic fungi, upstream of the *D. clematidis* mating-type locus was an ORF with homology to *ORF1*, and a partial ORF with homology to a DNA lyase was detected downstream of the mating-type locus (Fig. 1b).

The situation in *Pey. pinodes*, the other homothallic species examined, was completely different. This locus also contained an intact *MAT1-1-1* downstream on the same strand as *MAT1-2-1*. Interestingly, in between *MAT1-2-1* and *MAT1-1-1*, an additional copy of *ORF1* was found. Finally, the orientation of this (*MAT1-1-1*/*ORF1*/*MAT1-2-1*) fusion locus was inverted compared with the situation in the other species (Fig. 1b).

Characterization of mating-type genes

An analysis of all the mating-type genes indicated a high similarity between the different species. The length of the predicted *MAT1-1-1* genes varied between 1120 and 1148 nucleotides. All genes contained a single predicted intron varying between 46 and 56 nucleotides (Table 5). The length of the predicted *MAT1-2-1* genes varied between 1094 and 1115 nucleotides, and all genes contained a single intron of 55 or 56 nucleotides (Table 5) The predicted intron in *MAT1-1-1* was located within sequences encoding the characteristic α-domain motif. This position matches exactly an intron position found in all dothideomycetous *MAT1-1-1* genes examined. Similarly, the predicted intron in *MAT1-2-1* was located

Table 3 Isolates used in phylogenetic analyses.

Collection number*	Mating type†	Host	Origin	GenBank no. ITS‡
Didymella clematidis				
CBS 123705, PD 97/13460–1, ICMP 13664	**1/2**	*Clematis ligusticifolia*	USA	FJ515593
Didymella. vitalbina				
CBS 454.64	?	*Clematis vitalba*	France	FJ515605
CBS 911.87	**1**	*Clematis vitalba*	Germany	FJ515592
PD 75/294	**1**	*Clematis* sp.	Unknown	FJ515596
CBS 123707, PD 97/13460–2, ICMP 13664	**1**	*Clematis vitalba*	Switzerland	FJ515595
PD 04373904-2B	2	*Clematis vitalba*	Netherlands	FJ515603
CBS 123706, PD 04373904–5	2	*Clematis vitalba*	Netherlands	FJ515594
PD 04417700–3	1	*Clematis vitalba*	Netherlands	FJ515604
Peyronellaea pinodella				
CBS 116.28	2	Unknown	Unknown	**JF810508**
CBS 108.31	2	Unknown	USA	**JF810509**
CBS 110.32, MUCL 292	1	*Medicago sativa*	Netherlands	EU167565
CBS 351.34, MUCL 9927, MUCL 18217	2	Unknown	Unknown	**JF810510**
CBS 107.46	1	*Pisum sativum*	Netherlands	**JF810511**
CBS 108.46	1	*Pisum sativum*	Netherlands	**JF810512**
CBS 403.65, PD 57/90, IMI 116998	1	Unknown	Unknown	**JF810513**
CBS 531.66	2	*Trifolium pratense*	USA	FJ427052
CBS 317.90, PD 77/948	1	*Trifolium* sp.	Netherlands	**JF810514**
CBS 318.90, PD 81/729	2	*Pisum sativum*	Netherlands	FJ427051
CBS 319.90, PD 84/207	2	*Beta vulgaris* var. *rubra*	Netherlands	**JF810515**
CBS 133.92	2	*Glycine soja*	Hungary	**JF810516**
Peyronellaea pinodes				
CBS 206.28	1/2	*Pisum* sp.	Unknown	**JF810517**
CBS 249.47	1/2	*Pisum sativum*	Scotland	**JF810518**
CBS 250.47	1/2	*Pisum sativum*	Netherlands	**JF810519**
CBS 251.47	1/2	*Pisum sativum*	Netherlands	**JF810520**
CBS 252.47	1/2	*Pisum sativum*	Netherlands	**JF810521**
CBS 329.51	1/2	*Pisum* sp.	Germany	**JF810522**
CBS 235.55	1/2	*Pisum* sp.	Unknown	GU237805
CBS 525.77	1/2	*Pisum sativum*	Belgium	GU237883
CBS 159.78	1/2	*Pisum sativum*	Iraq	GU237786
CBS 374.84, PD 79/674	1/2	*Pisum sativum*	Netherlands	**JF810523**
Phoma clematidina				
CBS 201.49	**2**	*Clematis* sp.	Netherlands	FJ426991
CBS 195.64	1	*Clematis jackmannii*	Netherlands	FJ426990
CBS 102.66	**2**	*Clematis* sp.	England	FJ426988
CBS 520.66, PD 64/657	**2**	*Selaginella* sp.	Netherlands	FJ426992
CBS 108.79, PD 78/522	1	*Clematis* sp.	Netherlands	FJ426989
PD 80/683	1	*Clematis* sp.	Netherlands	FJ515597
PD 91/1865	1	*Clematis* sp.	Netherlands	FJ515598
PD 95/895	1	*Clematis* sp.	Netherlands	FJ515599
PD 97/12061	1	*Clematis* 'Purple spider'	Netherlands	FJ515600
PD 97/12062	1	*Clematis* 'New Dawn'	Netherlands	FJ515601
Phoma herbarum				
CBS 276.37, PD 92/332, MUCL 9920	?	Wood pulp	Sweden	**JF810524**
CBS 368.61	**2**	*Ulmus* sp.	Netherlands	**JF810525**
CBS 369.61	?	*Ulmus* sp.	Netherlands	**JF810526**
CBS 370.61	**2**	*Ulmus* sp.	Netherlands	**JF810527**
CBS 567.63, ATCC 15053, MUCL 9889	?	*Malus sylvestris*	USA	**JF810528**
CBS 615.75, PD 73/665, IMI 199779	**2**	*Rosa multiflora*	Netherlands	FJ427022
CBS 502.91, PD 86/276	?	*Nerium* sp.	Netherlands	GU237874
CBS 503.91, PD 87/499	**2**	*Thuja* sp.	Netherlands	**JF810529**
CBS 829.97	?	Ornithogenic soil	Antarctica	**JF810530**
CBS 830.97	?	Soil from foot of glacier	Antarctica	**JF810531**
CBS 100953	?	Soil near glacier	Antarctica	**JF810532**
CBS 101145, ATCC 12569, IMI 049948	?	White lead paint	UK	AY293803
PD 85/930	**2**	*Streptocarpus* sp.	Unknown	**JF810533**
PD 87/652	?	Soil	Netherlands	**JF810534**
PD 90/1454	**2**	*Lycopersicon esculentum*	Netherlands	**JF810535**

*Bold numbers indicate strains for which the mating-type locus is fully sequenced. ATCC, American Type Culture Collection, Manassas, VA, USA; CBS, CBS Fungal Biodiversity Centre, Utrecht, the Netherlands; ICMP, International Collection of Micro-organisms from Plants, Auckland, New Zealand; IMI, International Mycological Institute, CABI-Bioscience, Egham, Surrey, UK; MUCL, (agro)industrial fungi and yeast collection of the Belgian Co-ordinated Collections of Micro-organisms (BCCM), Louvain-la-Neuve, Belgium; PD, Dutch Plant Protection Service, Wageningen, the Netherlands.

†Isolates marked as mating-type 1 were positive in polymerase chain reaction (PCR) with the *Phoma*-specific α-box (*MAT1-1-1*) primers; isolates marked as mating-type 2 were positive in PCR with the *Phoma*-specific HMG-motif (*MAT1-2-1*) primers; isolates positive with both *Phoma*-specific PCRs are marked with '1/2' and isolates in which both *Phoma*-specific PCRs were negative are marked with '?'. Numbers in bold indicate isolates with a positive result in the initial α-box (*MAT1-1-1*) PCR or HMG motif (*MAT1-2-1*) PCR.

‡Bold numbers indicate sequences determined in this study.

Table 4 Idiomorph lengths of the heterothallic *Phoma clematidina*, *Didymella vitalbina*, *Peyronellaea pinodella* and *Phoma herbarum*.

Species	*MAT1-1**	*MAT1-2*
Phoma clematidina	1.8	2.1
Didymella vitalbina	1.9	2.2
Peyronellaea pinodella	2.4	2.8
Phoma herbarum	–	3.2

*Length in kb.

within the HMG domain encoding sequence and at a position perfectly conserved in dothideomycetous *MAT1-2-1* genes (data not shown) (Arzanlou *et al*., 2010; Barve *et al*., 2003; Groenewald *et al*., 2006; Stergiopoulos *et al*., 2007; Turgeon *et al*., 1993). In addition, the sizes of the deduced MAT1-1-1 (varying between 357 and 364 amino acids) and MAT1-2-1 (varying between 345 and 352 amino acids) proteins were highly similar and within the range observed for other dothideomycetous MAT1-1-1 and MAT1-2-1 proteins (data not shown).

Pairwise comparisons of the deduced protein sequences indicated that MAT1-1-1 and MAT1-2-1 of the heterothallic *Pey. pinodella* were more similar to those of the homothallic *Pey. pinodes* (91.9% and 92.6% identity, respectively) than to those of another heterothallic species. The same was observed for MAT1-1-1 and MAT1-2-1 of the heterothallic *D. vitalbina* and the homothallic *D. clematidis*, with 87.9% and 83.9% identity.

Phylogenetic analysis

On the basis of the publication of Aveskamp *et al*. (2010), *Didymella urticicola* (GenBank accession number GU237761) was used as outgroup in the ITS phylogeny. The ITS alignment contained 58 strains, including the outgroup strain, and had a total length of 455 characters, 13 of which were parsimony uninformative and 26 were parsimony informative. The heuristic search resulted in two most parsimonious trees [tree length, 50 steps; consistency index (CI) = 0.860; retention index (RI) = 0.983; rescaled consistency index (RC) = 0.845]. The Bayesian analysis resulted in 6202 trees from which the 50% majority rule consensus tree and posterior probabilities were calculated. In both phylogenetic analyses, five well-supported clades were obtained dividing *P. herbarum*, *P. clematidina*, *D. vitalbina* and *D. clematidis*, but the *Pey. pinodella* and *Pey. pinodes* strains all clustered in one clade with 99% bootstrap support/0.99 posterior probability (Fig. 2).

For the phylogeny based on the *MAT1-1-1* and *MAT1-2-1* alignments, part of the known sequences of *Pyrenophora teres* and *Pyr. graminae* (Table 2) were used as outgroup. The alignments consisted of 31/33 strains, respectively, including the outgroup strains, and had a total length of 405/235 characters, seven/six of which were parsimony uninformative and 122/130 were parsimony informative. The search resulted in four most parsimonious trees (tree length, 208 steps; CI = 0.803; RI = 0.948; RC = 0.761) for the partial *MAT1-1-1* alignment and in one most parsimonious tree (tree length, 272 steps; CI = 0.721; RI = 0.929; RC = 0.670) for the *MAT1-2-1* alignment. The Bayesian analyses resulted in 2702 and 2962 trees, respectively, from which the 50% majority rule consensus tree and posterior probabilities were calculated (Fig. 3). Both analyses indicated that all species used clustered in well-supported clades, with *Pey. pinodella* close to *Pey. pinodes* and *D. vitalbina* close to *D. clematidis*.

Similarities between heterothallic and homothallic mating-type loci

The phylogenetic analyses, as well as the pairwise comparisons of the deduced MAT1-1-1 and MAT1-2-1 proteins, indicated that the heterothallic *Pey. pinodella* was closely related to the homothallic *Pey. pinodes*. Similarly, the heterothallic *D. vitalbina* was closely related to the homothallic *D. clematidis*. This was also shown by BLAST2 pairwise alignment of the sequenced *MAT1-1* and *MAT1-2* regions of *P. clematidina*, *Pey. pinodella*, *D. vitalbina* and *P. herbarum* to the mating-type loci of *Pey. pinodes* and *D. clematidis*. A graphical analysis of the pairwise alignments with highest levels of similarity, *Pey. pinodella* versus *Pey. pinodes* and *D. vitalbina* versus *D. clematidis*, is shown in Fig. 4. This analysis shows that the *D. clematidis MAT1/2* locus appears to consist of a fusion between *MAT1-1* and *MAT1-2* sequences of *D. vitalbina*. The 5′ region of the *D. clematidis MAT1/2* locus is highly similar to the entire *MAT1-2* idiomorph of *D. vitalbina*, whereas the 3′ region of the *D. clematidis MAT1/2* locus is highly similar to large parts of the *D. vitalbina MAT1-1* idiomorph. At the *MAT1/2* fusion junction, a small stretch of sequence identity between *MAT1-1* and *MAT1-2* could be identified (Fig. 4a).

The *MAT1/2* locus of *Pey. pinodes* seemingly consists of an inverted fusion product between *MAT1-1* and *MAT1-2* sequences of *Pey. pinodella*. Within the *MAT1/2* fusion product, sequences highly related to *ORF1*, located outside the *Pey. pinodella MAT* loci, can also be found. The 5′ region of the *Pey. pinodes MAT1/2* locus is highly similar to the entire inverted *MAT1-1* idiomorph of *Pey. pinodella*, and the 3′ region of the *Pey. pinodes MAT1/2* locus is highly similar to most of the inverted *Pey. pinodella MAT1-2* idiomorph (Fig. 4b). The position of the *MAT1/2* fusion junction could not be identified. However, upstream of *MAT1-2-1* and downstream of *MAT1-1-1*, the boundaries of the inversion could be identified. Interestingly, the sequence found at the 3′ boundary is highly similar to the sequence found at the 5′ boundary when reverse complemented. In total, 10 of 18 nucleotides and eight of the first 11 positions are shared between the two motifs (Fig. 5a).

DISCUSSION

Recently, molecular and morphological studies were initiated to unravel the phylogeny of the anamorphic genus *Phoma* and

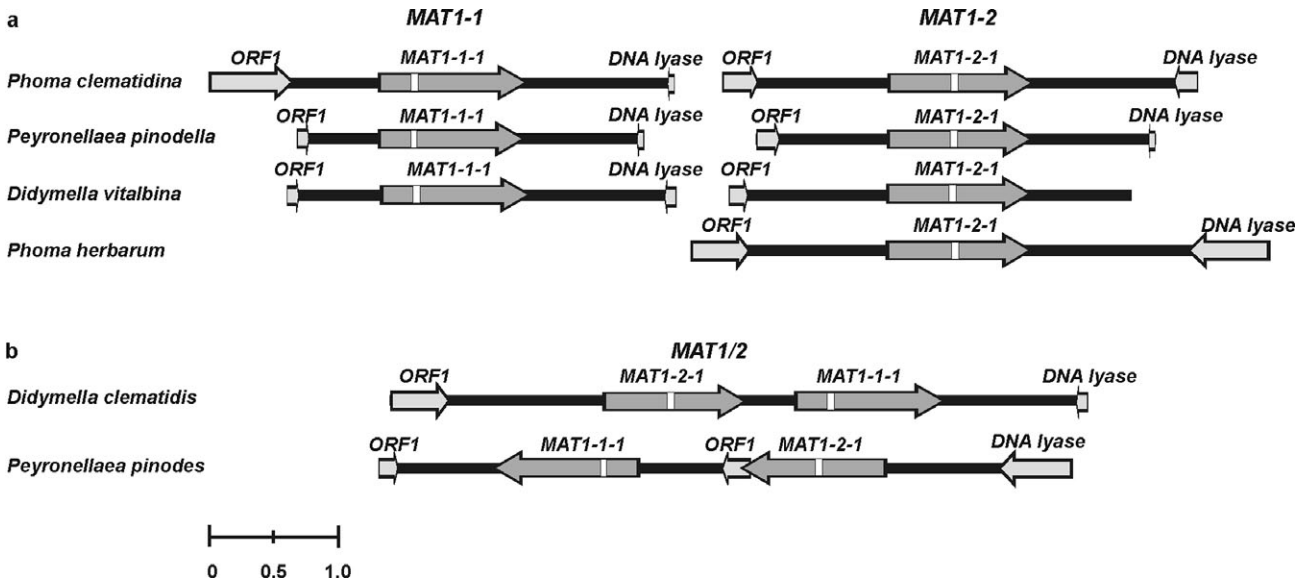

Fig. 1 Schematic representation of the organization of mating-type loci of the heterothallic *Phoma clematidina*, *P. herbarum*, *Peyronellaea pinodella* and *Didymella vitalbina* (a) and of the homothallic *Pey. pinodes* and *D. clematidis* (b). Mating-type genes are indicated by dark grey arrows, and other predicted (partial) gene models by light grey arrows. The positions of predicted introns are marked by white boxes. The marker at the bottom indicates a size of 1 kb.

Table 5 Comparison of the gene organization of *MAT1-1-1* and *MAT1-2-1* in idiomorphs of *Phoma clematidina*, *Didymella vitalbina*, *Didymella clematidis*, *Peyronellaea pinodella*, *Peyronellaea pinodes* and *Phoma herbarum*.

				*Mat1-1-1**			*Mat1-2-1**		
Species	Mating type	Gene size (nt)	Intron size (nt)	Intron position	Protein size (aa)	Gene size (nt)	Intron size (nt)	Intron position	Protein size (aa)
Phoma clematidina	Heterothallic	1143	48	245–292	364	1115	56	494–549	352
Didymella vitalbina	Heterothallic	1146	54	245–298	363	1106	56	484–540	349
Didymella clematidis	Homothallic	1148	56	245–300	363	1094	56	484–540	345
Peyronellaea pinodella	Heterothallic	1120	46	245–290	357	1108	55	488–542	350
Peyronellaea pinodes	Homothallic	1120	46	245–290	357	1111	55	488–542	351
Phoma herbarum	Heterothallic					1111	55	494–548	351

*aa, amino acids; nt, nucleotides.

associated genera, such as *Ascochyta*. This resulted in the establishment of a new family containing several *Phoma* and *Ascochyta* species (de Gruyter *et al*., 2009), the characterization of a disease complex occurring on *Clematis* sp. (Woudenberg *et al*., 2009) and the renaming of several species belonging to the disease complex causing Ascochyta blight (Aveskamp *et al*., 2010). These molecular analyses were based on sequences commonly used for fungal characterization, e.g. ITS, actin, β-tubulin. However, the use of mating-type genes in studies aimed at the elucidation of the phylogeny and species boundaries might provide better resolution. The mating-type genes are more variable than these barcode genes and evolve at a faster rate (Turgeon, 1998). Moreover, in addition to high inter-species variation, they generally exhibit low intra-species variation and therefore can be used to sort out species relationships in taxon-rich complexes. For example, the study of mating-type genes has helped to solve the phylogenetic relationship of the net and spot forms of *Pyrenophora teres* (Rau *et al*., 2005, 2007), the phylogeny of *Ascochyta* spp. associated with legumes (Barve *et al*., 2003), the relationship between oat- and wheat-infecting *Phaeosphaeria avenaria* (Ueng *et al*., 2003) and the recognition of species within the *Fusarium graminearum* complex (O'Donnell *et al*., 2004).

Our work, as well as that performed previously by others, has also clearly demonstrated the benefits of using mating-type sequences to determine species boundaries. *Peyronellaea pinodella* and *Pey. pinodes* could not be distinguished from each other on the basis of ITS sequences. However, the phylogeny based on *MAT1-1-1* and *MAT1-2-1* was capable of distinguishing between these two species (Fig. 3) (Barve *et al*., 2003), thus confirming the morphologically based characterization of the two species. This also demonstrates the risk associated with identification solely on the basis of molecular characters without real relevance for the lifestyle or biology of a species, e.g. ITS. This is especially relevant when studying/identifying quarantine

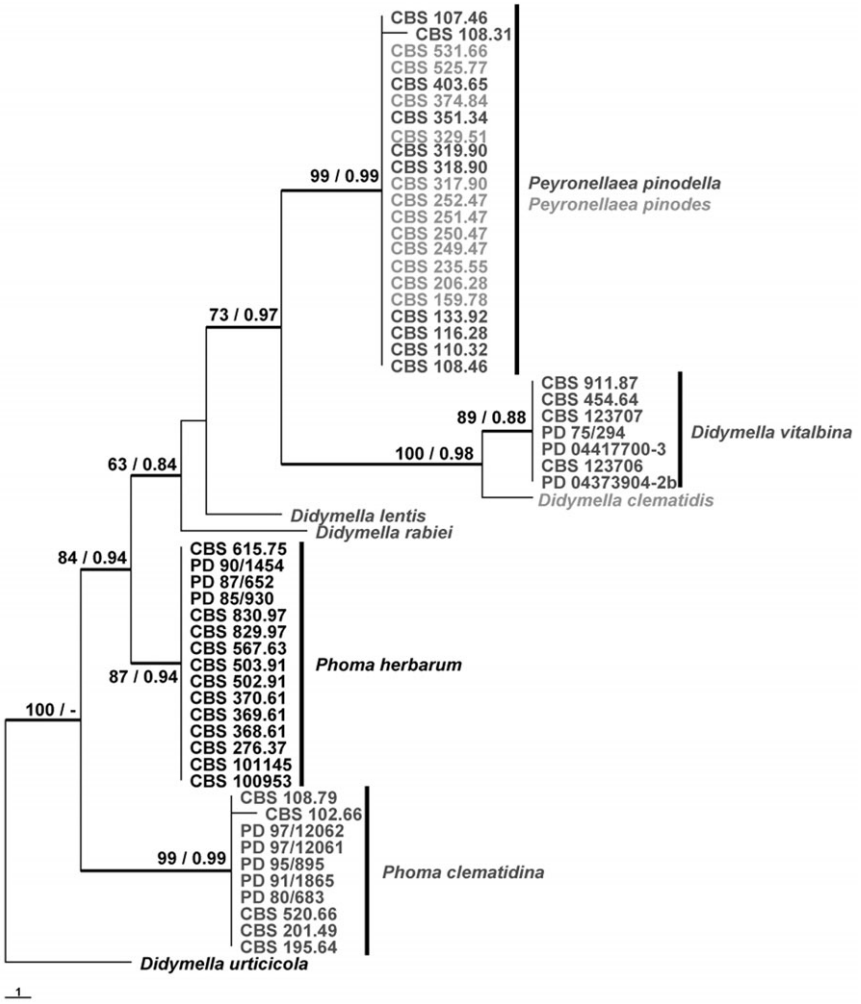

Fig. 2 One of the two most parsimonious trees obtained from a heuristic search of the internal transcribed spacer (ITS) sequence alignment. The scale bar shows one change, strict consensus branches are thickened and bootstrap support values from 1000 bootstrap replicates (BS) and posterior probabilities (PP) are shown at the nodes. BS/PP values below 60/0.6 are omitted. Confirmed heterothallic species are indicated in dark grey and confirmed homothallic species in light grey. The tree is rooted with *Didymella urticicola* GU237761. CBS, Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands; PD, Dutch National Reference Centre of the Plant Protection Service, Wageningen, the Netherlands.

organisms that are part of species complexes. Moreover, it also illustrates the enduring importance of classical morphology-based taxonomy.

Mating-type analysis can also provide a means to elucidate the sexual strategy of fungal plant pathogens. This is important as the reproductive strategy can influence the success of control (breeding) measurements. Many fungal species are classified as asexual within anamorphic genera because of the lack of a characterized teleomorph. Studies on mating-type loci of presumed asexual fungi have revealed that many of these species possess *MAT* genes, which may even be expressed (Kerenyi *et al*., 2004; Paoletti *et al*., 2005; Yun *et al*., 2000). This indicates that a lack of (obvious) sexual recombination is not a result of the absence of basal elements controlling the sexual reproductive machinery (Sharon *et al*., 1996). The fact that approximately one-fifth of all fungi still have no described sexual stage can be explained by the possibility that mating only occurs under specific environmental conditions and/or only within a time frame that exceeds normal laboratory crossing experiments (O'Gorman *et al*., 2008). In the case of cryptic heterothallic fungi, it can also be explained by the absence of one of the compatible mating types as a result of geographical separation, as has been reported for populations of *Didymella* (*Ascochyta*) *rabiei* (Barve *et al*., 2003; Kaiser and Kusmenoglu, 1997).

Our comparisons of the mating-type genes did not only distinguish between *Pey. pinodella* and *Pey. pinodes*, but also confirmed morphological studies, indicating that both species exhibit a completely different sexual strategy. The sexual state of *Pey. pinodella* has been described previously (Bowen *et al*., 1997) as heterothallic. In 1953, the homothallic nature of *Pey. pinodes* was confirmed by mono-ascospore cultures (Baumann, 1953). Our molecular studies confirm these conclusions. In addition, the predicted homothallic nature of *D. clematidis*, exhibiting both the teleomorph and anamorph states in pure culture (Woudenberg *et al*., 2009), is confirmed. On the basis of our molecular studies, we predict that *P. clematidina* and *D. vitalbina* are heterothallic species.

Amplification of the mating-type sequences from *P. herbarum* was only successful in six of the 15 isolates tested. None of the PCRs aimed at the amplification of *MAT1-1-1* sequences were

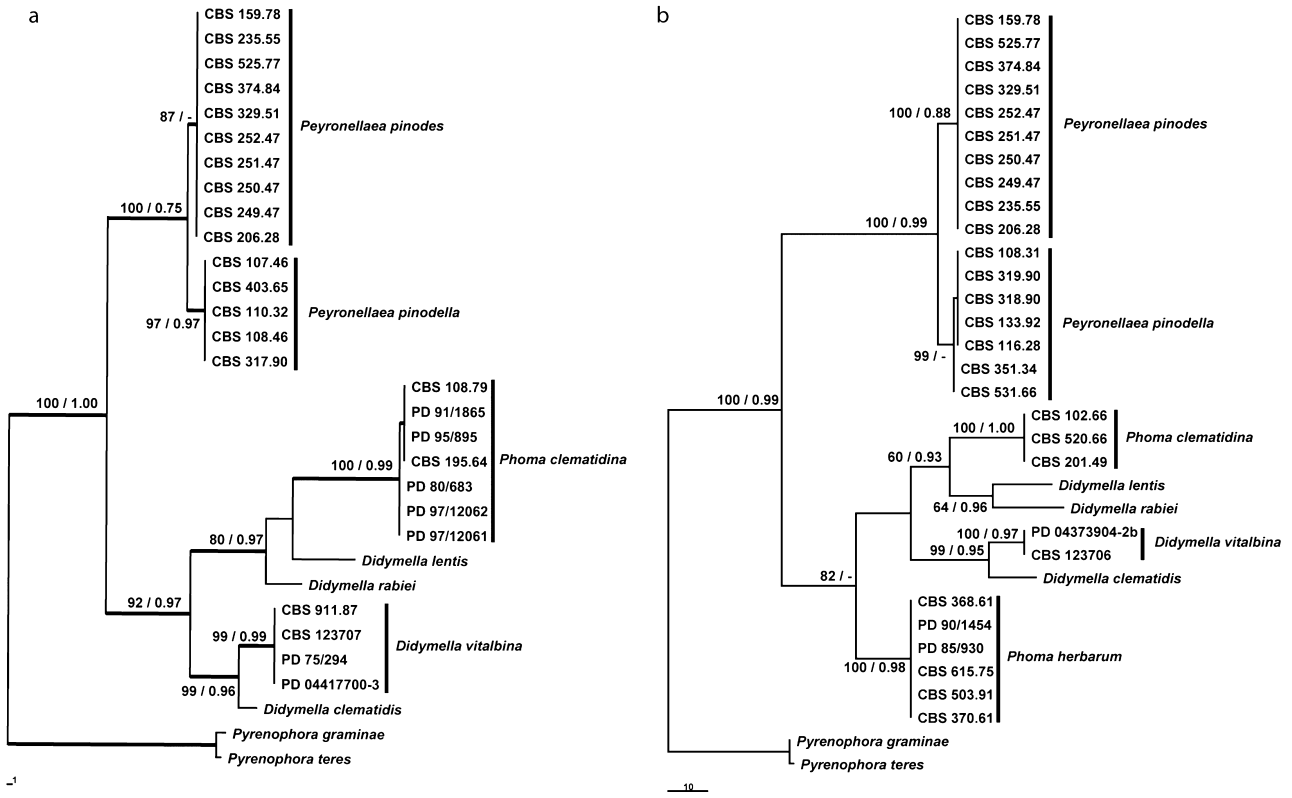

Fig. 3 Most parsimonious trees obtained from a heuristic search of the partial *MAT1-1-1* (a) and *MAT1-2-1* (b) sequence alignments. The scale bars show the changes; bootstrap support values from 1000 bootstrap replicates (BS) and posterior probabilities (PP) are shown at the nodes. BS/PP values below 60/0.6 are omitted. The trees are rooted with *Pyrenophora teres* and *Pyrenophora graminaea*. The first of four most parsimonious trees; the strict consensus branches are thickened. CBS, Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands; PD, Dutch National Reference Centre of the Plant Protection Service, Wageningen, the Netherlands.

successful and only *MAT1-2-1* sequences were obtained. The *MAT1-2* mating-type locus obtained exhibited the typical characteristics and organization observed for the heterothallic *P. clematidina*, *Pey. pinodella* and *D. vitalbina* (Fig. 1). The fact that the amplification of *MAT1-1-1* sequences remained unsuccessful despite the close phylogenetic relationship with the other examined species (Fig. 2) suggests that *MAT1-1-1* sequences in *P. herbarum* have either become absent or corrupted. Thus, these data suggest that *P. herbarum* might originally have been a functionally heterothallic fungus that lost its capacity for sexual reproduction and is now a genetically heterothallic, but functionally asexual, fungus. This is also supported by multilocus sequence analysis suggesting a clonal nature of *P. herbarum* isolates (M.M. Aveskamp, unpublished data).

A major question in fungal biology is whether homothallism has arisen from heterothallism or the other way round. Population genetics models have suggested that evolution from heterothallism to homothallism is the most likely scenario (Nauta and Hoekstra, 1992). Phylogenetic analyses and analyses of the *MAT* structure in *Cochliobolus* and *Stemphylium* species have shown that, in these species, heterothallism is indeed the ancestral state (Inderbitzin *et al*., 2005; Yun *et al*., 1999). In the genus *Aspergillus*, the ancestral state is still under debate. The predominance of known homothallic species over known heterothallic species, phylogenetic analyses and comparative genomics suggest that homothallism is the ancestral state in this genus (Galagan *et al*., 2005; Geiser *et al*., 1998; Varga *et al*., 2000). However, recent analysis of the *Neosartorya fischeri MAT* loci has suggested a heterothallic ancestral state (Rydholm *et al*., 2007).

In our study, the structural organization of the mating-type loci from all heterothallic species was conserved and similar to the organization of other loculoascomycetes (Chérif *et al*., 2006; Rau *et al*., 2005, 2007). In the two examined homothallic species, the mating-type locus contained both *MAT1-1-1* and *MAT1-2-1* (Fig. 1), whereas the genomic boundaries of the homothallic mating-type loci were identical to the flanking regions of the heterothallic *MAT* loci. Moreover, phylogenetic analysis showed that the homothallic mating-type genes did not cluster together, but clustered with heterothallic mating-type genes. All of these data strongly support the idea of an independent evolution of homothallism from a heterothallic ancestral state.

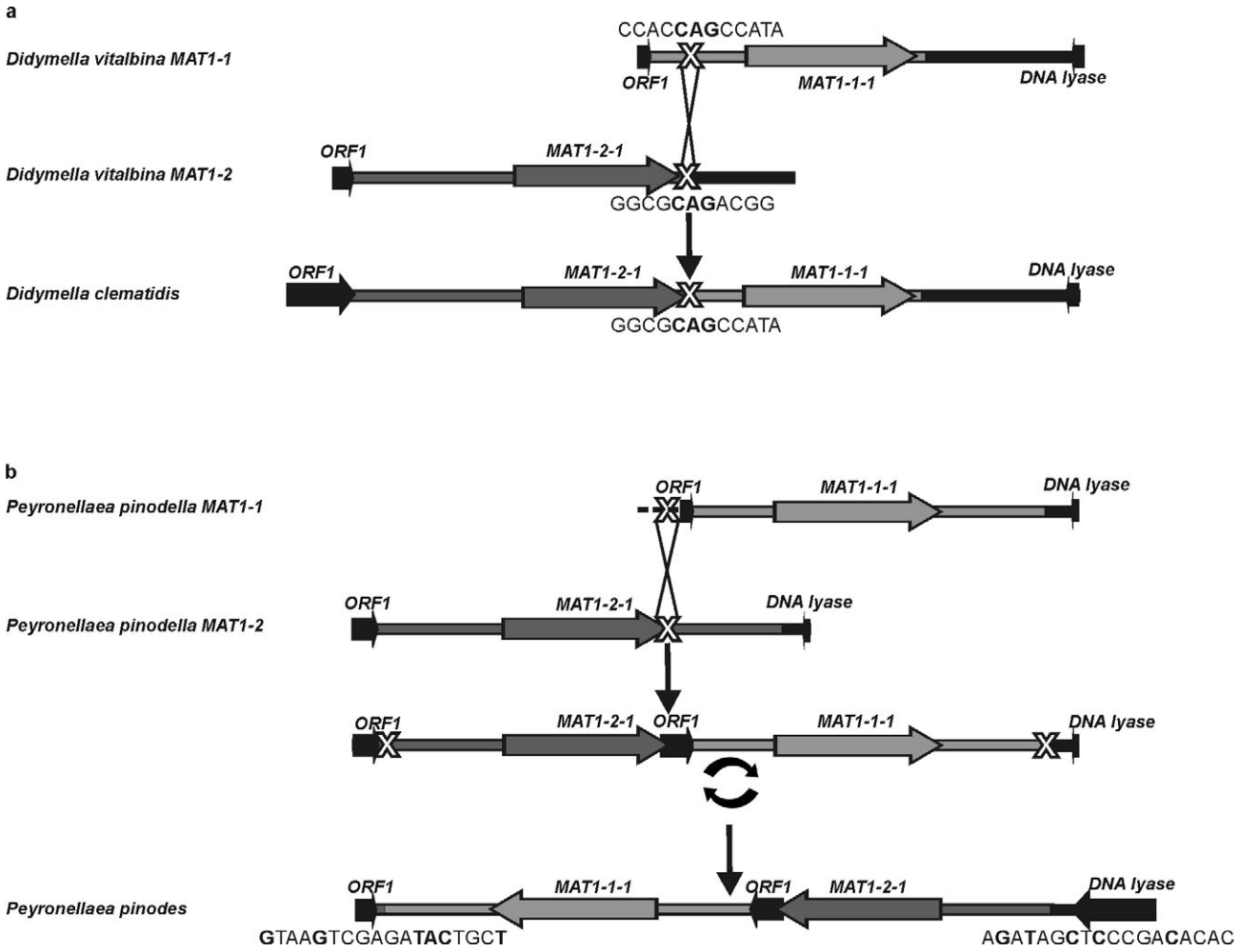

Fig. 4 Schematic overview of the sequence similarities between the mating-type loci of the heterothallic *Didymella vitalbina* and homothallic *D. clematidis* (a) and between the heterothallic *Peyronellaea pinodella* and homothallic *Pey. pinodes* (b). Also depicted are the putative recombination events responsible for the evolution of the homothallic species from heterothallic ancestors. Predicted gene models are indicated by arrows. Putative recombination spots are marked with a large 'X' and the putative inversion event is marked by a circular arrow. Sequences of the predicted junctions are indicated below the figure. All *MAT1-1*-derived areas are indicated in light grey; all *MAT1-2*-derived areas are indicated in dark grey; nonidiomorphic areas are indicated in black; sequences at fusion junctions that are shared between *MAT1-1* and *MAT1-2* are indicated in bold black.

The organization of the mating-type locus of the homothallic *D. clematidis* and the observed sequence similarity with the mating-type loci of the related heterothallic *D. vitalbina* could well be explained by a single crossover event between *MAT1-1* and *MAT1-2* sequences of heterothallic ancestors. The presence of a small stretch of sequence identity between *MAT1-1* and *MAT1-2* at the exact position of the *MAT1/2* fusion junction strongly suggests that the *D. clematidis MAT1/2* fusion locus originated from such a crossover event (Fig. 4a). The organization of the *D. clematidis MAT1/2* mating-type locus is very similar to the organization seen within homothallic *Crivellia*. In addition, in that species, the organization was explained by a crossover between a small stretch of identity shared between the mating-type loci of heterothallic ancestors (Inderbitzin *et al*., 2006). Moreover, the existence of several homothallic *Cochliobolus* sp. with *MAT* loci containing a (partial) *MAT1-1-1* fused to a (partial) *MAT1-2-1* has been explained by such recombination events (Yun *et al*., 1999)

The organization of the *MAT* locus of the homothallic *Pey. pinodes* is more complicated (Fig. 1) and, to some extent, resembles the organization observed in the homothallic *Cochliobolus kusanoi* (Yun *et al*., 1999). In *C. kusanoi*, part of a sequence, normally found downstream of the *MAT* loci in heterothallic species, is located between the mating-type genes. Similarly, in *Pey. pinodes*, *ORF1*, found upstream of the *MAT* locus in heterothallic species, is located in between *MAT1-1-1* and *MAT1-2-1* (Figs 1 and 4b). We propose that the organization of the *MAT1/2* locus of *Pey. pinodes* arose by a single crossover event followed by an inversion. The putative initial recombination event probably

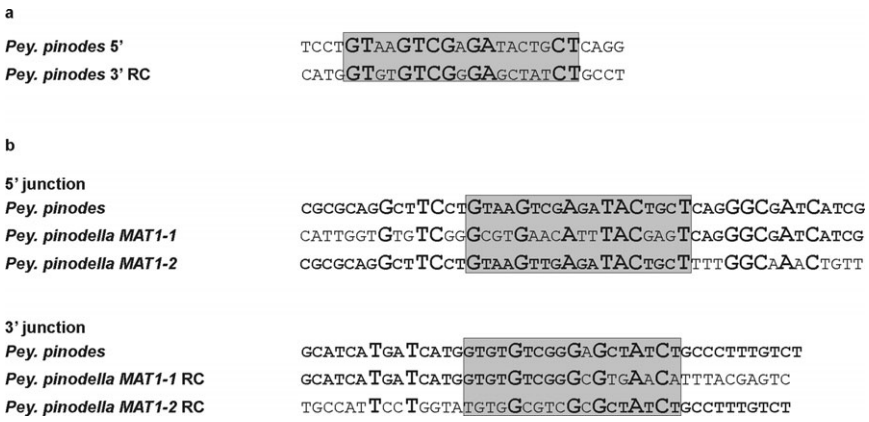

Fig. 5 Sequence analysis of inversion junctions in *Peyronellaea pinodes*. (a) Alignment of the 5′ junction of the putative inversion in *Pey. pinodes* to the reverse complemented (RC) sequence of the 3′ junction. The putative motif is indicated as a shaded box and identical nucleotides are marked in bold and have a larger font size. (b) Alignment of the 5′ and 3′ *Pey. pinodes* junctions to *Peyronellaea pinodella MAT1-1* and *MAT1-2* sequences. The sequences of the 3′ junctions are reverse complemented (RC) for better comparison of the putative motif (shaded box), as also indicated in (a). Identical nucleotides are indicated in bold and nucleotides shared by all three sequences have a larger font size.

occurred between sequences located at the 3′ end of *MAT1-2-1* and sequences found within *ORF1* upstream of the *MAT1-1* idiomorph. A subsequent inversion of this fused *MAT1-2/ORF1/MAT1-1* region could then have resulted in the observed *Pey. pinodes MAT1/2* organization. In contrast with *D. clematidis*, the position of the proposed initial recombination event (the *MAT1/2* fusion junction) could not be identified. This is because no sequence information about the putative crossover site in *ORF1* of *Pey. pinodella* was available. However, the junctions resulting from the proposed inversion events could clearly be identified (Figs. 4b and 5). The motif at the 5′ boundary of the proposed inversion is highly similar to the reverse complemented motif found at the 3′ boundary of the inversion. This suggests that, in an ancestor of *Pey. pinodes*, these regions of similarity formed a loop structure, resulting in crossover and subsequent inversion of the fused *MAT1/2* locus. Sequences resembling these motifs were also identified in both the *MAT1-1* and *MAT1-2* regions of *Pey. pinodella* (Fig. 5b), thus strengthening the hypothesis that *Pey. pinodes* originated from heterothallic ancestors, such as *Pey. pinodella*, after a single crossover event followed by an inversion. The proposed evolution of the homothallic *D. clematidis* and *Pey. pinodes* from heterothallic ancestors either resembling or identical to *D. vitalbina* and *Pey. pinodella*, is depicted in Fig. 4.

As mentioned previously, the phylogenetic analyses show that the mating-type genes of the heterothallic species *Pey. pinodella* and *D. vitalbina* are more related to the mating-type genes of the homothallic species *Pey. pinodes* and *D. clematidis*, respectively, than to the mating-type genes of other heterothallic phylogenetically related species. This observation suggests a common evolutionary history between *Pey. pinodella*/*Pey. pinodes*, on the one hand, and *D. vitalbina*/*D. clematidis* on the other. Interestingly, in both cases, the heterothallic species and the closely related homothallic species share the same host. *Peyronellaea pinodella* and *Pey. pinodes* are part of the Ascochyta blight complex on pea, whereas *D. vitalbina* and *D. clematidis* are both pathogens on *Clematis* spp. It has been shown that the presence of multiple closely related species on the same host can correlate with great evolutionary dynamics acting at mating-type loci (Arzanlou *et al*., 2010) with potential implications for speciation. The occurrence of recombinations as described above at *MAT* loci can lead to reproductive isolation between homothallic and heterothallic isolates, and result in the establishment of new species. Additionally, the co-occurrence of species on a single host could also lead to close physical interactions and potentially even to the exchange of genetic material through inter- and intraspecies mating, hybridization or anastomosis. This could also result in the establishment of novel species. Therefore, we postulate that the co-occurrence of multiple (related) species in both time and space on a single host (species complex) can be both the cause and consequence of (multiple) speciation events.

EXPERIMENTAL PROCEDURES

Isolates, culture and DNA extraction

All isolates used in this study are listed in Table 3. Strains of the species associated with the disease complex on *Clematis*, *D. clematidis*, *D. vitalbina* and *P. clematidina*, and the closely related species causing Ascochyta blight of pea, *Pey. pinodella* and *Pey. pinodes*, together with the type species of *Phoma*, *P. herbarum*, were selected. Freeze–dried strains were obtained from the culture collections of the Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands and the Dutch National Reference Centre of the Plant Protection Service (PD), Wageningen, the Netherlands, and were revived in 2 mL of malt/peptone (50%/50%) liquid medium. Subsequently, the cultures were transferred and maintained on oatmeal agar (OA) (Crous *et al*., 2009). DNA extractions were performed using an Ultraclean Microbial DNA Isolation Kit (Mobio Laboratories,

Carlsbad, CA, USA), according to the manufacturer's instructions. All DNA extracts were diluted 10 times in MilliQ water and stored at 4 °C before use.

Isolation of partial *MAT* sequences

The primers HMG-L and HMG-R, originally used for the amplification of the HMG motif of *A. rabiei* (teleomorph: *Didymella rabiei*) (Barve *et al*., 2003), as well as the primers 1 and 2 described for the amplification of the α-box of *L. maculans* (anamorph: *Phoma lingam*) (Cozijnsen and Howlett, 2003), were used in an attempt to amplify part of the mating-type genes of the isolates presented in Table 3. The PCR mixture contained 0.5 μL of diluted genomic DNA, 2 μM of each primer, 0.5 U of BIOTAQ DNA polymerase (Bioline, Luckenwalde, Germany), 0.1 mM deoxynucleoside triphosphates (dNTPs), 2.5 mM $MgCl_2$ and 1 × NH_4 reaction buffer (Bioline). Conditions for the amplification were an initial denaturation step of 5 min at 94 °C, followed by 40 cycles of 45 s at 94 °C, 45 s at 55 °C and 45 s at 72 °C, and a final elongation step of 7 min at 72 °C. The PCR products were visualized by electrophoresis and sequenced in both directions using PCR primers and the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA), following the manufacturer's instructions.

Chromosome walking, idiomorph PCR, specific HMG domain and *MAT-1* PCR

The partial *MAT* sequences obtained were aligned in Bionumerics v4.60 (Applied Maths, Sint-Martens-Latem, Belgium). Primer3 v0.4.0 (Rozen and Skaletsky, 2000) and the program Vector NTI Advanced 10 (Invitrogen, Carlsbad, CA, USA) were used to create additional primers that were subsequently employed to determine additional sequences up- and downstream of the partial *MAT* sequences, and, finally, the complete *MAT* idiomorph. This chromosome walking was performed using the DNA Walking Speedup Kit (Seegene Inc., Rockville, MD, USA), according to the manufacturer's instructions. The products obtained by chromosome walking were ligated into pGEM®-T easy vector (Promega, Madison, WI, USA) according to the manufacturer's instructions. The vector with insert was transformed into JM109-competent *Escherichia coli* cells (Promega). Recombinants were analysed by colony PCR with the universal M13F and M13R primers. The colony PCR mixture contained 1.5 μL of liquid colony, 0.2 μM of each primer, 0.4 U BIOTAQ DNA polymerase (Bioline), 0.03 mM dNTPs, 1.5 mM $MgCl_2$ and 1 × NH_4 reaction buffer (Bioline). Conditions for the amplification were an initial denaturation step of 5 min at 94 °C, followed by 35 cycles of 20 s at 94 °C, 20 s at 55 °C and 100 s at 72 °C, and a final elongation step of 7 min at 72 °C. The PCR products were visualized on gels and sequenced as described above using M13F and M13R universal primers.

The multiple sequence products obtained by the chromosome walking procedure were assembled and edited in Bionumerics v4.60 (Applied Maths). BLASTX analysis of the assembled sequences against the National Center for Biotechnology Information (NCBI) nonredundant protein database (Altschul *et al*., 1997) was used to predict the end of the idiomorphs.

New primers were designed on the basis of the flanking regions of the idiomorphs and five reference sequences (Table 2), and employed in an attempt to amplify directly the entire idiomorph of the species used in this study (Table 1). The idiomorph PCR mixture contained 1.0 μL of diluted DNA, 0.2 μM of each primer, 1 U BIOTAQ DNA polymerase (Bioline), 0.1 mM dNTPs, 2 mM $MgCl_2$ and 1 × NH_4 reaction buffer (Bioline). Conditions for the amplification were an initial denaturation step of 5 min at 94 °C, followed by 35 cycles of 45 s at 94 °C, 45 s at 58 °C and 3 min at 72 °C, and a final elongation step of 10 min at 72 °C. The products were cloned as described above and subsequently fully sequenced.

Based on the newly sequenced idiomorphs, *Phoma*-specific *MAT1* and HMG motif primers were designed (Table 1). The PCR mixture in this *Phoma*-specific PCR contained 1 μL of diluted genomic DNA, 0.2 μM of each primer, 0.5 U BIOTAQ DNA polymerase (Bioline), 0.06 mM dNTPs, 2.4 mM $MgCl_2$ and 1 × NH_4 reaction buffer (Bioline). Conditions for the amplification were an initial denaturation step of 5 min at 94 °C, followed by 10 cycles of 30 s at 94 °C and 30 s at 65 °C with a decrease of 0.7 °C every cycle, and 30 s at 72 °C, followed by 30 cycles with an annealing temperature of 58 °C, and a final elongation step of 7 min at 72 °C.

For the species for which no *MAT1-1* and/or *MAT1-2* idiomorphs had been obtained, the newly obtained HMG motif or partial *MAT1-1* PCR sequences were used to perform additional chromosome walking as described above.

The gene-finding software FGENESH (Softberry Inc., Mount Kisco, NY, USA) was used to predict the gene structure of all mating-type genes. Lasergene Seqbuilder v7.2.1(DNASTAR Inc., Madison, WI, USA) software was used to make a graphical representation of the structural organization of the mating genes.

Phylogenetic analysis

Phylogenetic analyses were performed on parts of the ITS1, ITS2 and 5.8S rRNA gene (ITS), and on parts of the *MAT1-1-1* and *MAT1-2-1* sequences obtained using the *Phoma*-specific primers. The primers V9G (de Hoog and Gerrits van den Ende, 1998) and ITS4 (White *et al*., 1990) were used for the amplification of the ITS region, as described previously (Woudenberg *et al*., 2009). The multiple sequence alignments were made with MAFFT v6.850b (http://mafft.cbrc.jp/alignment/server/index.html), using the L-INS-i setting (Katoh *et al*., 2005). Phylogenetic analyses of the sequence data consisted of a parsimony analysis conducted in PAUP v4.0b10 (Swofford, 2003) and a Bayesian analysis conducted with MrBayes v3.1.2 (Huelsenbeck and Ronquist, 2001). In the parsimony analyses, the heuristic search option with 100 random taxa additions was used, with tree bisection and reconstruction (TBR) as the branch-swapping algorithm. Alignment gaps were treated as missing. The robustness of the parsimony tree was evaluated by 1000 bootstrap replicates (Hillis and Bull, 1993). The Bayesian analyses were run with a GTR model with gamma-distributed rate variation for the ITS alignment and an HKY model with gamma-distributed rate variation for the *MAT1-1-1* and *MAT1-2-1* alignments; models were selected using Findmodel (http://www.hiv.lanl.gov/content/sequence/findmodel/findmodel.html). Further settings included a 'temperature' value of 0.05, five million generations and a sample frequency of 100. The run was automatically stopped as soon as the average standard deviation of split frequencies dropped below 0.01. The resulting trees were printed with Treeview v1.6.6 (Page, 1996)

Nucleotide sequence accession numbers

All sequences generated were deposited in GenBank. ITS sequences were deposited with accession numbers JF810508–JF810535. *MAT1-1*

mating-type loci of *P. clematidina*, *Pey. pinodella* and *D. vitalbina* were deposited with accession numbers JF815528, JF815529 and JF815527, respectively. The sequences of the *MAT1-2* mating-type loci of *P. clematidina*, *Pey. pinodella*, *D. vitalbina* and *P. herbarum* were deposited with accession numbers JF815530, JF815531, JF815532 and JF815526, respectively. The sequences of the mating-type loci of *Pey. pinodes* and *D. clematidis* were deposited with accession numbers JF815533 and JF815534, respectively.

ACKNOWLEDGEMENTS

This research was financially supported by the Dutch Ministry of Agriculture, Nature and Food Quality through an endowment of the Fonds Economische Structuurversterking programme 'Versterking Infrasctructuur Plantgezondheid'. The authors would like to thank J. Z. Groenewald (CBS-KNAW Fungal Biodiversity Centre, Utrecht, the Netherlands) for guidance in the phylogenetic analyses and two anonymous reviewers for critical reading of the manuscript and helpful comments.

REFERENCES

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res*. **25**, 3389–3402.

Arzanlou, M., Crous, P.W. and Zwiers, L.-H. (2010) Evolutionary dynamics of mating-type loci of *Mycosphaerella* spp. occurring on banana. *Eukaryot. Cell*, **9**, 164–172.

Aveskamp, M.M., De Gruyter, J. and Crous, P.W. (2008) Biology and recent developments in the systematics of *Phoma*, a complex genus of major quarantine significance. *Fungal Divers.* **31**, 1–18.

Aveskamp, M.M., Verkley, G.J.M., de Gruyter, J., Murace, M.A., Perello, A., Woudenberg, J.H.C., Groenewald, J.Z. and Crous, P.W. (2009a) DNA phylogeny reveals polyphyly of *Phoma* section *Peyronellaea* and multiple taxonomic novelties. *Mycologia*, **101**, 363–382.

Aveskamp, M.M., Woudenberg, J.H.C., De Gruyter, J., Turco, E., Groenewald, J.Z. and Crous, P.W. (2009b) Development of taxon-specific sequence characterized amplified region (SCAR) markers based on actin sequences and DNA amplification fingerprinting (DAF): a case study in the *Phoma exigua* species complex. *Mol. Plant Pathol*. **10**, 403–414.

Aveskamp, M.M., de Gruyter, J., Woudenberg, J.H.C., Verkley, G.J.M. and Crous, P.W. (2010) Highlights of the *Didymellaceae*: a polyphasic approach to characterise *Phoma* and related pleosporalean genera. *Stud. Mycol*. **65**, 1–60.

Barve, M.P., Arie, T., Salimath, S.S., Muehlbauer, F.J. and Peever, T.L. (2003) Cloning and characterization of the mating type (MAT) locus from *Ascochyta rabiei* (teleomorph: *Didymella rabiei*) and a MAT phylogeny of legume-associated *Ascochyta* spp. *Fungal Genet. Biol*. **39**, 151–167.

Baumann, G. (1953) Untersuchungen zur Biologie van *Mycosphaerella pinodes* (Berk. et. Blox.) Stone. *Kühn-Archiv*. **67**, 305–383.

Begerow, D., Nilsson, H., Unterseher, M. and Maier, W. (2010) Current state and perspectives of fungal DNA barcoding and rapid identification procedures. *Appl. Microbiol. Biotechnol*. **87**, 99–108.

Boerema, G.H. (1997) Contributions towards a monograph of *Phoma* (coelomycetes). 5. Subdivision of the genus in sections. *Mycotaxon*, **64**, 321–333.

Boerema, G.H. and Bollen, G.J. (1975) Conidiogenesis and conidial septation as differentiating criteria between *Phoma* and *Ascochyta*. *Persoonia*, **8**, 111–144.

Boerema, G.H., De Gruyter, J., Noordeloos, M.E. and Hamers, M.E.C. (2004) *Phoma Identification Manual. Differentiation of Specific and Infra-Specific Taxa in Culture*. Wallingford, CT: CABI Publishing.

Bowen, J.K., Lewis, B.G. and Matthews, P. (1997) Discovery of the teleomorph of *Phoma medicaginis* var. *pinodella* in culture. *Mycol. Res*. **101**, 80–84.

Brewer, J.G. and Boerema, G.H. (1965) Electron microscope observations on the development of pycnidiospores in *Phoma* and *Ascochyta* spp. *Proc. K. Ned. Akad. Wet. C*, **68**, 86–97.

Buchanan, P.K. (1987) A reappraisal of *Ascochytula and Ascochytella* (coelomycetes). In: *Mycological Papers*, Vol. 156, pp. 1–83. Kew, Surrey: CABI Publishing.

Chérif, M., Chilvers, M., Akamatsu, H., Peever, T.L. and Kaiser, W. (2006) Cloning of the mating type locus from *Ascochyta lentis* (teleomorph: *Didymella lentis*) and development of a multiplex PCR mating assay for *Ascochyta* species. *Curr. Genet*. **50**, 203–215.

Chilvers, M.I., Rogers, J.D., Dugan, F.M., Stewart, J.E., Chen, W. and Peever, T.L. (2009) *Didymella pisi* sp. nov., the teleomorph of *Ascochyta pisi*. *Mycol. Res*. **113**, 391–400.

Coppin, E., Debuchy, R., Arnaise, S. and Picard, M. (1997) Mating types and sexual development in filamentous ascomycetes. *Microbiol. Mol. Biol. Rev*. **61**, 411–428.

Cozijnsen, A.J. and Howlett, B.J. (2003) Characterisation of the mating-type locus of the plant pathogenic ascomycete *Leptosphaeria maculans*. *Curr. Genet*. **43**, 351–357.

Crous, P.W., Verkley, G.J.M., Groenewald, J.Z. and Samson, R.A. (eds) (2009) *CBS Laboratory Manual Series 1: Fungal Biodiversity*. Utrecht: CBS-KNAW Fungal Biodiversity Centre.

Davidson, J.A., Hartley, D., Priest, M., Krysinska-Kaczmarek, M., Herdina, McKay, A. and Scott, E.S. (2009) A new species of *Phoma* causes ascochyta blight symptoms on field peas (*Pisum sativum*) in South Australia. *Mycologia*, **101**, 120–128.

Faris-Mokaiesh, S., Boccara, M., Denis, J., Derrien, A. and Spire, D. (1996) Differentiation of the '*Ascochyta* complex' fungi of pea by biochemical and molecular markers. *Curr. Genet*. **29**, 182–190.

Fatehi, J., Bridge, P.D. and Punithalingam, E. (2003) Molecular relatedness within the '*Ascochyta pinodes*-complex'. *Mycopathologia*, **156**, 317–327.

Galagan, J.E., Calvo, S.E., Cuomo, C., Ma, L.-J., Wortman, J.R., Batzoglou, S., Lee, S.-I., Basturkmen, M., Spevak, C.C., Clutterbuck, J., Kapitonov, V., Jurka, J., Scazzocchio, C., Farman, M., Butler, J., Purcell, S., Harris, S., Braus, G.H., Draht, O., Busch, S., D'Enfert, C., Bouchier, C., Goldman, G.H., Bell-Pedersen, D., Griffiths-Jones, S., Doonan, J.H., Yu, J., Vienken, K., Pain, A., Freitag, M., Selker, E.U., Archer, D.B., Penalva, M.A., Oakley, B.R., Momany, M., Tanaka, T., Kumagai, T., Asai, K., Machida, M., Nierman, W.C., Denning, D.W., Caddick, M., Hynes, M., Paoletti, M., Fischer, R., Miller, B., Dyer, P., Sachs, M.S., Osmani, S.A. and Birren, B.W. (2005) Sequencing of *Aspergillus nidulans* and comparative analysis with *A. fumigatus* and *A. oryzae*. *Nature*, **438**, 1105–1115.

Geiser, D.M., Frisvad, J.C. and Taylor, J.W. (1998) Evolutionary relationships in *Aspergillus* section *Fumigati* inferred from partial β-tubulin and hydrophobin DNA sequences. *Mycologia*, **90**, 831–845.

Groenewald, M., Groenewald, J.Z., Harrington, T.C., Abeln, E.C.A. and Crous, P.W. (2006) Mating type gene analysis in apparently asexual *Cercospora* species is suggestive of cryptic sex. *Fungal Genet. Biol*. **43**, 813–825.

de Gruyter, J., Aveskamp, M.M., Woudenberg, J.H.C., Verkley, G.J.M., Groenewald, J.Z. and Crous, P.W. (2009) Molecular phylogeny of *Phoma* and allied anamorph genera: towards a reclassification of the *Phoma* complex. *Mycol. Res*. **113**, 508–519.

de Gruyter, J., Woudenberg, J.H.C., Aveskamp, M.M., Verkley, G.J.M., Groenewald, J.Z. and Crous, P.W. (2010) Systematic reappraisal of species in *Phoma* section *Paraphoma*, *Pyrenochaeta* and *Pleurophoma*. *Mycologia*, **102**, 1066–1081.

Hillis, D.M. and Bull, J.J. (1993) An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. *Syst. Biol*. **42**, 182–192.

de Hoog, G.S. and Gerrits van den Ende, A.H. (1998) Molecular diagnostics of clinical strains of filamentous Basidiomycetes. *Mycoses*, **41**, 183–189.

Huelsenbeck, J.P. and Ronquist, F. (2001) MRBAYES: bayesian inference of phylogenetic trees. *Bioinformatics*, **17**, 754–755.

Inderbitzin, P., Harkness, J., Turgeon, B.G. and Berbee, M.L. (2005) Lateral transfer of mating system in *Stemphylium*. *Proc. Natl. Acad. Sci. USA*, **102**, 11 390–11 395.

Inderbitzin, P., Shoemaker, R.A., O'Neill, N.R., Turgeon, B.G. and Berbee, M.L. (2006) Systematics and mating systems of two fungal pathogens of opium poppy: the heterothallic *Crivellia papaveracea* with a *Brachycladium penicillatum* asexual state and a homothallic species with a *Brachycladium papaveris* asexual state. *Can. J. Bot*. **84**, 1304–1326.

Kaiser, W.J. and Kusmenoglu, I. (1997) Distribution of mating types and the teleomorph of *Ascochyta rabiei* on chickpea in Turkey. *Plant Dis*. **81**, 1284–1287.

Katoh, K., Kuma, K.-I., Toh, H. and Miyata, T. (2005) MAFFT version 5: improvement in accuracy of multiple sequence alignment. *Nucleic Acids Res*. **33**, 511–518.

Kerenyi, Z., Moretti, A., Waalwijk, C., Olah, B. and Hornok, L. (2004) Mating type sequences in asexually reproducing *Fusarium* species. *Appl. Environ. Microbiol*. **70**, 4419–4423.

Metzenberg, R.L. and Glass, N.L. (1990) Mating type and mating strategies in *Neurospora*. *Bioessays*, **12**, 53–59.

Nauta, M.J. and Hoekstra, R.F. (1992) Evolution of reproductive systems in filamentous ascomycetes. 1. Evolution of mating types. *Heredity*, **68**, 405–410.

O'Donnell, K., Ward, T.J., Geiser, D.M., Kistler, H.C. and Aoki, T. (2004) Genealogical concordance between the mating type locus and seven other nuclear genes supports formal recognition of nine phylogenetically distinct species within the *Fusarium graminearum* clade. *Fungal Genet. Biol.* **41**, 600–623.

O'Gorman, C.M., Fuller, H.T. and Dyer, P.P.S. (2008) Discovery of a sexual cycle in the opportunistic fungal pathogen *Aspergillus fumigatus*. *Nature*, **457**, 471–474.

Onfroy, C., Tivoli, B., Corbière, R. and Bouznad, Z. (1999) Cultural, molecular and pathogenic variability of *Mycosphaerella pinodes* and *Phoma medicaginis* var. *pinodella* isolates from dried pea (*Pisum sativum*) in France. *Plant Pathol.* **48**, 218–229.

Page, R.D.M. (1996) TreeView: an application to display phylogenetic trees on personal computers. *Comput. Appl. Biosci.* **12**, 357–358.

Paoletti, M., Rydholm, C., Schwier, E.U., Anderson, M.J., Szakacs, G., Lutzoni, F., Debeaupuis, J.-P., Latge, J.-P., Denning, D.W. and Dyer, P.S. (2005) Evidence for sexuality in the opportunistic fungal pathogen *Aspergillus fumigatus*. *Curr. Biol.* **15**, 1242–1248.

Peever, T. (2007) Role of host specificity in the speciation of *Ascochyta* pathogens of cool season food legumes. *Eur. J. Plant Pathol.* **119**, 119–126.

Peever, T.L., Barve, M.P. and Stone, L.J. (2007) Evolutionary relationships among *Ascochyta* species infecting wild and cultivated hosts in the legume tribes Cicereae and Vicieae. *Mycologia*, **99**, 59–77.

Punithalingam, E. (1979) *Graminicolous Ascochyta* species. In: *Mycological Papers*, Vol. 142, pp. 1–214. Kew, Surrey: CABI Publishing.

Rau, D., Maier, F.J., Papa, R., Brown, A.H., Balmas, V., Saba, E., Schaefer, W. and Attene, G. (2005) Isolation and characterization of the mating-type locus of the barley pathogen *Pyrenophora teres* and frequencies of mating-type idiomorphs within and among fungal populations collected from barley landraces. *Genome*, **48**, 855–869.

Rau, D., Attene, G., Brown, A., Nanni, L., Maier, F., Balmas, V., Saba, E., Schäfer, W. and Papa, R. (2007) Phylogeny and evolution of mating-type genes from *Pyrenophora teres*, the causal agent of barley 'net blotch' disease. *Curr. Genet.* **51**, 377–392.

Rozen, S. and Skaletsky, H.J. (2000) Primer 3 on the WWW for general users and for biologist programmers. In: *Bioinformatics Methods and Protocols: Methods in Molecular Biology* (Krawetz, S. and Misener, S., eds), pp. 365–386. Totowa, NJ: Humana Press.

Rydholm, C., Dyer, P.S. and Lutzoni, F. (2007) DNA sequence characterization and molecular evolution of MAT1 and MAT2 mating-type loci of the self-compatible ascomycete mold *Neosartorya fischeri*. *Eukaryot. Cell*, **6**, 868–874.

Seifert, K.A. (2009) Progress towards DNA barcoding of fungi. *Mol. Ecol. Resour.* **9**, 83–89.

Sharon, A., Yamaguchi, K., Christiansen, S., Horwitz, B.A., Yoder, O.C. and Turgeon, B.G. (1996) An asexual fungus has the potential for sexual development. *Mol. Gen. Genet.* **251**, 60–68.

Stergiopoulos, I., Groenewald, M., Staats, M., Lindhout, P., Crous, P.W. and De Wit, P.J.G.M. (2007) Mating-type genes and the genetic structure of a world-wide collection of the tomato pathogen *Cladosporium fulvum*. *Fungal Genet. Biol.* **44**, 415–429.

Swofford, D.L. (2003) *PAUP*: Phylogenetic Analysis Using Parsimony (* and Other Methods). Version 4*. Sunderland, MA: Sinauer Associates.

Turgeon, B.G. (1998) Application of mating type gene technology to problems in fungal biology. *Annu. Rev. Phytopathol.* **36**, 115–137.

Turgeon, B.G. and Yoder, O.C. (2000) Proposed nomenclature for mating type genes of filamentous ascomycetes. *Fungal Genet. Biol.* **31**, 1–5.

Turgeon, B.G., Bohlmann, H., Ciuffetti, L.M., Christiansen, S.K., Yang, G., Schafer, W. and Yoder, O.C. (1993) Cloning and analysis of the mating-type genes from *Cochliobolus heterostrophus*. *Mol. Gen. Genet.* **238**, 270–284.

Ueng, P.P., Dai, Q., Cui, K., Czembor, P.C., Cunfer, B.M., Tsang, H., Arseniuk, E. and Bergstrom, G. (2003) Sequence diversity of mating-type genes in *Phaeosphaeria avenaria*. *Curr. Genet.* **43**, 121–130.

Varga, J., Vida, Z., Toth, B., Debets, F. and Horie, Y. (2000) Phylogenetic analysis of newly described *Neosartorya* species. *Anton. Leeuw. Int. J. G.* **77**, 235–239.

Waalwijk, C., Mendes, O., Verstappen, E.C.P., de Waard, M.A. and Kema, G.H.J. (2002) Isolation and characterization of the mating-type idiomorphs from the wheat septoria leaf blotch fungus *Mycosphaerella graminicola*. *Fungal Genet. Biol.* **35**, 277–286.

White, T.J., Bruns, T., Lee, S. and Taylor, J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols: A Guide to Methods and Applications* (Innis, M.A., Gelfand, D.H., Sninsky, J.J. and White, T.J., eds), pp. 315–322. San Diego, CA: Academic Press.

Wollenweber, H.W. and Hochapfel, H. (1936) Beiträge zur Kenntnis parasitärer und saprophytotischer Pilze. I. *Phomopsis*, *Dendrophoma*, *Phoma* und *Ascochyta* und ihre Beziehung zur Fruchtfäule. *Z. Parasitenkd.* **8**, 561–605.

Woudenberg, J.H.C., Aveskamp, M.M., de Gruyter, J., Spiers, A.G. and Crous, P.W. (2009) Multiple *Didymella* teleomorphs are linked to the *Phoma clematidina* morphotype. *Persoonia*, **22**, 56–62.

Yun, S.H., Berbee, M.L., Yoder, O.C. and Turgeon, B.G. (1999) Evolution of the fungal self-fertile reproductive life style from self-sterile ancestors. *Proc. Natl. Acad. Sci. USA*, **96**, 5592–5597.

Yun, S.-H., Arie, T., Kaneko, I., Yoder, O.C. and Turgeon, B.G. (2000) Molecular organization of mating type loci in heterothallic, homothallic, and asexual *Gibberella*/*Fusarium* species. *Fungal Genet. Biol.* **31**, 7–20.

