

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. zae-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. zae-maydis* and *C. zeina* were found to be dissimilar. The presence of both mating types in approximately even proportions in *C. beticola*, *C. zae-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.

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1. Introduction

The genus *Cercospora* was described by Fresenius (Fuekel, 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 sensu 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*, *Mohuccella*, *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 phylogenetic 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. zae-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. zaeae-maydis* and *C. zeina*. The description of *C. zeina* (Crous et al., 2006) has resolved some of the taxonomic uncertainty surrounding groups in *C. zaeae-maydis*. The previously described *C. zaeae-maydis* group II is now *C. zeina*, whereas group I is *C. zaeae-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. zaeae-maydis* (Latterell and Rossi, 1977). Wang et al. (1998) were unable to find evidence of the *MAT-2* idiomorph in isolates of *C. zaeae-maydis*, and *in vitro* pairing studies with isolates of *C. zaeae-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. zaeae-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 coerulea* 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. apii-cola*, *C. beticola*, *C. zea-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. apii-cola*) 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. zea-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 (DNA-STAR, 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'-CATTNGCNCATCCCTTTG-3' and MgMfSpMat1-1r2 5'-GGCTTNGANACCATGGTGAG-3') and the other in a conserved region of the *MAT1-2* (MgMfSpMat1-2f2 5'-CAAAGAANGCNTTCNTGATCT-3' and MgMfSpMat1-2r1 5'-TTCTTCTCNGATGGCTTGC-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 MgCl₂ 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. apii-cola*, *C. zea-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. <i>MAT1-1-1</i> ; <i>MAT1-2</i>
<i>C. acaciae-mangii</i> CPC 10527	<i>Acacia</i>	Thailand	K. Pongpanich	—;DQ264749
<i>C. achyranthis</i> CPC 10091	<i>Achyranthes</i>	Korea	H.D. Shin	DQ264733; —
<i>C. apii</i> CPC 5057; CBS 257.67	<i>Helianthemum</i>	Romania	O. Constantinescu	DQ264734; —
CPC 5086; CBS 119.25	<i>Apium</i>	—	G.H. Coons	DQ264735; —
^b CPC 11556; CBS 116455	<i>Apium</i>	Germany	K. Schrameyer	DQ264736; —
<i>C. “apii”</i> CPC 5329; CBS 115536	<i>Cajanus</i>	South Africa	L. van Jaarsveld	—;DQ264750
CPC 5365; CBS 114817	<i>Fuchsia</i>	New Zealand	C.F. Hill	DQ264737; —
CPC 5366; CBS 115060	<i>Gaura</i>	New Zealand	C.F. Hill	—;DQ264751
<i>C. apiicola</i> CPC 10266	<i>Apium</i>	Venezuela	N. Pons	—;DQ264753
^b CPC 10267; CBS 116457	<i>Apium</i>	Venezuela	N. Pons	—;DQ264752
<i>C. berteroeae</i> CPC 5090; CBS 538.71	<i>Berteroea</i>	Romania	O. Constantinescu	—;DQ264754
<i>C. beticola</i> CPC 5065; CBS 548.71	<i>Malva</i>	Romania	O. Constantinescu	—;DQ264755
CPC 5069; CBS 125.31	<i>Beta</i>	Japan	—	—;DQ264756
CPC 5128	<i>Beta</i>	New Zealand	C.F. Hill	—;DQ264757
CPC 5125	<i>Beta</i>	New Zealand	C.F. Hill	DQ264738; —
^b CPC 12190	<i>Beta</i>	Germany	S. Mittler	—;DQ192582
^b CPC 12191	<i>Beta</i>	Germany	S. Mittler	DQ192581; —
<i>C. canescens</i> CPC 1138; CBS 111134	<i>Vigna</i>	South Africa	S. van Wyk	DQ264739; —
<i>C. erysimi</i> CPC 5361; CBS 115059	<i>Erysimum</i>	New Zealand	C.F. Hill	DQ264740; —
<i>C. ipomoeae-pedis-caprae</i> CPC 10094	<i>Ipomoea</i>	Korea	H.D. Shin	—;DQ264758
<i>C. kikuchii</i> CPC 5067; CBS 135.28	<i>Glycine</i>	Japan	H.W. Wollenweber	DQ264741; —
<i>C. lactucae-sativae</i> CPC 10082	<i>Ixeris</i>	Korea	H.D. Shin	—;DQ264759
<i>C. malvacearum</i> CPC 5066; CBS 126.26	<i>Malva</i>	—	C. Killian	DQ264742; —
<i>C. modiolae</i> CPC 5115	<i>Modiola</i>	New Zealand	C.F. Hill	—;DQ264760
<i>C. penzigii</i> CPC 4001	<i>Citrus</i>	Swaziland	M.C. Pretorius	DQ264743; —
CPC 4410; CBS 115482	<i>Citrus</i>	South Africa	M.C. Pretorius	DQ264744; —
<i>C. polygonaceae</i> CPC 10117	<i>Persicaria</i>	Korea	H.D. Shin	DQ264745; —
<i>C. violae</i> CPC 5079; CBS 251.67	<i>Viola</i>	Romania	O. Constantinescu	DQ264746; —
<i>C. zae-maydis</i> ^b CBS 117758	<i>Zea</i>	Iowa, U.S.A.	B. Fleener	DQ264747; —
^b CBS 117760	<i>Zea</i>	Pennsylvania, U.S.A.	B. Fleener	—;DQ264761
<i>C. zeina</i> ^b CPC 11995	<i>Zea</i>	South Africa	P. Caldwell	—;DQ264762
^b CPC 11998	<i>Zea</i>	South Africa	P. Caldwell	DQ264748; —

Table 1 (continued)

Accession Nos. ^a	Host genus	Origin	Collector	GenBank No. <i>MAT1-1-1</i> ; <i>MAT1-2</i>
<i>Cercospora</i> sp.				
CPC 5126	<i>Oenothera</i>	New Zealand	C.F. Hill	—;DQ264763
CPC 10627	<i>Delairea</i>	South Africa	C.L. Lennox	—;DQ264764
CPC 12062	<i>Zea</i>	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. zea-maydis*, and *C. zeina*. The aligned *MAT1-1-1* sequences included *C. beticola*, *C. apii*, *C. zea-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. zea-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'-CTTGCACTGAGGACATGG-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. zea-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/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,

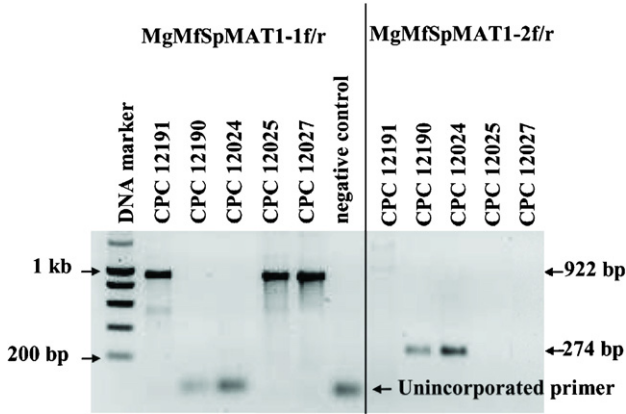


Fig. 1. Amplification products obtained from *Cercospora beticola* isolates containing the *MAT1-1-1* (922 bp) and *MAT1-2* (274 bp) genes using the degenerate primer pairs MgMfSpMAT1-1 and MgMfSpMAT1-2, respectively.

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. zea-maydis* and *C. zeina* isolates during the first round of amplification using the MgMfSpMAT11f1 and MgMfSpMAT11r2 degener-

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

	<i>C. zea-maydis</i>	<i>C. zeina</i>	<i>C. apiicola</i>	<i>C. apii</i>	<i>C. beticola</i>
<i>C. zea-maydis</i>	—	92.6	NA	87.4	87.3
<i>C. zeina</i>	74.5	—	NA	87.3	87.2
<i>C. apiicola</i>	70.3	90.8	—	NA	NA
<i>C. apii</i>	NA	NA	NA	—	99.9
<i>C. beticola</i>	90.2	70.6	76.4	NA	—

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

ate primers. The *C. apiicola* population of 47 isolates, as well as 11 additional *C. apiicola* isolates, that were obtained from Greece, Korea and Venezuela 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.

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

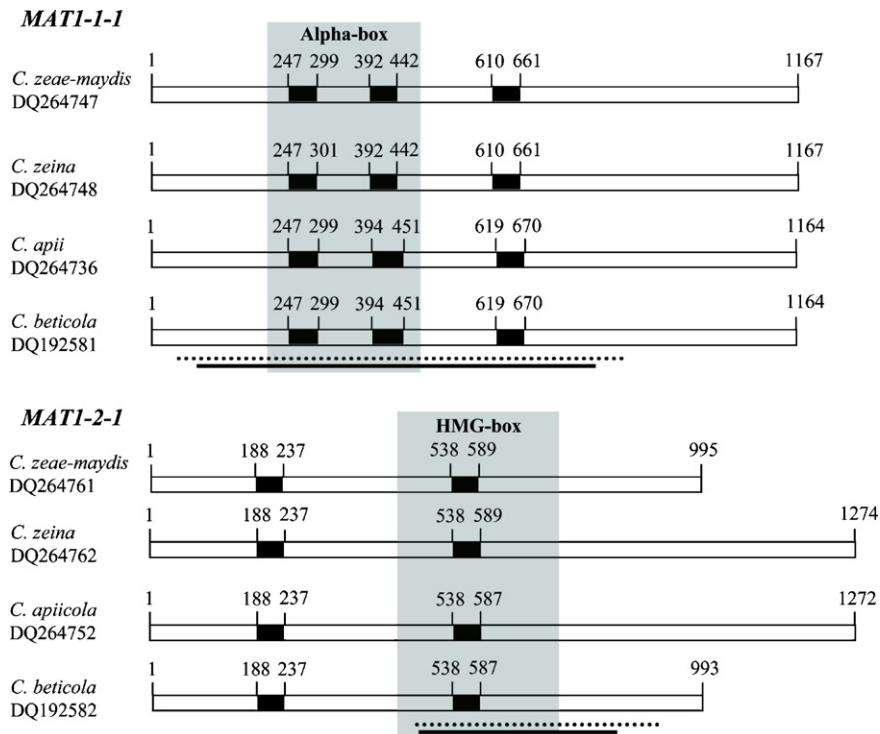


Fig. 2. Diagrammatic representation of the full-length mating type genes of *Cercospora zea-maydis*, *C. zeina*, *C. apiicola*, *C. apii* and *C. beticola*. The predicted sites of exons (white bars), and introns (black bars) are shown, and their locations (nucleotide position) are indicated. The lines 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).

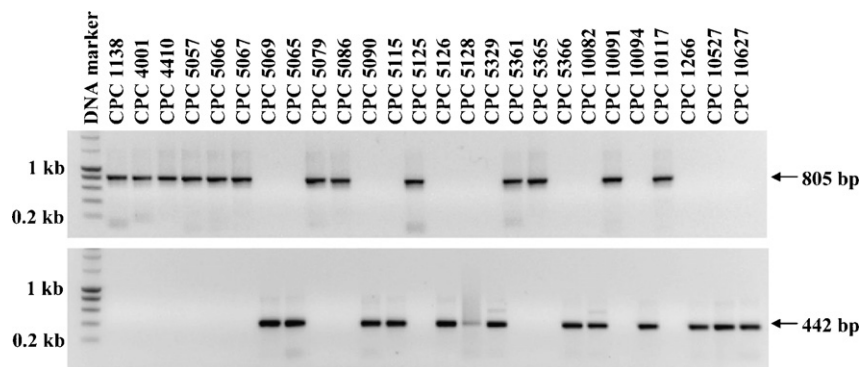


Fig. 3. Different *Cercospora* species screened using the CercosporaMat1 primer set (805 bp fragment; top part of photo) and the same *Cercospora* isolates screened with the CercosporaMat2 primer set (442 bp fragment; lower part of photo).

ariat 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. zeaemaydis* 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. zeaemaydis* 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. zeaemaydis* 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. zeaemaydis* 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. zeaemaydis* 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,

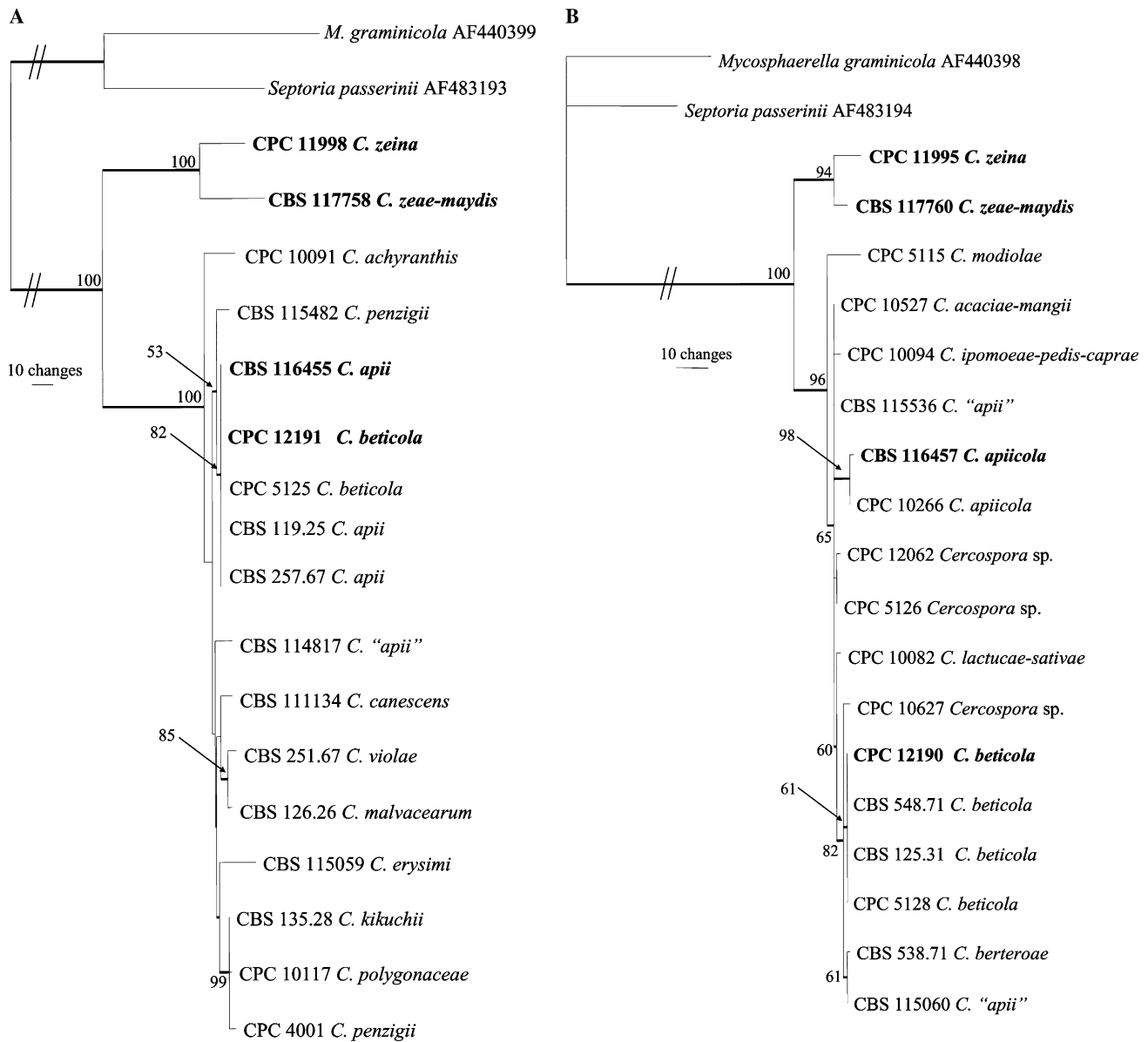


Fig. 4. (A) One of five most parsimonious trees obtained from the *MATI-1-1* sequence alignment. Bootstrap support values from 1000 replicates are shown at the nodes. The tree was rooted to *Mycosphaerella graminicola* (AF440399) and *Septoria passerinii* (AF483193) (tree length = 622 steps; CI = 0.904; RI = 0.857 and RC = 0.774). (B) One of three most parsimonious trees obtained from the *MATI-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 *MATI-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 *MATI-1-1* sequences, and three most parsimonious trees were obtained from the *MATI-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% (*MATI-1-1*) and 96% (*MATI-2*). The trees obtained for both the *MATI-1-1* and *MATI-2* datasets showed that the two isolates that do not belong to the *C. apii* complex, namely *C. zae-maydis* and *C. zeina*, group together with a 100% bootstrap support for *MATI-1-1* and 94% bootstrap support for *MATI-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).

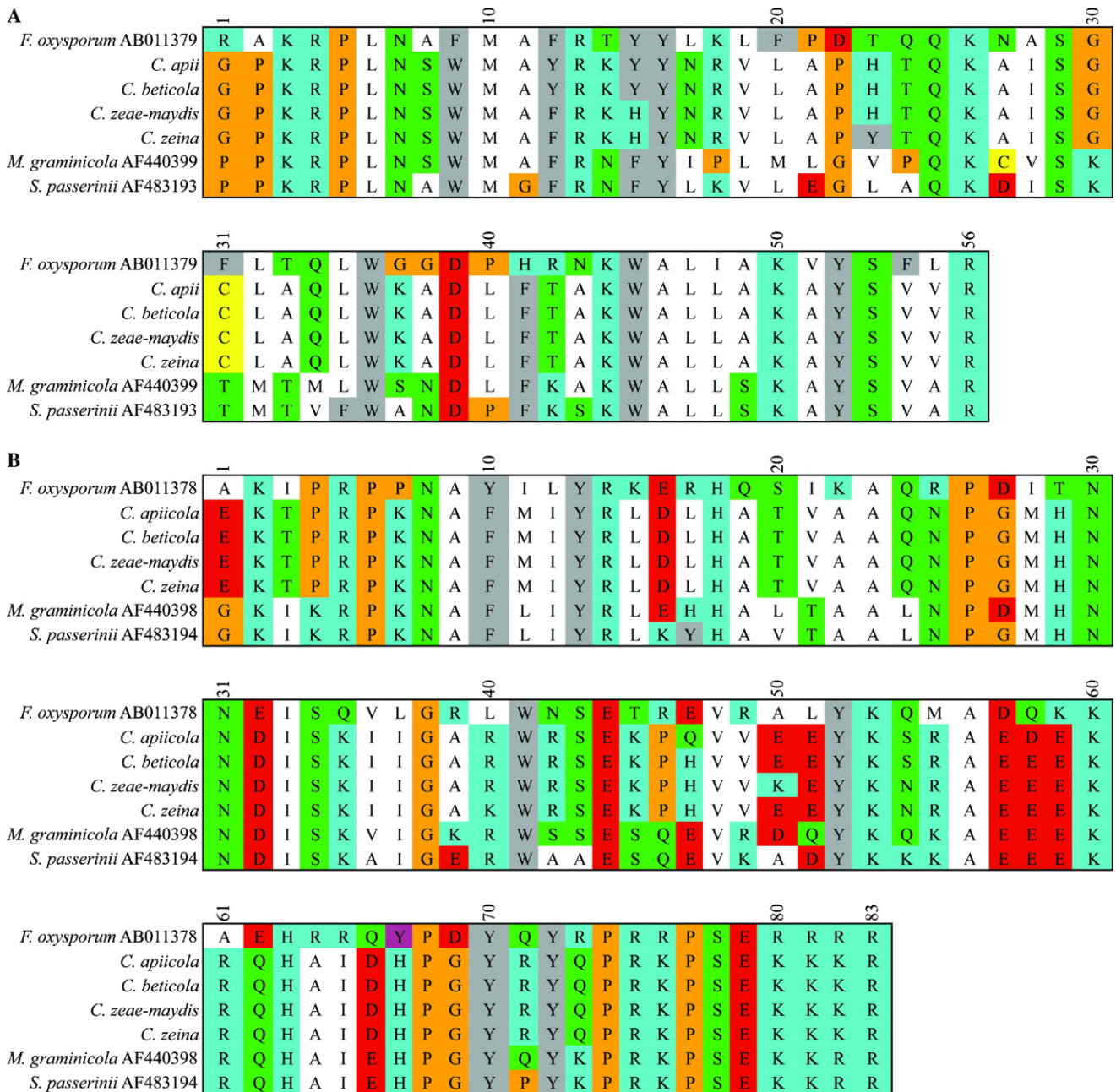


Fig. 5. Protein sequence alignments of the conserved A, alpha domain and B, HMG domain of the mating type genes of *Cercospora* species and closely related fungi.

The *MATI-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 *MATI-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 *MATI-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 *MATI-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. zea-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. zea-maydis*, *C. apiicola* and *C. zeina* (Fig. 5B). The *C. beticola* predicted amino acid sequences showed moderate identity (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 *MATI-1-1* and *MATI-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 *Cercospora*MAT1 (805 bp fragment) and *Cercospora*MAT2 (442 bp fragment). Each tested isolate showed either the 442 bp fragment or the 805 bp fragment of the respective *MATI-1-1* or *MATI-2* genes, and no isolate showed both fragments. The *MATI-1-1* and *MATI-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 *MATI-1-1*:*MATI-2* ratio calculated for each of the populations tested.

A total of 43 *C. zea-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 *MATI-1-1* and *MATI-2* genes were distributed in the *C. zea-maydis* and *C. zeina* populations at observed *MATI-1-1*:*MATI-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

MATI-2, whereas all the *C. apii* isolates obtained from Germany were found to be *MATI-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. zea-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 *MATI-1-1* and/or *MATI-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 *MATI-1-1* gene characterized during this study contains an area that corresponds to a putative alpha domain of *MATI-1-1*, and DNA sequences in the *MATI-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 *MATI-1-1* and *MATI-2* genes of other ascomycetes. Species containing only one

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

Populations (country; region)	N ^a	<i>MATI-1-1</i>	<i>MATI-2</i>	Ratio ^b	χ^2 ^c	P ^d
<i>C. beticola</i> (France; Longvic)	46	25 (0.54)	21 (0.46)	1.19	0.35	0.55
<i>C. beticola</i> (Germany; Niedersachsen)	41	22 (0.54)	19 (0.46)	1.16	0.22	0.64
<i>C. beticola</i> (Italy; Ravenna)	33	11 (0.33)	22 (0.67)	0.50	3.77	0.05
<i>C. beticola</i> (Netherlands; Bergen op Zoom)	48	22 (0.46)	26 (0.54)	0.85	0.33	0.57
<i>C. beticola</i> (Iran; Pakajik)	50	26 (0.52)	24 (0.48)	1.08	0.08	0.78
<i>C. beticola</i> (New Zealand; Unknown)	37	19 (0.51)	18 (0.49)	1.06	0.03	0.86
<i>C. zea-maydis</i> (USA; Iowa)	43	21 (0.49)	22 (0.51)	0.95	0.02	0.89
<i>C. zeina</i> (South Africa; KwaZulu-Natal)	49	30 (0.61)	19 (0.39)	1.58	2.5	0.11
<i>C. apiicola</i> (Venezuela; Caripe)	47	0 (0)	47 (1)	e	62.67	<0.001
<i>C. apii</i> (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 *MATI-1-1*:*MATI-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 *MATI-1-1* was not detected in *C. apiicola*.

^f *MATI-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. apicola* 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* 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* 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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Indirect evidence for sexual reproduction in *Cercospora beticola* populations from sugar beet

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Cercospora beticola is the main causal agent of cercospora leaf spot on sugar beet and has a large negative impact on the yield and quality of sugar beet production worldwide. Previous studies have shown that both mating type idiomorphs of *C. beticola* are present in natural populations, suggesting that *C. beticola* is heterothallic and may be reproducing sexually. *Cercospora beticola* isolates are diverse in the morphology of their conidia, onset of disease symptoms and fungicide resistance. To find the source of this diversity and to determine if sexual reproduction occurs in this fungus, *C. beticola* populations were collected from Western Europe, Iran and New Zealand. The mating types of these isolates were determined and AFLP analyses were used to study the genetic diversity in these populations. The mating type ratios did not deviate significantly from a 1:1 ratio in most of the populations and AFLP analyses showed high levels of genetic variation within and between the populations, with 86.4% of the isolates having unique genotypes. All populations were in significant linkage disequilibrium but levels of disequilibrium were low, and loci from only one primer pair were in significant gametic equilibrium in populations from the Netherlands and Italy. From these results there is the possibility that *C. beticola* reproduces sexually. High levels of gene flow among the samples from Europe demonstrated a single panmictic European population. This study confirms *C. beticola* to be a genetically highly diverse species, supporting the assumption that some populations are reproducing sexually.

Keywords: AFLP, *Beta vulgaris*, gene flow, genetic diversity, mating type idiomorphs, population structure

Introduction

More than 3000 species have been named in the genus *Cercospora* (Pollack, 1987), which is currently regarded as one of the largest genera of hyphomycetes. Following the recent revision by Crous & Braun (2003), this number was significantly reduced to 659 species, with a further 281 species that are treated as morphologically indistinguishable from *C. apii sensu lato*. *Cercospora beticola* belongs to the *C. apii* complex (Crous & Braun, 2003) and is the main causal agent of cercospora leaf spot of sugar beet (Saccardo, 1876; Groenewald *et al.*, 2005, 2006a). Some confusion existed in the past about whether *C. beticola* and *C. apii*, the main leaf spot causing agent of *Apium* species, are synonymous. Groenewald *et al.* (2005) conducted a detailed study of the cultural characteristics, cardinal temperature requirements for growth

and molecular analyses to demonstrate that these two *Cercospora* species are indeed distinct.

Cercospora beticola is considered to be one of the most destructive foliar pathogens of sugar beet, causing yield losses of up to 40% (Shane & Teng, 1992; Holschulte, 2000). For most *Cercospora* species, including *C. beticola*, no sexual stage is known from nature and *in vitro* pairing studies have not been successful in producing a teleomorph for *C. beticola* (unpublished data). The genus *Cercospora* is a well-established anamorph of *Mycosphaerella* (Crous & Braun, 2003), and phylogenetic analyses on a variety of *Cercospora* species have placed them as a well-defined clade within *Mycosphaerella* (Crous *et al.*, 2001, 2006a, 2006b; Goodwin *et al.*, 2001). Therefore, if a sexual stage does exist for *C. beticola*, it would be a species of *Mycosphaerella*.

A wide array of phenotypic diversity has been described for *C. beticola* that includes variation in spore morphology and production, cultural characteristics, pathogenicity and fungicide resistance (Rossi, 1995; Moretti *et al.*, 2004). In fungi, gene diversity is not necessarily affected by the mating structure (McDonald, 1997), but sexually

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reproducing fungi usually have high levels of genotypic diversity and alleles among loci should be randomly associated (Milgroom, 1996). Even though phenotypic markers indicate high levels of variation, little is known about the genetic structure of *C. beticola* populations.

Recently a few studies attempted to determine the population genetic structure of *C. beticola* and a substantial amount of genetic variation was found within *Cercospora* strains isolated from sugar beet fields in Italy (Moretti *et al.*, 2006), and genetic variation was also observed in *C. beticola* isolates from lesions of the same plant (Moretti *et al.*, 2004). This is in contrast to the data available for other *Cercospora* species which have low levels of genetic diversity, e.g. *C. sorghii* (Okori *et al.*, 2004). This species also shows low genetic differentiation between populations from Uganda, suggesting a close genetic relatedness among populations (Okori *et al.*, 2004). Similarly, genetic variation among isolates of *C. zea-maydis* from Africa (Okori *et al.*, 2003) and the United States (Wang *et al.*, 1998; Crous *et al.*, 2006a) was also found to be low, with little genetic differentiation either within or between populations.

Mating type genes are often under frequency-dependent selection in randomly mating populations (Milgroom, 1996; May *et al.*, 1999). Mating type genes (*MAT1-1-1* and *MAT1-2*) of *C. beticola* were isolated and characterized to show that the fungus has a bipolar mating system (Groenewald *et al.*, 2006b). However, the putative intron splicing sites, gene predictions and functionality of these genes in *C. beticola* have not yet been confirmed and additional studies are necessary to show whether these genes are functional. Ascomycetes that are heterothallic have a single locus, two allele mating system which requires two nuclei of opposite mating types to fuse in order for sexual reproduction to occur (Kronstad & Staben, 1997). *Cercospora* mating type-specific primers were developed for use in a multiplex PCR to determine the frequencies of these idiomorphs in field populations (Groenewald *et al.*, 2006b). They found that mating types occurred in similar frequencies in *C. beticola* field populations, a phenomenon that is commonly accepted as indicative of random mating, such as in *Mycosphaerella graminicola* (Waalwijk *et al.*, 2002; Zhan *et al.*, 2002). Groenewald *et al.* (2006b) therefore suggested that some *Cercospora* species cannot be strictly asexual and that another method of reproduction has to occur to account for the frequency-dependent selection of the mating type genes observed within field populations.

Although previous studies showed that high levels of genotypic variation could be found in populations of *C. beticola* (Moretti *et al.*, 2004, 2006), these studies were all based on small sample sizes ($N \leq 13$ per population). Knowledge of the distribution of the mating types, together with the amount of genotypic variation observed within a specific fungal population, can provide a strong indication whether or not sexual reproduction is likely to occur. The main objectives of this study were therefore to (i) determine the genetic structure of *C. beticola* populations with AFLPs, including genotypic diversity and

gametic disequilibrium, and (ii) to determine whether there is frequency-dependent selection on mating types. This knowledge will provide indirect evidence for the possible presence of a sexual cycle occurring in this fungus. In order to achieve these objectives populations from Western Europe, Iran and New Zealand were analysed.

Materials and methods

Fungal isolation and DNA extraction

Beta vulgaris leaves were sampled during the 2003 growing season from single sugar beet fields in four European countries (Netherlands, Germany, France and Italy) as well as in New Zealand (Table 1). The samples from Iran were collected during the 2004 growing season. The sampling was done in an X figure across each field. For each population, leaves with symptoms were collected from 10 plants in each leg of the cross. Single-spore isolations were made and cultures were established on 2% malt extract agar (MEA). The isolates were examined morphologically to confirm their identity as *C. apii sensu lato* as described by Crous & Braun (2003). All isolates were also screened with *C. beticola*-specific primers to confirm that they were truly *C. beticola* before being included in the analyses (Groenewald *et al.*, 2005). Isolates were cultured on MEA plates for 8 days at 24°C, and 200–400 mg mycelium were used in the DNA extraction using the FastDNA kit (BIO 101, Carlsbad) according to the manufacturer's instructions.

Screening of markers

Degenerate mating type idiomorph primers designed by Groenewald *et al.* (2006b) were used to screen all isolates from the six *C. beticola* populations as described previously. AFLP analyses were performed according to Vos *et al.* (1995), with minor modifications as described by Groenewald *et al.* (2005). Genomic DNA (30 ng) from 250 isolates was digested with the restriction enzymes *EcoRI* and *MseI* and ligated to the corresponding adaptors. Four selective primer combinations were used, namely *EcoRI*-A-[FAM]/*MseI*-CT, *EcoRI*-AT-[JOE]/*MseI*-C, *EcoRI*-AG-[NED]/*MseI*-C and *EcoRI*-G-[JOE]/*MseI*-CG (Applied Biosystems), for the final amplification step. To test the reproducibility of the AFLP profiles, separate DNA extractions, PCR amplifications and AFLP analyses were performed in duplicate on 10 isolates (using the four

Table 1 *Cercospora beticola* populations included in this study

Country of origin	Sample size	Location	Collector
France (Fr)	46	Longvic	S. Garressus
Germany (Ger)	39	Niedersachsen	S. Mittler
Italy (It)	32	Ravenna	V. Rossi
Netherlands (Neth)	48	Bergen op Zoom	Unknown
New Zealand (NZ)	35	Unknown	C.F. Hill
Iran (Ir)	50	Pakajik	A.A. Ravanlou

primer combinations). An error rate of 1% (1 to 2 bands difference per isolate among 206 loci) was observed. Only polymorphic loci (78) were included in the analyses.

Data analyses

The presence and absence of bands obtained from AFLP analyses were scored as 1 and 0, respectively, and these results were combined for the statistical analyses. Isolates were considered members of the same clone or clonal lineage if they had 99% similar bands. Clones identified with AFLPs which had different mating type idiomorphs were considered different haplotypes. To quantify genotypic variation within populations, the genotype richness was measured with a Shannon-Wiener index (Grünwald & Hoheisel, 2006).

To evaluate the associations among loci in each sample, the index of association (I_A) and an unbiased estimate of multilocus linkage disequilibrium (\bar{r}_d) were used. I_A and \bar{r}_d values were calculated by using Multilocus 1.3 software, and 1000 artificially recombined data sets were used to determine the statistical values of the test (Agapow & Burt, 2001). Significant departures from an expected 1:1 ratio in mating type frequencies were tested with a chi-squared test.

TFPGA (Miller, 1997) and POPGENE v1.32 (Yeh *et al.*, 1997) were used to analyse the 0/1 matrix. The population genetic analyses program TFPGA was used to calculate the gene diversity (Nei, 1978), percentage of polymorphic loci, F -statistics, genetic distances and the exact tests. The percentage polymorphic loci were based on 99% criteria. The population differentiation was calculated using the method of Weir & Cockerham (1984), jackknife over loci was done with 10 000 iterations using a confidence interval (C.I.) of 95%. Genetic distances between the populations were calculated using Wright's (1978) modification of Rogers' (1972) distance. For this study, a value of < 0.1 indicates small genetic distances, 0.10–0.15 indicates moderate genetic distances, 0.15–0.2 indicates high genetic distances and > 0.2 indicates very large genetic distances. A graphical representation of the genetic distance data (Nei, 1978) was done using the UPGMA algorithm. Bootstrap support values were calculated over all the loci using 1000 repetitions. The exact test was used to determine if significant differences in allele frequencies exist between populations (Sokal & Rohlf, 1995). The Markov Chain Monte Carlo approach that was used to calculate the exact test values gives an approximation of the exact probability of the observed differences in allele frequencies (Raymond & Rousset, 1995).

POPGENE was used to calculate the gene flow (Nm) between any two populations, between the four Western European populations, between the five Eurasian populations and between all six populations. The grouping of populations into major geographic areas of Asia (Iran), Europe (Netherlands, France, Italy and Germany) and New Zealand allowed the analysis of variation (analysis of molecular variance, or AMOVA) at three levels: within

individual populations, between populations within geographic regions, and between geographic regions. All calculations, including random-permutation procedures to assess statistical significance, were performed using the GenALEX 6 package (Peakall & Smouse, 2005).

Results

AFLP markers

Moderate levels of polymorphism were obtained from the four AFLP primer combinations used in this study (Table 2). In total, 208 bands could be scored unambiguously. The number of polymorphic bands obtained from all six populations varied from 15 to 22 (Table 2) and the band sizes ranged from 50 to 500 base pairs. The AFLP primer sets *EcoRI*-AG/*MseI*-C amplified the largest number of polymorphic bands (22) whereas AFLP primer pair *EcoRI*-G/*MseI*-CG amplified the lowest number of polymorphic bands (15) (Table 2). The percentage polymorphic loci ranged from 20.9% in the New Zealand population to 30.6% in the German population (Table 2).

Population genetic analyses

Genotypic diversity (H) ranged from 3.25 (New Zealand) to 3.82 (France, Table 3). Among 250 isolates, 217 (86.4%) unique genotypes were obtained. Unique genotypes refer to isolates with dissimilar AFLP profiles, but also to isolates with identical AFLP profiles but different mating types.

Gene diversity (H) is lowest in the New Zealand population (0.19) and highest in the German and Italian populations (0.27) (Table 3). The theta value shows high population differentiation (0.17) across the six populations, and moderate population differentiation across the four European populations (0.07) and five Eurasian populations (0.07) (Table 4). The pairwise comparisons of population differentiation between the New Zealand population and other populations was high (theta = 0.33–0.41), even though the New Zealand population had only two private alleles. The theta values from pairwise comparisons between the remaining populations varied between 0.02 (Dutch/Italian) and 0.13 (French/German).

Table 2 The number of polymorphic bands analysed with four AFLP primer combinations on 250 *Cercospora beticola* isolates

Primer pair	No. of bands	No. of polymorphic bands ^a						All
		NZ	Fr	Ger	Ir	It	Neth	
<i>EcoRI</i> -A/ <i>MseI</i> -CT	54	14	14	16	14	16	16	21
<i>EcoRI</i> -AG/ <i>MseI</i> -C	52	11	16	17	17	16	15	22
<i>EcoRI</i> -G/ <i>MseI</i> -CG	52	8	11	13	12	12	12	15
<i>EcoRI</i> -AT/ <i>MseI</i> -C	48	10	11	17	9	14	16	21
Total	206	43	52	63	52	58	59	79
% Polymorphic loci		20.9	25.2	30.6	25.2	28.6	28.6	38.3

^aNZ = New Zealand, Fr = France, Ger = Germany, Ir = Iran, It = Italy, Neth = Netherlands, All = total of all six populations.

Population	<i>n</i>	<i>H</i> ^a	<i>H</i> ^b	MAT frequency		χ^2 ^c	<i>I</i> _A ^d	\bar{r}_d ^d
				Mat1-1-1	Mat1-2			
NZ	35 (27)	3.25	0.19	0.52	0.48	0.037	1.530*	0.037*
Fr	46 (46)	3.82	0.23	0.54	0.46	0.347	0.729*	0.015*
Ger	39 (32)	3.39	0.27	0.59	0.41	1.125	1.934*	0.032*
Ir	50 (43)	3.67	0.24	0.49	0.51	0.023	1.025*	0.021*
It	32 (32)	3.47	0.27	0.31	0.69	4.500*	0.377*	0.006*
Neth	48 (37)	3.29	0.25	0.57	0.43	0.675	0.214*	0.004*
Total	250 (217)	–	–	0.51	0.49	6.668	1.135*	0.016*

^aShannon – Wiener index for genotype richness.

^bGene diversity (Nei, 1978).

^c χ^2 value based on 1:1 ratio and 1 degree of freedom for clone corrected populations and 5 degrees of freedom for the contingency χ^2 analyses of the total data set. *Indicates mating type frequencies which are significantly different at $P < 0.05$.

^d*indicates significant *I*_A and \bar{r}_d values at $P < 0.01$.

Table 4 Gene flow (*Nm*) (below diagonal) and theta (population differentiation, above diagonal) for pair-wise comparisons among the six *Cercospora beticola* populations, among all combined populations, and among populations from Europe or Eurasia

	NZ	Fr	Ger	Ir	It	Neth
NZ	–	0.41*	0.33*	0.40*	0.36*	0.36*
Fr	1.3	–	0.13*	0.08*	0.06	0.06
Ger	1.8	5.7	–	0.10	0.05	0.06
Ir	1.4	8.5	6.6	–	0.06	0.09
It	1.6	11.1	10.9	11.3	–	0.02
Neth	1.6	11.4	10.4	8.0	19.1	–
<i>Nm</i> (all populations)	2.2					
<i>Nm</i> (European)	6.8					
<i>Nm</i> (Eurasian)	5.8					
theta (all populations)	0.17*					
theta (European)	0.07*					
theta (Eurasian)	0.07*					

* $P \leq 0.01$, *P*-values obtained with 1000 randomizations in Multilocus v1.3.

The high gene flow (*Nm*) values of 6.8 and 5.8 across the four European and five Eurasian populations, respectively, indicate high genetic exchange between these populations, but *Nm* was low when the New Zealand population was included in the calculation (*Nm* = 2.2) (Table 4). Low *Nm* values (1.3–1.8) were observed between the New Zealand population and every other population analysed. The highest *Nm* values were obtained in pairwise comparisons between Italy and the Netherlands (*Nm* = 19.1), followed by Netherlands/France (*Nm* = 11.4) (Table 4).

AMOVA analyses revealed that the percentage of genetic variation among individuals within populations was 75%. Only 4% of the variation was due to differences among populations within a region (European populations) and 21% to differences among geographic regions.

In 14 cases, isolates with the same multilocus AFLP haplotype had different mating type idiomorphs. Mating type ratios did not deviate significantly from a 1:1 ratio

Table 3 The number of isolates, (number of haplotypes), genotypic and gene diversity, tests of multilocus association and mating type frequencies of *Cercospora beticola* clone corrected populations

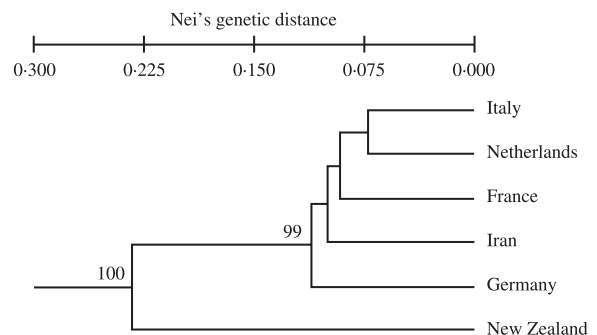


Figure 1 Graphical representation of the genetic distance data (Nei, 1978) generated by UPGMA clustering in the software TFPGA. The scale bar shows the genetic distance, and bootstrap support values in percentage from 1000 replicates are shown at the nodes (only bootstrap support values of 70% and higher are shown).

suggesting frequency-dependent selection, except in the population from Italy where MAT1-2 isolates were more predominant (Table 3). Multilocus measures of association (*I*_A = index of association and \bar{r}_d = multilocus linkage disequilibrium) were significant ($P < 0.01$) for all populations (Table 3). All four loci showed significant ($P < 0.01$) *I*_A and \bar{r}_d values for the New Zealand, German and Iranian populations. Loci from only two primer combinations were in gametic disequilibrium for the population from France, and only one primer combination A/CT was in significant ($P < 0.01$) gametic disequilibrium for the *C. beticola* populations from Italy and the Netherlands (data not shown).

Cluster analysis

Figure 1 represents the genetic distance data obtained between populations using the TFPGA program with UPGMA clustering, and bootstrap support values from 1000 replicates are shown. Genetic distances between the New Zealand population and all other populations were high (0.22–0.25) (Fig. 1). The genetic distance values between the remaining populations were lower and varied

between 0.07 and 0.13. The Exact test showed significant differences between the New Zealand populations and the rest ($P < 0.001$) as well as for the pair-wise comparison between populations of France/Germany ($P = 0.02$).

Discussion

This study is the first to report on the genetic structure and mating type distribution of *C. beticola* populations from different geographic localities. The results obtained from population differentiation, gene flow and genetic distance analyses suggest that the populations from Europe and Iran are genetically similar, whereas the New Zealand population is significantly different. High levels of genetic variation were found among the *C. beticola* isolates tested. This variation, illustrated by the high number of distinct haplotypes obtained with the AFLP analyses, compares well with earlier studies that also reported high levels of genetic variation among isolates obtained from the same lesion on a sugar beet plant in Italy (Moretti *et al.*, 2004), and between isolates from Italy (Moretti *et al.*, 2006). Most of the isolates that were obtained from one plant during the present study also had a distinct multilocus AFLP haplotype (data not shown). The sampling allowed partitioning of genetic variation and showed that most variation could be found within populations (75%), whereas only 4% of the variation was due to differences among populations within a region (European populations) and 21% to differences among geographic regions.

To date no teleomorph has been found for *C. beticola* (Groenewald *et al.*, 2006b) and the reproductive structure of this pathogen has been considered clonal. However, this study found high levels of genotypic diversity in all six populations analysed. It is known that populations that regularly undergo sexual reproduction should have many genotypes that result in higher levels of genotypic diversity compared to those that reproduce only asexually (Milgroom, 1996). This type of genetic structure is seen in most populations of *M. graminicola* (Linde *et al.*, 2002; Zhan *et al.*, 2003; Zhan & McDonald, 2004). Thus, the genotypic diversity observed for *C. beticola* is exceptionally high for a presumed asexually reproducing organism.

Milgroom (1996) and Zhan *et al.* (2002) found that a combination of high levels of genetic diversity and the equal distribution of mating types in a given population indicates that sexual recombination occurs. This study therefore screened for the presence and frequency of the mating type idiomorphs in the populations. The equal distribution of mating types in most populations (except Italy) suggests frequency-dependent selection and thus random mating. Both mating types could also be found on the same plant (data not shown), providing opportunity for genetic exchange. Thus, the high levels of genotypic diversity together with equal mating type ratios indicate that this fungus reproduces sexually. If *C. beticola* was strictly asexual, one would expect that, over time, there would be a skewed distribution of the mating types, or that only one mating type would be present, as was found for other *Cercospora* species such as *C. apii* and *C. apiicola*

(Groenewald *et al.*, 2006b). *Cercospora beticola* has been observed to form spermatogonia on leaf tissues collected during this study, which is also indicative of a possible sexual cycle, although any sexual stage that may exist is, so far, not readily observed in nature nor induced under laboratory conditions.

Tests for multilocus associations (I_A and \bar{r}_d) showed that all six populations were in gametic disequilibrium. This suggests that asexual production is predominant and that random mating occurs only rarely, if at all. However, although significant, the values of I_A and \bar{r}_d were low for populations from Italy, France and the Netherlands. Furthermore, \bar{r}_d was similar or even lower in *C. beticola* (0.004–0.037) than that estimated for *Pyrenophora teres* f.sp. *teres* (0.037–0.039), which is known to undergo regular sexual recombination (Rau *et al.*, 2003). Furthermore, only one primer combination was in significant gametic disequilibrium in the population from Italy and the Netherlands. This contradicts results on frequency-dependent selection and levels of genotypic diversity which suggest populations undergo regular sexual recombination. There are two possible explanations for gametic disequilibrium in these populations. First, frequent population expansions during epidemics can result in populations dominated by closely related individuals (Maynard-Smith *et al.*, 2000). During epidemics, even though populations are recombining, genotypes may arise that are strongly favoured by selection. These genotypes will increase in frequency, generating disequilibrium until recombination has had time to randomize the genetic background (Maynard-Smith *et al.*, 2000), presumably at the end of the growing season when sexual reproduction is known to occur as a survival mechanism for many plant pathogens. Unless mating type idiomorphs are linked to pathogenicity factors or fungicide resistance, their frequency should by chance follow a 1:1 ratio during the epidemic. However, the AFLP loci used in this study were selectively neutral.

A second explanation for the observed gametic disequilibrium lies with the type of marker used. AFLPs often represent hypervariable regions that include dispersed repetitive elements (reviewed in Wong *et al.*, 2001), resulting in a co-dominant marker. Thus, conventional population genetic approaches to analyse AFLP data will underestimate the variability at each locus and overestimate the number of loci analysed, since each allele will be taken as an independent locus (Wong *et al.*, 2001). In a comparison between RFLP and hypervariable AFLP markers, Yan *et al.* (1999) showed that heterozygosity was underestimated in the yellow fever mosquito by AFLP markers, resulting in Hardy-Weinberg disequilibrium. The present results suggest that at least one AFLP primer pair (A/CT) amplified hypervariable regions since it was the only primer combination that showed significant gametic disequilibrium in all *C. beticola* populations analysed. Furthermore, in populations from Italy and the Netherlands, this was the only primer combination that resulted in loci (20 out of 78) in gametic disequilibrium. It is therefore suggested that at least the *C. beticola* populations from Italy and the Netherlands are in gametic equilibrium.

The high level of genotypic variation in *C. beticola* can also be explained by other factors. First, it is possible that *C. beticola* reproduced sexually prior to modern agricultural practices (e.g. burying of plant material during soil cultivation) which prevents sexual reproduction at the end of the growing season. Secondly, Weiland & Koch (2004) showed that the genome of *C. beticola* can undergo chromosome changes after repetitive subculturing. These changes were observed after chromosome separation by gel electrophoreses. Although the authors studied only two isolates and did not mention the number of times the sub-culturing was repeated before these rearrangements were observed, the possibility that such rearrangements can influence results obtained using marker systems, such as AFLPs, has to be taken into account. In order to limit these chromosomal rearrangements in isolates, sub-culturing during this study was kept to a minimum and the DNA was extracted from the cultures directly after the original isolation. It is therefore concluded that the genetic variation observed in the populations screened during this study occurred during the life cycle of the fungus in its natural field environment.

Genetic diversity within a species can also be caused by asexual events that include hyphal anastomosis (Molnar *et al.*, 1990), selfing (Anderson & Kohn, 1995), normal mutations (Koenig *et al.*, 1997; Bentley *et al.*, 1998; O'Donnell *et al.*, 1999) and events occurring during parasexual cycles (Kuhn *et al.*, 1995; Taylor *et al.*, 1999). There is no evidence for parasexual recombination as an important generator of genetic diversity *in vivo* for any fungal system. The high levels of genetic diversity observed in *C. beticola* cannot be explained by mutation only, thus it is proposed that, apart from asexual recombination, a sexual cycle must be present for this pathogen.

No geographic boundaries could be enforced on the European populations based on the country of isolation because of the low population subdivision and low genetic distances between them, and because of shared haplotypes. Also, the Iranian population was not differentiated from the European populations. Sharing of haplotypes among geographic populations could be explained by man-mediated dispersal, as import and export of host material between countries in the European Union readily occurs because of the open borders. The high gene flow and low genetic distance and differentiation values observed between European populations and Iran indicate that genotype transfer also readily takes place between these countries. Based on genetic distance analysis, the Iranian genotypes are intermingled with European isolates, but this was not found for the New Zealand isolates. Therefore, it can be concluded that the European populations and the population from Iran are panmictic.

Pennycook (1989) recorded *C. beticola* on sugar beet in New Zealand, and during the last few years it has been isolated from different localities in New Zealand (New Zealand Fungi Database, 2002). The population from New Zealand is readily distinguished from other populations because of its low gene diversity, high genetic distances and population subdivision. This genetic differentiation

could either be due to a founder event, or the New Zealand populations might represent a different species of *Cercospora*. Groenewald *et al.* (2006a) included New Zealand isolates in a multi-gene phylogeny and could not distinguish them from the other *C. beticola* isolates. Also, the *C. beticola*-specific primers (Groenewald *et al.*, 2005) amplified a product of the correct size for the New Zealand isolates. Only two private AFLP alleles and two null alleles were found to be specific to the New Zealand populations during this study. From these data it is concluded that the *Cercospora* isolates obtained from sugar beet in New Zealand are indeed *C. beticola*. Small population sizes and genetic drift during founder events could have resulted in genetic subdivision, as has been found for other *Mycosphaerella* populations (Boileau *et al.*, 1992; Hayden *et al.*, 2003). However, the specific origin of *C. beticola* in New Zealand is unknown. The first strain that was designated a type of *C. beticola* was described from *Beta cicla* in Italy in 1875 (Saccardo, 1876) and it is most likely that earlier sugar beet trade introduced *C. beticola* to New Zealand from Europe.

Several studies have reported high levels of variation during the onset and progression of cercospora leaf spot on sugar beet (Wolf & Verreet, 2002, 2005), and that *C. beticola* has become resistant or has developed an increased tolerance to fungicides (Karaoglanidis *et al.*, 2000; Weiland & Koch, 2004). Variation in fungicide resistance and variability in disease symptoms on resistant sugar beet plants make effective disease management difficult. It is likely that the high levels of genetic variation that exists within *C. beticola* plays a role in the variation in pathogenicity that has been reported.

Previous studies showed that some genetic variation exists within *C. beticola*, but it was not known whether this variation was due to sexual recombination. The results here indicate that the genetic variation observed in the isolates studied was most likely caused by recombination events. It is suggested that *C. beticola* has both an asexual and sexual reproduction system and that it is unlikely that only asexual reproduction occurs in *C. beticola*. The high levels of genotypic variation and the equal distribution of the mating types within populations suggest that sexual recombination events most likely play an important role in the reproductive cycle of this species.

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