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Mating type gene analysis in apparently asexual *Cercospora* species is suggestive of cryptic sex

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Abstract

The genus *Cercospora* consists of numerous important, apparently asexual plant pathogens. We designed degenerate primers from homologous sequences in related species to amplify part of the *C. apii*, *C. apiicola*, *C. beticola*, *C. zeae-maydis* and *C. zeina* mating type genes. Chromosome walking was used to determine the full length mating type genes of these species. Primers were developed to amplify and sequence homologous portions of the mating type genes of additional species. Phylogenetic analyses of these sequences revealed little variation among members of the *C. apii* complex, whereas *C. zeae-maydis* and *C. zeina* were found to be dissimilar. The presence of both mating types in approximately even proportions in *C. beticola*, *C. zeae-maydis* and *C. zeina* populations, in contrast to single mating types in *C. apii* (MAT1) and *C. apiicola* (MAT2), suggests that a sexual cycle may be active in some of these species.

Keywords: Allele frequency; *Cercospora* leaf spot; Geographic distribution; Mating types; *Mycosphaerella*; PCR amplification

1. Introduction

The genus *Cercospora* was described by Fresenius (Fuckel, 1863) and is one of the largest genera of hyphomycetes. More than 3000 names were listed by Pollack (1987), but Crous and Braun (2003) revised the genus and reduced many species to synonymy, leaving a total of 659 *Cercospora* species. There are 281 morphologically indistinguishable *Cercospora* species, infecting a wide range of plant genera and families, listed as synonyms under *C. apii senso lato* (Crous and Braun, 2003).

Cercospora apii is the main causal agent of *Cercospora* leaf spot on celery, although it has also been confirmed to occur on additional host genera such as *Beta*, *Helianthemum*, *Moluccella*, *Plantago* and *Plumbago* (Crous and Braun, 2003; Groenewald et al., 2005, 2006). A second *Cercospora* species, *C. apiicola*, has also been found to cause *Cercospora* leaf spot on celery (Groenewald et al., 2005, 2006). A multi-gene phylogeny revealed *C. apiicola* to be distinct from *C. apii* (Groenewald et al., 2005, 2006). This species is morphologically similar, but not identical, to *C. apii*, and has thus far only been isolated from celery in Venezuela, Korea and Greece.

Cercospora beticola, which causes *Cercospora* leaf spot on sugar beet (Groenewald et al., 2005; Saccardo, 1876), is morphologically identical to *C. apii*. Although these two species were considered to be synonymous in the past (Crous and Braun, 2003), a multi-gene phylogenic comparison and cultural characteristics revealed them to be distinct species (Groenewald et al., 2005). *C. beticola* has also been confirmed from additional host genera such as *Apium*, *Chrysanthemum*, *Limonium*, *Malva*, and *Spinacia* (Crous and Braun, 2003; Groenewald et al., 2006).

Three *Cercospora* species have been linked to grey leaf spot on maize, namely *C. zeae-maydis*, *C. zeina*, and an

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unnamed *Cercospora* sp. (Crous et al., 2006), though it appears that other *Cercospora* species may also occur on this host (Wang et al., 1998). The unnamed *Cercospora* sp. reported by Crous et al. (2006) appeared to be morphologically and phylogenetically more similar to isolates in the *C. apii* complex than to *C. zeae-maydis* and *C. zeina*. The description of *C. zeina* (Crous et al., 2006) has resolved some of the taxonomic uncertainty surrounding groups in *C. zeae-maydis*. The previously described *C. zeae-maydis* group II is now *C. zeina*, whereas group I is *C. zeae-maydis sensu stricto* (Crous et al., 2006; Dunkle and Levy, 2000; Goodwin et al., 2001).

No teleomorphs are known for the *Cercospora* species causing leaf spot on celery, sugar beet or maize, although there was an unconfirmed report of a teleomorph for *C. zeae-maydis* (Latterell and Rossi, 1977). Wang et al. (1998) were unable to find evidence of the *MAT-2* idiomorph in isolates of *C. zeae-maydis*, and *in vitro* pairing studies with isolates of *C. zeae-maydis* and *C. zeina* have thus far proven unsuccessful in producing a teleomorph (Crous et al., 2006). Wang et al. (1998) reported that there is little genotypic variation in populations of Group I and Group II (*C. zeae-maydis* and *C. zeina*, respectively), which might be expected for asexual species. In contrast, high levels of genetic variation have been reported within and among *C. beticola* field populations, as well as among isolates from the same leaf lesion (Große-Herrenthey, 2001; Moretti et al., 2004). Phylogenetic analyses using the ITS sequences of a variety of *Cercospora* species have resolved *Cercospora* as a well-defined monophyletic clade within the teleomorph genus *Mycosphaerella* (Crous et al., 2000, 2001, 2004; Goodwin et al., 2001; Pretorius et al., 2003; Stewart et al., 1999). Based on these data, it is clear that if sexual states do exist for these species, they would reside in *Mycosphaerella*.

In the absence of a known sexual stage, several approaches can be used to test for evidence of sexual reproduction. Populations that regularly undergo sexual reproduction should have many more genotypes that result in higher levels of genotypic diversity compared to those with only asexual reproduction (Milgroom, 1996). This type of genetic structure is seen in most populations of *M. graminicola* (Linde et al., 2002; Zhan and McDonald, 2004; Zhan et al., 2003). Another method to test for the possibility of sexual reproduction is to establish the occurrence and frequency of the mating type genes. Both mating types have been characterized for filamentous ascomycetes such as *Alternaria alternata* and *Fusarium oxysporum*, for which only asexual reproduction have been observed (Arie et al., 1997, 2000). Therefore, the presence of the mating type idiomorphs in a given species alone is insufficient to prove that a sexual stage exists. However, it is probable that sexual recombination does take place if the two mating types occur in approximately equal frequencies within a given population (Halliday et al., 1999; Linde et al., 2003; Milgroom, 1996; Waalwijk et al., 2002).

The fact that different mating types are necessary for sexual reproduction was first recognized for the genus *Rhizopus* by Blakeslee (1904); and the first molecular characterization of the mating type idiomorphs was achieved for the yeast *Saccharomyces cerevisiae* (Astell et al., 1981). *Neurospora crassa* was the first filamentous ascomycete for which the mating type genes (*MAT1-1-1* and *MAT1-2*) were cloned and sequenced (Glass et al., 1988). The direct target genes of the mating type proteins have not yet been described, although there is evidence for the control of some genes such as pheromone genes (Bobrowicz et al., 2002). The DNA and amino acid sequences of mating type genes show no obvious similarities, although the mating type locus is surrounded by common flanking regions (Turgeon et al., 1993). Except for the high mobility group (HMG)- and the alpha domains, the similarity of homologous mating type genes is usually very low between different species (Turgeon, 1998). Regions with similarities of up to 90% can be found in the HMG domain, and these homologous regions have been used to design degenerative primers for amplification and cloning of the *MAT1-2* gene (Arie et al., 1997).

Four *MAT1-1* genes have been observed in ascomycetes (Pöggeler, 2001). Three of these genes can be distinguished from one another by the specific domain they contain. The *MAT1-1-1* gene contains an alpha domain, the *MAT1-1-2* gene has a MAT A-2 domain, and the *MAT1-1-3* gene has a HMG domain, whereas the *MAT1-1-4* encodes for a metallothionein protein (Kronstad and Staben, 1997; Turgeon, 1998). Only a single gene, *MAT1-2*, is known to confer the MAT2 phenotype. The formal mating type gene nomenclature proposed by Turgeon and Yoder (2000) will be used to define the mating type locus and genes from the *Cercospora* species.

The *MAT1-2* nucleotide sequences show high variability among species but low variability within species (Du et al., 2005; Paoletti et al., 2005). Sequences of the HMG domain of the *MAT1-2* gene have been used to investigate the phylogenetic relationships among closely related species in the *Gibberella fujikuroi* complex (Steenkamp et al., 2000), the *Ceratocystis coerulescens* complex (Witthuhn et al., 2000), *Fusarium graminearum* (O'Donnell et al., 2004), the *Ophiostoma ulmi* complex (Paoletti et al., 2005), and *Colletotrichum* species (Du et al., 2005). Most of these studies concluded that sequences of the HMG domain gave the same and sometimes even greater resolution and stronger support for most branches in a phylogenetic tree than the sequences of the more frequently used internal transcribed spacer regions of nuclear ribosomal DNA.

Sexual reproduction frequently results in genetic recombination and this has a major impact on the dynamics and fitness of a species. The teleomorphs of the *Cercospora* leaf spot pathogens are unknown, and have thus far not been successfully induced by crosses in the laboratory. As a first step to understanding the reproduction cycle in the apparently asexual species of the genus *Cercospora*, our objectives are to identify which mating type(s) are present in *Cercospora* species and to characterize the mating type gene(s). To achieve this objective, we (1) sequence and char-

acterize the full-length mating type genes of *C. apii*, *C. apiicola*, *C. beticola*, *C. zeae-maydis*, and *C. zeina* using PCR-based techniques, (2) amplify and sequence portions of the *MAT1-1-1* and *MAT1-2* genes of other *Cercospora* species for comparison, and (3) develop a multiplex PCR method for rapid identification of the *MAT1-1-1* and *MAT1-2* genes to determine the frequencies of the mating types in different *Cercospora* populations.

2. Materials and methods

2.1. Fungal isolation and DNA extraction

Single conidial cultures were established from *Cercospora* leaf spots associated with celery leaves collected in Venezuela (*C. apiicola*) on 23 June 2002 and in Germany (*C. apii*) on 10 August 2004. Isolations were also made from symptomatic sugar beet leaves obtained from The Netherlands, Germany, Italy, France and New Zealand in 2003 and from Iran in 2004. Symptomatic maize leaves were collected from fields in South Africa (*C. zeina*) in the beginning of 2005 and from Pioneer 3394, a gray leaf spot susceptible hybrid of *Zea mays*, in the USA (*C. zeae-maydis*) on 2 August 2005. Sampling was done in an X figure across each field to ensure consistency. For each population, 50 symptomatic leaves were collected: 10 of each leg and 10 from the center plant. Isolates collected were used to screen for mating type distribution. Additional isolates used during this study were obtained from the Centraalbureau voor Schimmelcultures (CBS) culture collection in Utrecht, the Netherlands. DNA analyses were done on all isolates listed in Table 1. The FastDNA kit (BIO 101, Carlsbad, California) was used according to the manufacturer's instructions to isolate genomic DNA from 200 to 400 mg fungal mycelia grown on MEA plates for 8 days at 24 °C.

2.2. Degenerate primer development and screening of Cercospora isolates

The primer pairs, MAT1-1F/R, and MAT1-2F/R, described by Waalwijk et al. (2002) for the screening of the *MAT1-1-1* and *MAT1-2* genes, respectively, of *M. graminicola*, as well as the degenerate *MAT1-2* primers, ChHMG1 and ChHMG2 described by Arie et al. (1997), were used in an attempt to amplify part of the mating type genes of *C. beticola*. The amplifications were done according to the authors' instructions, and additional annealing temperatures (47 and 50 °C) were tested.

The *MAT1-1-1* sequences of *M. graminicola* (GenBank Accession No. AF440399), *S. passerinii* (GenBank Accession No. AF483193) and *M. fijiensis* (Abeln, *unpublished data*) and the *MAT1-2* sequences of *M. graminicola* (GenBank Accession No. AF440398), *S. passerinii* (GenBank Accession No. AF483194) and *M. fijiensis* (Abeln, *unpublished data*) were aligned using MegAlign from the Lasergene package (DNASTAR, Madison, WI). Two sets of degenerate primers were designed from this alignment, one set in a conserved region of the *MAT1-1-1* (MgMfSpMat1-1f1 5′-CATTNGCNCATCC CTTTG-3′ and MgMfSpmat1-1r2 5′-GGCTTNGANACC ATGGTGAG-3′) and the other in a conserved region of the *MAT1-2* (MgMfSpmat1-2f2 5′-CAAAGAANGCNTTCNT GATCT-3′ and MgMfSpmat1-2r1 5′-TTCTTCTCNGATG GCTTGC-3′) gene. Initially, five randomly selected *C. beticola* isolates from the German population were screened with these two primer sets in order to amplify a partial region of the *MAT1-1-1* or *MAT1-2* genes.

The same PCR conditions were used for the amplification of both partial mating type genes. The reaction mixtures had a total volume of 12.5 µl and contained 0.7 µl of diluted gDNA, 1× PCR buffer (Bioline, London, UK), 48 µM of each of the dNTPs, 8 pmol of each degenerate primer, 1.5 mM $MgCl_2$ and 0.7 units *Taq* polymerase (Bioline). The amplification reactions were done on a GeneAmp PCR System 9600 (Applied Biosystems, Foster City, CA). The initial denaturation step was done at 94 °C for 5 min, followed by 15 cycles of 94 °C (20 s), 52 °C (20 s) and 72 °C (50 s), followed by 25 cycles of 94 °C (20 s), 50 °C (20 s), and 72 °C (50 s). A final elongation step at 72 °C (5 min) was included in the run. The PCR products obtained were separated by electrophoresis at 80 V for 1 h on a 1% (w/v) agarose gel containing 0.1 µg/ml ethidium bromide in 1× TAE buffer (0.4 M Tris, 0.05 M NaAc, and 0.01 M EDTA, pH 7.85) and visualized under UV-light. Amplicons were sequenced in both directions using the PCR primers and a DYEnamic ET Terminator Cycle Sequencing kit (Amersham Biosciences, Roosendaal, The Netherlands) according to the manufacturer's recommendations. The products were analyzed on an ABI Prism 3700 DNA Sequencer (Applied Biosystems). A consensus sequence was computed from the forward and reverse sequences with SeqMan from the Lasergene package.

The degenerate primers and the amplification and sequencing conditions described above were also used to screen *C. apii*, *C. apiicola*, *C. zeae-maydis* and *C. zeina* isolates to obtain portions of their mating type genes.

2.3. Isolation and characterization of Cercospora MAT1-1-1 and MAT1-2 genes

Internal primers were designed in the partially sequenced *MAT1-1-1* and *MAT1-2* genes for each of the species. These internal primers were used together with the appropriate primers from the DNA walking speedup kit (Seegene Inc., Rockville, USA) to determine additional sequences upstream and downstream of the partial sequences in order to obtain the full-length genes. In total, 57 primers were designed and used for the chromosome walking. Blastx (Altschul et al., 1997) was used to compare the sequences obtained from the five *Cercospora* species with protein sequences of other fungi present in the NCBI non-redundant protein database. The geneid v1.2 web server (http://www1.imim.es/geneid.html—Research Unit on Biomedical Informatics of IMIM, Barcelona, Spain)

Table 1
Cercospora isolates included in this study

Accession Nos.[a]	Host genus	Origin	Collector	GenBank No. *MAT1-1-1; MAT1-2*
C. acaciae-mangii				
CPC 10527	*Acacia*	Thailand	K. Pongpanich	— ;DQ264749
C. achyranthis				
CPC 10091	*Achyranthes*	Korea	H.D. Shin	DQ264733; —
C. apii				
CPC 5057; CBS 257.67	*Helianthemum*	Romania	O. Constantinescu	DQ264734; —
CPC 5086; CBS 119.25	*Apium*	—	G.H. Coons	DQ264735; —
[b]CPC 11556; CBS 116455	*Apium*	Germany	K. Schrameyer	DQ264736; —
C. "apii"				
CPC 5329; CBS 115536	*Cajanus*	South Africa	L. van Jaarsveld	— ;DQ264750
CPC 5365; CBS 114817	*Fuchsia*	New Zealand	C.F. Hill	DQ264737; —
CPC 5366; CBS 115060	*Gaura*	New Zealand	C.F. Hill	— ;DQ264751
C. apiicola				
CPC 10266	*Apium*	Venezuela	N. Pons	— ;DQ264753
[b]CPC 10267; CBS 116457	*Apium*	Venezuela	N. Pons	— ;DQ264752
C. berteroae				
CPC 5090; CBS 538.71	*Berteroa*	Romania	O. Constantinescu	— ;DQ264754
C. beticola				
CPC 5065; CBS 548.71	*Malva*	Romania	O. Constantinescu	— ;DQ264755
CPC 5069; CBS 125.31	*Beta*	Japan	—	— ;DQ264756
CPC 5128	*Beta*	New Zealand	C.F. Hill	— ;DQ264757
CPC 5125	*Beta*	New Zealand	C.F. Hill	DQ264738; —
[b]CPC 12190	*Beta*	Germany	S. Mittler	— ;DQ192582
[b]CPC 12191	*Beta*	Germany	S. Mittler	DQ192581; —
C. canescens				
CPC 1138; CBS 111134	*Vigna*	South Africa	S. van Wyk	DQ264739; —
C. erysimi				
CPC 5361; CBS 115059	*Erysimum*	New Zealand	C.F. Hill	DQ264740; —
C. ipomoeae-pedis-caprae				
CPC 10094	*Ipomoea*	Korea	H.D. Shin	— ;DQ264758
C. kikuchii				
CPC 5067; CBS 135.28	*Glycine*	Japan	H.W. Wollenweber	DQ264741; —
C. lactucae-sativae				
CPC 10082	*Ixeris*	Korea	H.D. Shin	— ;DQ264759
C. malvacearum				
CPC 5066; CBS 126.26	*Malva*	—	C. Killian	DQ264742; —
C. modiolae				
CPC 5115	*Modiola*	New Zealand	C.F. Hill	— ;DQ264760
C. penzigii				
CPC 4001	*Citrus*	Swaziland	M.C. Pretorius	DQ264743; —
CPC 4410; CBS 115482	*Citrus*	South Africa	M.C. Pretorius	DQ264744; —
C. polygonaceae				
CPC 10117	*Persicaria*	Korea	H.D. Shin	DQ264745; —
C. violae				
CPC 5079; CBS 251.67	*Viola*	Romania	O. Constantinescu	DQ264746; —
C. zeae-maydis				
[b]CBS 117758	*Zea*	Iowa, U.S.A.	B. Fleener	DQ264747; —
[b]CBS 117760	*Zea*	Pennsylvania, U.S.A.	B. Fleener	— ;DQ264761
C. zeina				
[b]CPC 11995	*Zea*	South Africa	P. Caldwell	— ;DQ264762
[b]CPC 11998	*Zea*	South Africa	P. Caldwell	DQ264748; —

Table 1 (*continued*)

Accession Nos.[a]	Host genus	Origin	Collector	GenBank No. *MAT1-1-1; MAT1-2*
Cercospora sp.				
CPC 5126	*Oenothera*	New Zealand	C.F. Hill	—;DQ264763
CPC 10627	*Delairea*	South Africa	C.L. Lennox	—;DQ264764
CPC 12062	*Zea*	South Africa	—	—;DQ264765

[a] CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CPC: Collection of Pedro Crous, housed at CBS.
[b] Strains used for characterization of full-length *MAT1-1-1* and *MAT1-2* sequences.

was used to predict the gene and intron/exon boundaries using the genetic code of *Neurospora crassa*. The conversion of DNA sequences to putative amino acid sequences was done using the translate tool of ExPASy (Gasteiger et al., 2003). The percentage identities between the predicted *MAT1-1-1* and *MAT1-2* gene sequences for the different *Cercospora* species were calculated using the alignment tool of ALIGN (Pearson et al., 1997).

2.4. Obtaining partial MAT sequences of additional Cercospora isolates

Cercospora-specific primers for the mating type genes were designed from the aligned sequences of *C. apii*, *C. apiicola*, *C. beticola*, *C. zeae-maydis*, and *C. zeina*. The aligned *MAT1-1-1* sequences included *C. beticola*, *C. apii*, *C. zeae-maydis* and *C. zeina* (GenBank Accession Nos. DQ192581, DQ264736, DQ264747 and DQ264748, respectively). The aligned *MAT1-2* sequences included those of *C. beticola*, *C. apiicola*, *C. zeae-maydis* and *C. zeina* (GenBank Accession Nos. DQ192582, DQ264752, DQ264761 and DQ264762, respectively). The sequences of each gene were aligned using MegAlign from the Lasergene package (DNASTAR). To robustly amplify partial Cercospora mating type genes, the primers CercosporaMat1f (5′-CTTGCAGTGAGGACATGG-3′) and CercosporaMat1r (5′-GAGGCCATGGTGAGTGAG-3′) were designed from the conserved regions of the *MAT1-1-1* gene, and primers CercosporaMat2f (5′-GATNTACCNTCTCGA CCTC-3′) and CercosporaMat2r (5′-CTGTGGAGCAGTG GTCTC-3′) were designed from the conserved regions of the *MAT1-2* gene. Twenty-six additional *Cercospora* isolates representing species that belong to the *C. apii* complex (Table 1) were screened with the CercosporaMat1 and CercosporaMat2 primer sets in two separate amplification reactions.

For amplification of the *MAT1-1-1* and *MAT1-2* gene regions, primer concentrations were halved and the other reagent concentrations were as described above. The initial denaturation was done at 94 °C for 5 min, followed by 20 cycles of 94 °C (20 s), 58 °C (20 s) and 72 °C (50 s), followed by 20 cycles of 94 °C (20 s), 55 °C (20 s) and 72 °C (50 s). A final elongation step at 72 °C (5 min) was included. The obtained PCR products were visualized and sequenced as described above.

2.5. Phylogenetic analyses and protein alignment

The partial *MAT1-1-1* and *MAT1-2* sequences of the Cercospora isolates were analyzed using the mating type gene sequences of *M. graminicola* (GenBank Accession Nos. AF440399 and AF440398, respectively) and *S. passerinii* (GenBank Accession Nos. AF483193 and AF483194, respectively) as outgroup taxa. All phylogenetic analyses were done in PAUP (Phylogenetic Analysis Using Parsimony) v4.0b10 (Swofford, 2003). Maximum parsimony and neighbor joining analyses were conducted as described by Groenewald et al. (2005). All sequences generated were deposited in GenBank (Table 1), and the alignments and trees were deposited in TreeBASE (TreeBASE Accession No. SN2529.

Amino acid sequences of the alpha domain (MAT1) and/or HMG domain (MAT2) of *M. graminicola* and *S. passerinii* were downloaded from NCBI's GenBank database. The downloaded amino acid sequences of both of the mating type proteins were aligned to that of the five *Cercospora* species using Sequence Alignment Editor v2.0a11 (Rambaut, 2002).

2.6. Mating type distribution in Cercospora populations

The two primer sets, CercosporaMat1 and CercosporaMat2, were used in a multiplex PCR to screen for the presence of the two mating type genes in the *C. apii*, *C. apiicola*, *C. beticola*, *C. zeae-maydis* and *C. zeina* populations. Reagent concentrations were as described above and all four primers were present at equal concentrations. The initial denaturation step was done at 94 °C for 5 min, followed by 40 cycles of 94 °C (20 s), 60 °C (30 s) and 72 °C (50 s); a final elongation step at 72 °C (5 min) was included. The products were separated on a 1% agarose gel and visualized as described above. The mating type frequency and the *MAT1-1-1*/*MAT1-2* ratios were calculated for each population.

3. Results

3.1. MAT1-1-1 isolation and characterization in Cercospora species

The MAT1-1F and MAT1-1R primers that were designed to amplify part of the *MAT1-1-1* of *M. graminicola* (Waalwijk et al., 2002) were not successful in amplifying the mating type 1 region of *C. beticola*. The degenerate primers, MgMfSpMAT1-1f1 and MgMfSpMAT1-1r2, designed from the *M. graminicola*, *S. passerinii* and *M. fijiensis* sequences, amplified a fragment of 922 bp for three of the five *C. beticola* isolates tested (Fig. 1). The fragment obtained from strain CPC 12191 was sequenced,

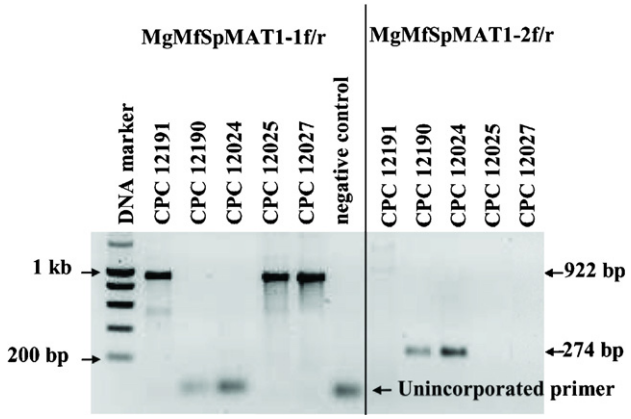

Fig. 1. Amplification products obtained from *Cercospora beticola* isolates containing the *MAT1-1-1* (922 bp) and *MAT1-2* (274 bp) genes using the degenerate primer pairs MgMfSpMAT1-1 and MgMfSpMAT1-2, respectively.

and the translated sequence showed 77% identity to a 57 amino acid region of the *S. passerinii* MAT1 protein and 54% identity to a 57 amino acid region as well as 34% identity to a 82 amino acid region of the *M. graminicola* MAT1 protein using Blastx on the GenBank database. This confirmed that the 922 bp fragment is part of the *MAT1-1-1* gene of *C. beticola*. A homologous fragment was also obtained from *C. apii*, *C. zeae-maydis* and *C. zeina* isolates during the first round of amplification using the MgMfSpMAT11f1 and MgMfSpMAT11r2 degenerate primers. The *C. apiicola* population of 47 isolates, as well as 11 additional *C. apiicola* isolates, that were obtained from Greece, Korea and Venezuella and used in previous studies by Groenewald et al. (2005, 2006), were screened for the presence of the mating type genes, but all isolates were found to only contain the *MAT1-2* gene.

Table 2
Percentage nucleotide identity across the whole *MAT1-1-1* (upper right triangle) and *MAT1-2* (lower left triangle) genes between the *Cercospora* species studied

	C. zeae-maydis	*C. zeina*	*C. apiicola*	*C. apii*	*C. beticola*
C. zeae-maydis	—	92.6	NA	87.4	87.3
C. zeina	74.5	—	NA	87.3	87.2
C. apiicola	70.3	90.8	—	NA	NA
C. apii	NA	NA	NA	—	99.9
C. beticola	90.2	70.6	76.4	NA	—

NA = not available due to the absence of the specific gene in the isolates tested.

The full-length *MAT1-1-1* gene sequences for all four *Cercospora* species were obtained by chromosome walking. The geneid software predicted that the *MAT1-1-1* sequences of all four species contain four exons (Fig. 2). Although the number of amino acids was the same for all three species (335 aa), several differences were observed between the *MAT1-1-1* of the two maize pathogens and that of *C. apii* and *C. beticola*. The predicted length of the genes, as well as exon and intron positions are illustrated in Fig. 2 and the percentage sequence similarities between the different *Cercospora* species are listed in Table 2. Perfect

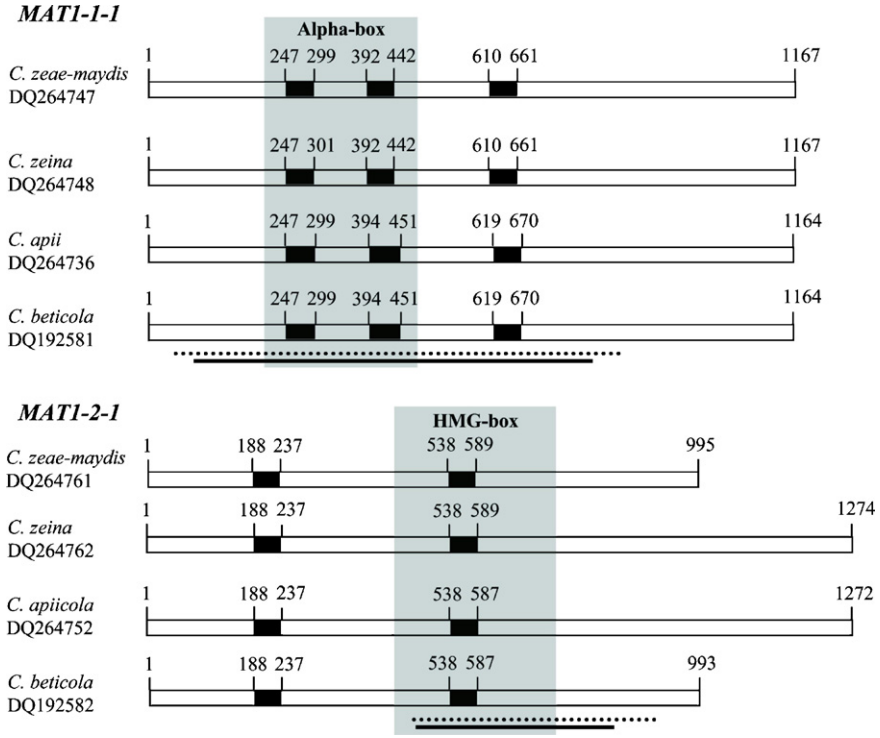

Fig. 2. Diagrammatic representation of the full-length mating type genes of *Cercospora zeae-maydis*, *C. zeina*, *C. apiicola*, *C. apii* and *C. beticola*. The predicted sites of exons (white bars), and introns (black bars) are shown, and their locations (nucleotide position) are indicated. The lines at the bottom of each diagram indicate the area amplified by the CercosporaMat1 and CercosporaMat2 primer sets (dotted line) and the area used for the phylogenetic analyses (solid black line).

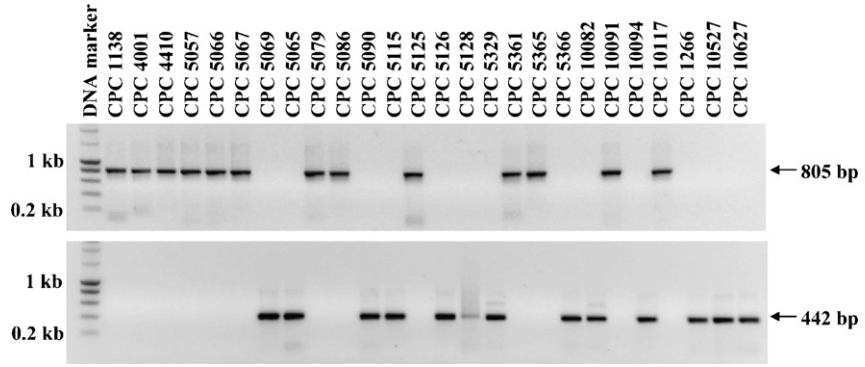

Fig. 3. Different *Cercospora* species screened using the CercosporaMat1 primer set (805 bp fragment; top part of photo) and the same *Cercospora* isolates screened with the CercosporaMat2 primer set (442 bp fragment; lower part of photo).

lariat sequences (RCTRAC) (Bruchez et al., 1993) were present in the introns of all four *Cercospora* species, except in the first intron of *C. beticola* and *C. apii*, that contained a GCTGAT sequence starting at 16 nt upstream from the likely 3′ splice site. The number of predicted introns (two) in the conserved alpha domain region of the *Cercospora* species studied correlates with the number predicted for the same region in *M. graminicola* (Waalwijk et al., 2002) and *S. passerinii* (Goodwin et al., 2003).

3.2. MAT1-2 isolation and characterization in Cercospora species

The *MAT1-2* region in the *C. beticola* genome could not be amplified using the MAT1-2F and MAT1-2R primers of *M. graminicola* (Wang et al., 1998) nor using the degenerate ChHMG1 and ChHMG2 primers of Arie et al. (1997). The degenerate primers (MgMfSpMAT1-2f2 and MgMfSpMAT1-2r1) designed in this study resulted in a 274 bp PCR product in those *C. beticola* isolates of the test panel which did not amplify with the MgMfSpMAT1-1f1 and MgMfSpMAT1-1r2 primers (Fig. 1). The fragment obtained from CPC 12190 was sequenced and the translated sequence showed 59% identity to a 76 amino acid region of the *S. passerinii* MAT2 protein and 61% identity to a 76 amino acid region of the *M. graminicola* MAT2 protein using Blastx. This confirmed that a part of the *MAT1-2* gene of *C. beticola* had been amplified using the newly developed degenerate primers.

A 274 bp fragment was also amplified in three of the additional four *Cercospora* species (*C. apiicola*, *C. zeae-maydis* and *C. zeina*) using the degenerate primers. A *C. apii* population of 32 isolates as well as 17 additional *C. apii* isolates, that were obtained from different countries and used in previous studies by Groenewald et al. (2005, 2006), were screened for the presence of the mating type genes, but only the *MAT1-1-1* gene was found. The sequence of these products corresponded with the *MAT1-2* sequence found for *C. beticola*. Chromosome walking enabled us to obtain the full-length *MAT1-2* genes of *C. apiicola*, *C. beticola*, *C. zeae-maydis* and *C. zeina*. The predicted length of the genes, as well as exon and intron positions are illustrated in Fig. 2. Both introns in all four *MAT1-2* genes contain a perfect lariat sequence (RCTRAC). The predicted presence of a single intron in the conserved HMG domain region of the *Cercospora* species corresponded with the predicted intron for the same region in *M. graminicola* (Waalwijk et al., 2002) and *S. passerinii* (Goodwin et al., 2003).

The percentage sequence identities between the different *Cercospora* species are listed in Table 2. Because the putative *MAT1-2* gene of *C. beticola* and *C. zeae-maydis* is much shorter than that of the other species, the similarities among the *MAT1-2* sequences vary greatly. The high similarity (90.2%) between *C. zeae-maydis* and *C. beticola* is largely due to their similarity in number of nucleotides. The number of amino acids predicted for the MAT2 protein of *C. beticola* and *C. zeae-maydis* was 299, whereas for *C. zeina* and *C. apiicola* it was 392 amino acids.

3.3. Partial MAT1-1-1 and MAT1-2 sequences from additional Cercospora species

The *Cercospora*-specific mating type primer sets CercosporaMat1 and CercosporaMat2 were successful in amplifying a portion (location indicated with a dashed black line in Fig. 2) of the *MAT1-1-1* or the *MAT1-2* genes, respectively, of 26 additional *Cercospora* isolates representing 17 putative species. The primer pair CercosporaMat1f and CercosporaMat1r amplified a fragment of approximately 805 bp in half of the isolates tested, and the CercosporaMat2f and CercosporaMat2r primer set a 442 bp fragment in the rest of the isolates (Fig. 3). These sequences, which included the alpha and the HMG domain, respectively, were aligned with the corresponding *MAT* regions of the *Cercospora* species characterized in this study. The sequences were of relatively high similarity, even in the variable regions flanking the conserved domains (alignments available in TreeBASE Accession No. SN2529).

3.4. Phylogenetic analyses of nucleic acid sequences

The *MAT1-1-1* alignment (TreeBASE Accession No. SN2529) contained 19 taxa, including the two outgroups, and 702 characters, including alignment gaps. Of these characters,

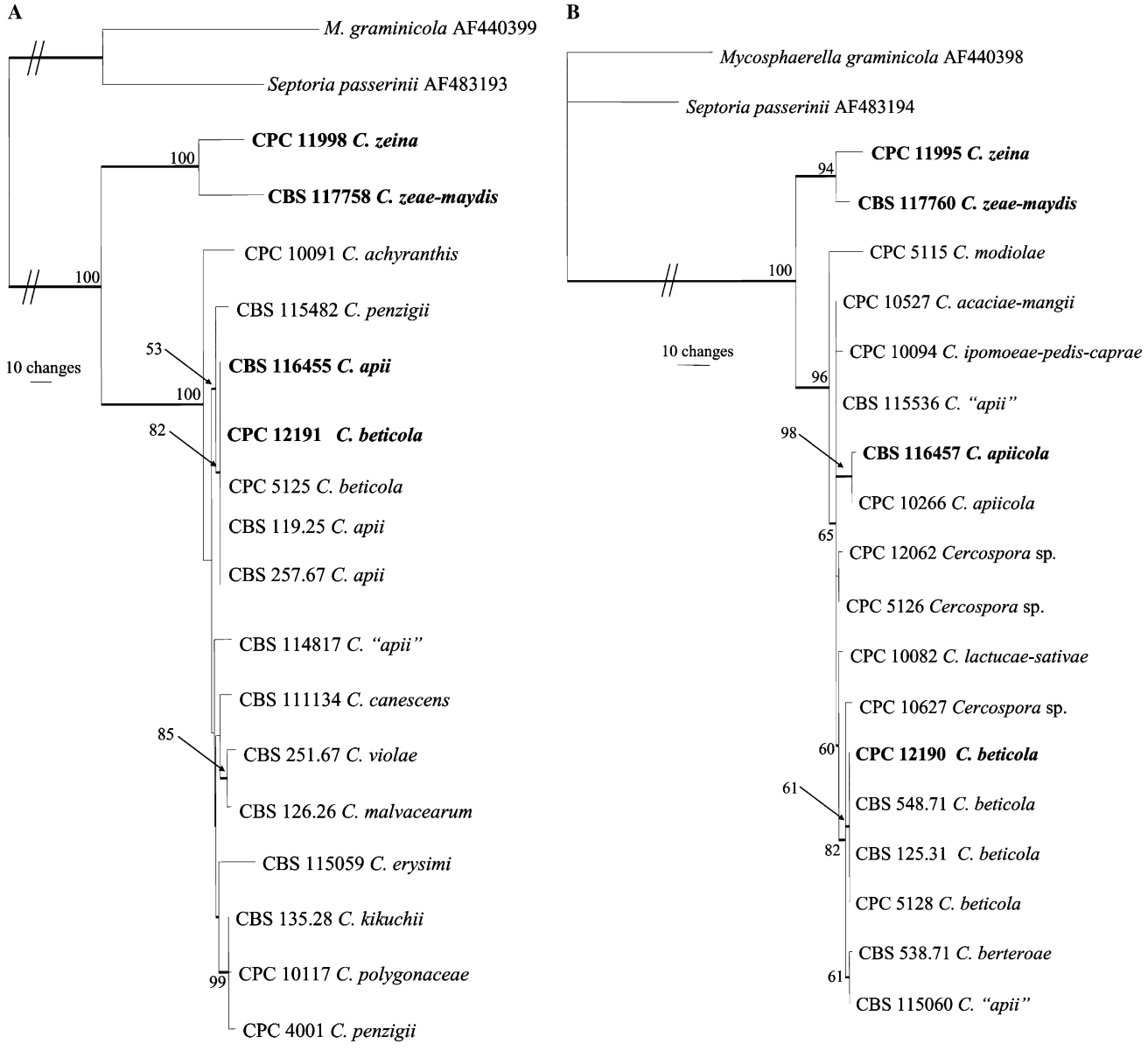

Fig. 4. (A) One of five most parsimonious trees obtained from the *MAT1-1-1* sequence alignment. Bootstrap support values from 1000 replicates are shown at the nodes. The tree was rooted to *Mycosphaerella graminicola* (AF440399) and *Septoria passerinii* (AF483193) (tree length = 622 steps; CI = 0.904; RI = 0.857 and RC = 0.774). (B) One of three most parsimonious trees obtained from the *MAT1-2* sequence alignment. Bootstrap support values from 1000 replicates are shown at the nodes. The tree was rooted to *M. graminicola* (AF440398) and *S. passerinii* (AF483194) (tree length = 247 steps; CI = 0.943; RI = 0.917 and RC = 0.865). Thickened lines indicate the strict consensus branches. Labels in bold represent species for which full-length genes were sequenced.

290 were constant, 139 were variable and parsimony-uninformative, and 273 characters were parsimony-informative. The *MAT1-2* alignment (TreeBASE Accession No. SN2529) contained 20 taxa, including the two outgroups, and 362 characters, including alignment gaps. Of these characters, 181 were constant, 68 were variable and parsimony-uninformative, and 113 characters were parsimony-informative.

Similar trees were obtained irrespective of whether neighbor joining or parsimony was used. Five most parsimonious trees were obtained from the *MAT1-1-1* sequences, and three most parsimonious trees were obtained from the *MAT1-2* sequences. The most parsimonious trees differed somewhat in the arrangement of the taxa within the clade containing the *C. apii* complex (Fig. 4). Limited variation was observed among the isolates belonging to the *C. apii* complex, and these isolates clustered together with bootstrap support values of 100% (*MAT1-1-1*) and 96% (*MAT1-2*). The trees obtained for both the *MAT1-1-1* and *MAT1-2* datasets showed that the two isolates that do not belong to the *C. apii* complex, namely *C. zeae-maydis* and *C. zeina*, group together with a 100% bootstrap support for *MAT1-1-1* and 94% bootstrap support for *MAT1-2*. The phylogenetic trees obtained from these sequences are congruent with the main groupings of the housekeeping gene trees published for the *Cercospora* species (Crous et al., 2006; Groenewald et al., 2005, 2006).

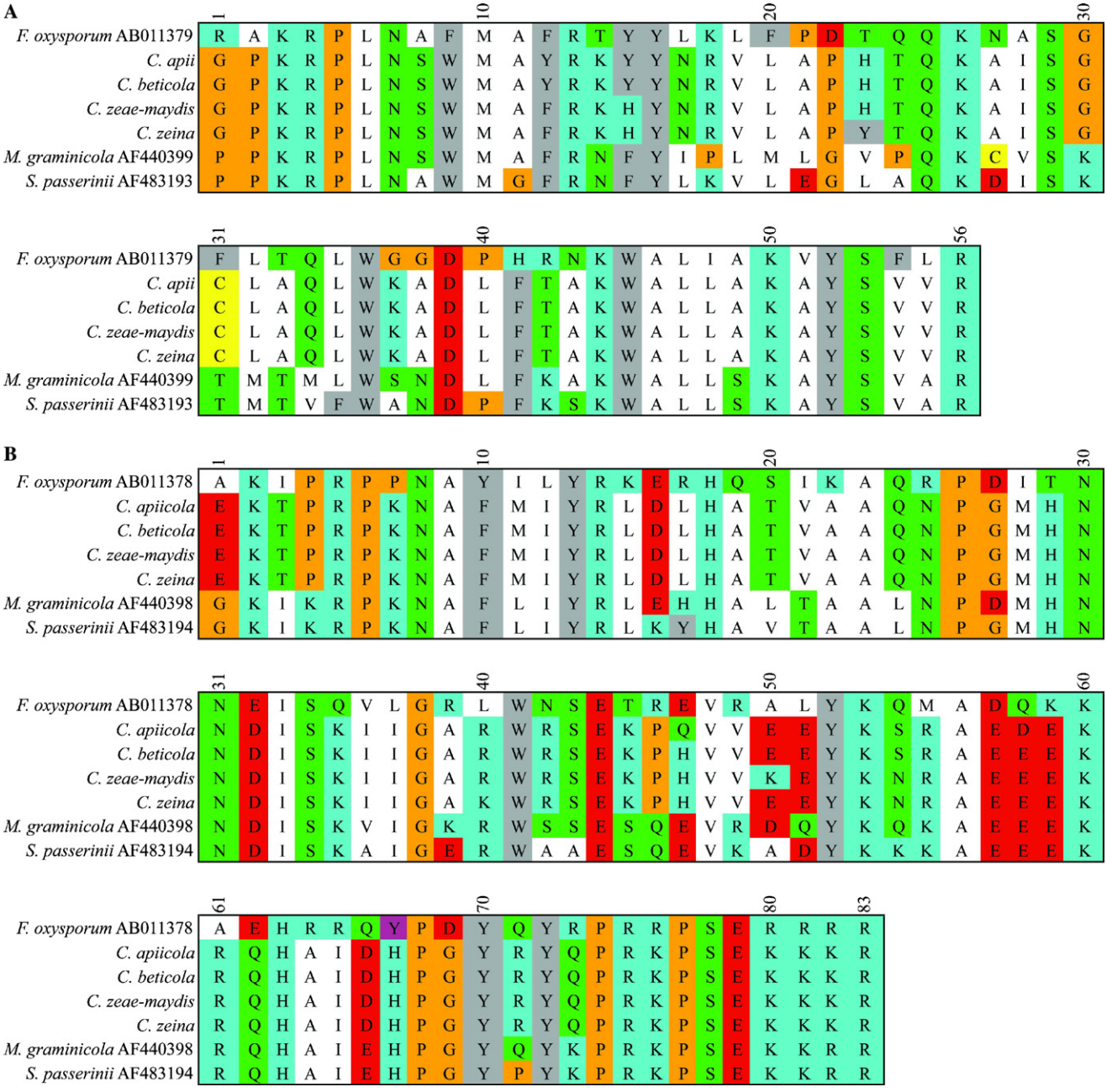

Fig. 5. Protein sequence alignments of the conserved A, alpha domain and B, HMG domain of the mating type genes of *Cercospora* species and closely related fungi.

The *MAT1-1-1* phylogeny showed that all the isolates from *C. apii* (CBS 116455, CBS 119.25 and CBS 257.67) and *C. beticola* (CPC 5125 and CPC 12191) group together with a bootstrap support value of 82% (Fig. 4A). The unnamed *Cercospora* sp. from maize (CPC 12062) did not group with the other maize isolates in the *MAT1-2* analysis, but it did group with the rest of the *Cercospora* isolates with a bootstrap support value of 96% (Fig. 4A). The analyses of the *MAT1-1-1* sequences showed that the isolate from *Helianthemum* (CBS 257.67) identified as *C. apii* in an earlier study (Groenewald et al., 2006) grouped together with the other *C. apii* isolates obtained from celery (CBS 116455 and CBS 119.25) (Fig. 4B). The analysis using the *MAT1-2* dataset showed that the isolate from *Malva* (CBS 548.71) and identified as *C. beticola* using sequence data (Groenewald et al., 2006) grouped with the *C. beticola* isolates (CBS 125.31, CPC 5128, CPC 12190) from sugar beet (Fig. 4B).

3.5. Comparison of predicted amino acid sequences

The predicted amino acid sequences in the alpha (MAT1) and HMG (MAT2) domain showed very high similarity among the four *Cercospora* species (Fig. 5A). For the alpha domain only three amino acid changes were detected between *C. beticola* and *C. zeina*, and only two

between *C. beticola* and *C. zeae-maydis*. The amino acid compositions of the alpha domain of *C. beticola* and *C. apii* were identical. For the HMG domain, two amino acid changes were predicted between *C. beticola* and each of *C. zeae-maydis*, *C. apiicola* and *C. zeina* (Fig. 5B). The *C. beticola* predicted amino acid sequences showed moderate identitiy (Fig. 5) to the alpha domain (MAT2) and HMG domain (MAT2) regions of *S. passerinii* (53.6% and 67.5%, respectively) and *M. graminicola* (57.1% and 67.5%, respectively).

3.6. Distribution of *MAT1-1-1* and *MAT1-2* in *Cercospora* populations

A total of 255 *C. beticola* isolates (46 from France, 41 from Germany, 33 from Italy, 48 from The Netherlands, 50 from Iran and 37 from New Zealand) were screened with a multiplex PCR assay using primer pairs CercosporaMAT1 (805 bp fragment) and CercosporaMAT2 (442 bp fragment). Each tested isolate showed either the 442 bp fragment or the 805 bp fragment of the respective *MAT1-1-1* or *MAT1-2* genes, and no isolate showed both fragments. The *MAT1-1-1* and *MAT1-2* genes were equally distributed in most of the *C. beticola* populations. The ratios were in most cases near to 1.00 (0.85–1.19), except for the Italian population, in which a ratio of 0.50 was found (Table 3). There was no significant deviation ($P < 0.05$) from a 1:1 ratio for the *MAT1-1-1*:*MAT1-2* ratio calculated for each of the populations tested.

A total of 43 *C. zeae-maydis*, 49 *C. zeina*, 32 *C. apii* and 47 *C. apiicola* isolates were screened for the presence of the mating type genes, and no isolate showed both fragments. The *MAT1-1-1* and *MAT1-2* genes were distributed in the *C. zeae-maydis* and *C. zeina* populations at observed *MAT1-1-1*:*MAT1-2* ratios of 0.95 and 1.58, respectively, which did not differ ($P < 0.05$) from the expected 1:1 ratio based on Chi-square analyses (Table 3). All of the *C. apiicola* isolates obtained from Venezuela were found to be *MAT1-2*, whereas all the *C. apii* isolates obtained from Germany were found to be *MAT1-1-1*.

4. Discussion

Very little is known about the occurrence or importance of sex in apparently asexual species of *Cercospora*. During this study the mating type genes of a sugar beet pathogen, *C. beticola*, two celery pathogens, *C. apii* and *C. apiicola*, and two maize pathogens, *C. zeae-maydis* and *C. zeina*, were sequenced and characterized. The degenerate primer sets MgMfSpMAT1-1 and MgMfSpMAT1-2 successfully amplified a portion of the mating type genes, and these sequences led to the characterization of the full-length *MAT1-1-1* and/or *MAT1-2* sequences of *Cercospora* species. Preliminary data reveal that these degenerate primer sets can also amplify the corresponding areas within the mating type genes of other species belonging to the *Mycosphaerellaceae* and allied *Davidiellaceae*. These species include some important pathogens of pines (*Dothistroma pini*, *D. septosporum*), tomatoes (*Passalora fulva*), bananas (*M. musicola, M. musae*), eucalypts (*M. marksii*, *M. thailandica*), or are important as agents in human health or food spoilage (*Cladosporium herbarum*), and will be treated elsewhere in future studies.

The *MAT1-1-1* gene characterized during this study contains an area that corresponds to a putative alpha domain of *MAT1-1-1*, and DNA sequences in the *MAT1-2* gene correspond to the HMG domain described from other ascomycetes. As illustrated in this and other studies, these two domains are also found in the mating type genes of a wide range of ascomycetes. The putative introns in these domains of the *Cercospora* mating type genes are also found in *M. graminicola* and *S. passerinii* (Goodwin et al., 2003; Waalwijk et al., 2002). However, additional introns are predicted in the areas flanking the conserved boxes of each of the respective genes for *Cercospora*. The number of putative introns also varies for the *MAT1-1-1* and *MAT1-2* genes of other ascomycetes. Species containing only one

Table 3
Occurrence and frequency of the *MAT1-1-1* and *MAT1-2* genes in *Cercospora* populations

Populations (country; region)	*N*[a]	*MAT1-1-1*	*MAT1-2*	Ratio[b]	χ^{2c}	*P*[d]
C. beticola (France; Longvic)	46	25 (0.54)	21 (0.46)	1.19	0.35	0.55
C. beticola (Germany; Niedersachsen)	41	22 (0.54)	19 (0.46)	1.16	0.22	0.64
C. beticola (Italy; Ravenna)	33	11 (0.33)	22 (0.67)	0.50	3.77	0.05
C. beticola (Netherlands; Bergen op Zoom)	48	22 (0.46)	26 (0.54)	0.85	0.33	0.57
C. beticola (Iran; Pakajik)	50	26 (0.52)	24 (0.48)	1.08	0.08	0.78
C. beticola (New Zealand; Unknown)	37	19 (0.51)	18 (0.49)	1.06	0.03	0.86
C. zeae-maydis (USA; Iowa)	43	21 (0.49)	22 (0.51)	0.95	0.02	0.89
C. zeina (South Africa; KwaZulu-Natal)	49	30 (0.61)	19 (0.39)	1.58	2.5	0.11
C. apiicola (Venezuela; Caripe)	47	0 (0)	47 (1)	[e]	62.67	<0.001
C. apii (Germany; Baden Württemberg)	32	32 (1)	0 (0)	[f]	58.33	<0.001

The numbers in brackets represent the frequency of the gene.
[a] Number of isolates analyzed.
[b] *MAT1-1-1*:*MAT1-2* ratio.
[c] χ^2 value for the deviation from the expected 1:1 ratio.
[d] Probability of a greater χ^2 value under the null hypothesis of 1:1 ratio (1 degree of freedom).
[e] *MAT1-1-1* was not detected in *C. apiicola*.
[f] *MAT1-2* was not detected in *C. apii*.

putative intron in both of these genes include *Alternaria alternata* (Arie et al., 2000), *Ascochyta rabiei* (Barve et al., 2003), *Cochliobolus heterostrophus* (Turgeon et al., 1993) and *Pyrenopeziza brassicae* (Singh and Ashby, 1998; Singh and Ashby, 1999). *Fusarium oxysporum* (Arie et al., 2000), *Giberella fujikuroi* and *G. zeae* (Yun et al., 2000) have two introns in the *MAT1-2* region, whereas *Ophiostoma novo-ulmi* has one intron in the *MAT1-2* gene (Paoletti et al., 2005). The putative intron splicing sites and gene predictions of only a few filamentous ascomycetes, e.g., *A. alternata* (*MAT1-1-1* and *MAT1-2*), *F. oxysporum* (*MAT1-1-1* and *MAT1-2*) and *O. novo-ulmi* (*MAT1-2*), have been confirmed by mRNA studies. Further studies at the mRNA and protein level are necessary to confirm the exact length of the coding regions as well as the intron and exon boundaries for the mating type genes of the *Cercospora* species.

The predicted length of the encoded proteins among different *MAT1-1-1* and *MAT1-2* genes of ascomycetes varies greatly (Goodwin et al., 2003; Pöggeler, 2001). Usually the MAT1 protein is much larger than the MAT2 protein of the same species. However, this is not the case for *M. graminicola*, where the predicted MAT1 protein (296 amino acids) is smaller than the predicted MAT2 protein (394 amino acids) (Waalwijk et al., 2002), and for *C. zeina* (predicted MAT1 = 339 amino acids and MAT2 protein = 392 amino acids).

Most protein coding genes used in previous taxonomic studies of *Cercospora* lack resolution to distinguish closely related *Cercospora* species (Groenewald et al., 2005, 2006). This study is the first to conduct phylogenetic analyses of partial mating type genes to determine whether they have sufficient discriminatory resolution between closely related *Cercospora* species, particularly those included in the *C. apii* complex. The *Cercospora* mating type-specific primer sets (CercosporaMat1 and CercosporaMat2) amplifies the three introns of *MAT1-1-1* and the intron that is present in the HMG domain of the *MAT1-2*. One of the biggest problems encountered when using *MAT* genes in phylogenetic analyses is that sometimes only one mating type is known in the species, or only one isolate of a species is available, and this isolate carries only one of the two mating type genes. This was the case for most of the *Cercospora* species tested, and these taxa could only be compared to taxa with sequences of the same mating type. Another problem is that the *MAT* gene sequences differ a great deal among different genera and even among species of the same genus. This may restrict analyses to related species and to only a small portion of the gene, specifically, to the more conserved regions (alpha or HMG domains) of these genes. The conserved regions may lack the resolution to distinguish among closely related species, as was the case within the group of isolates belonging to the *C. apii* complex and it is clear that the *MAT1-1-1* sequences cannot separate *C. apii* and *C. beticola*. Mating type genes therefore do not appear to represent promising loci for phylogenetic studies aimed at distinguishing cryptic species belonging to the *C. apii* complex.

Both mating type genes have been isolated from strains of *C. beticola*, *C. zeae-maydis* and *C. zeina*. The *Cercospora* mating type-specific primer sets (CercosporaMat1 and CercosporaMat2) can be used in a multiplex PCR assay for amplification of these two genes in *Cercospora* populations. The two mating types are approximately evenly distributed within the six sampled populations of *C. beticola* as well as in the *C. zeae-maydis* population in the USA and in the *C. zeina* population in South Africa, suggesting that the genes may be functional in these populations. If *C. beticola*, *C. zeae-maydis* and *C. zeina* were strictly asexual, we would expect that with time there would be a skewed distribution of the mating types, or perhaps only a single mating type would be found. Also, if these populations arose from a human introduction of a single genotype, we might expect only one mating type to be present, as was found for the *C. apii* and *C. apiicola* populations. The presence of both mating type genes in the USA population of *C. zeae-maydis* and the South African population of *C. zeina* further strengthens the hypothesis (Crous et al., 2006; Dunkle and Levy, 2000) that these species are native to North America and Africa, respectively. Though the teleomorph has not been confirmed for these three *Cercospora* species, we would expect their teleomorphs to be in the genus *Mycosphaerella*. Detailed analyses have been done on the distribution of the mating types of *M. graminicola* and an equal distribution of the mating types were found in different populations of this sexually reproducing fungus (Waalwijk et al., 2002; Zhan et al., 2002). It is therefore probable that these *Cercospora* species that contain both mating types, are also able to reproduce sexually, but that the teleomorph is not readily observed in nature nor induced under laboratory conditions. However, Halliday and Carter (2003) found segregation of the mating types in natural populations of *Cryptococcus gattii* but, on studying the population structure using AFLP fingerprinting, did not find any evidence supporting genetic exchange between members of the population. These results indicated a clonal population structure even though both mating types were present. All attempts to obtain successful matings between these isolates failed, and the authors concluded that heterogeneity in genome composition resulted in mating incompatibility which gave rise to the clonal population structure (Halliday and Carter, 2003). Contrary to Halliday et al. (1999), who found severely skewed distributions of up to 30:1 for the mating types of some *Cryptococcus gattii* populations, all the *Cercospora* populations we sampled containing both mating types favored a 1:1 ratio, being more consistent with the distribution pattern observed for the sexually reproducing *M. graminicola*. A detailed study on the genetic population structure and the genome composition (for example chromosome number and genome size) of the *Cercospora* species characterized in this study is needed to further evaluate the effect of mating type distribution in these species.

Only the *MAT1-2* gene was present in the *C. apiicola* isolates tested, including isolates from Korea and Greece that were used in previous studies (Groenewald et al., 2005, 2006), as well as a field population of 47 isolates from Venezuela. Although it is possible that a *MAT1-1-1* gene may exist for this species, these data suggest that it would rarely occur, if it were to be present. Without sexual recombination, a species may not be able to rapidly evolve, and it is subsequently more difficult for these species to easily adapt to different environmental conditions. Alternatively, *C. apiicola* may be native to another part of the world, and the sampled populations may be introductions of a single mating type. The tested isolates of *C. apii sensu stricto* contained only the *MAT1-1-1* gene. Based on our current sampling, we predict that *C. apii* is asexual. However, more populations need to be studied, but due to the cultivation of celery under controlled greenhouse conditions we were unsuccessful in obtaining more populations. Unlike *C. apiicola*, *C. apii* has an extremely wide host range (Crous and Braun, 2003; Groenewald et al., 2006). The geographic origin of *C. apii* is Western Europe, whereas *C. apiicola* was originally described from Korea and Venezuela (Groenewald et al., 2005). Recently, Groenewald et al. (2006) showed that *C. apiicola* also occurs in Europe (i.e. Greece). As only one mating type has until now been found for *C. apii* (MAT1) and *C. apiicola* (MAT2), it is possible that these two species lack the ability to reproduce sexually due to the absence of the opposite mating type. If these species are homothallic, they will still be able to reproduce sexually. Our attempts to induce mating between isolates of *C. apii* have failed. In the sexually reproducing basidiomycetous yeast *Cryptococcus neoformans*, laboratory matings produce offspring with an equal distribution of the mating types (Kwon-Chung, 1976). However, in environmental and clinical isolates the majority of isolates belong to one mating type; yet they still retain their sexual reproductive potential by means of fruiting, a process of diploidization followed by reduction to haploid basidiospores which results in a high rate of recombination (Lin et al., 2005). Similar methods of sexual recombination have not yet been observed or reported for the *Cercospora* species characterized here, and strictly asexual reproduction can not be ruled out.

Mating type genes play an important part in the biology and evolution of fungal species. Knowledge of these genes can provide insight in the potential prevalence of sex in species of *Cercospora*, the majority of which are currently thought to be asexual. The primers that were developed during this study allowed us to determine and characterize the mating type genes of several agronomically important *Cercospora* species. The even distribution of the mating types for most species studied here do not favor asexual reproduction; however, further studies are needed to determine whether recombination is taking place. The primers designed here will allow the identification and characterization of mating type genes, or portions thereof, of other important *Cercospora* species and other members of the *Mycosphaerellaceae*.

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