ELSEVIER
Available online at www.sciencedirect.com
ScienceDirect
Fungal Genetics and Biology 44 (2007) 415–429
FUNGAL GENETICS AND BIOLOGY
www.elsevier.com/locate/yfgbi

Mating-type genes and the genetic structure of a world-wide collection of the tomato pathogen *Cladosporium fulvum*

Ioannis Stergiopoulos [a], Marizeth Groenewald [a,b], Martijn Staats [a],
Pim Lindhout [c,1], Pedro W. Crous [a,b], Pierre J.G.M. De Wit [a,*]

[a] *Laboratory of Phytopathology, Wageningen University and Research Centre, Binnenhaven 5, 6709 PD Wageningen, The Netherlands*
[b] *Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands*
[c] *Laboratory of Plant Breeding, Wageningen University and Research Centre, Droevendaalsesteeg 1, 6708 PB Wageningen, The Netherlands*

Received 30 June 2006; accepted 7 November 2006
Available online 18 December 2006

Abstract

Two mating-type genes, designated *MAT1-1-1* and *MAT1-2-1*, were cloned and sequenced from the presumed asexual ascomycete *Cladosporium fulvum* (syn. *Passalora fulva*). The encoded products are highly homologous to mating-type proteins from members of the Mycosphaerellaceae, such as *Mycosphaerella graminicola* and *Cercospora beticola*. In addition, the two MAT idiomorphs of *C. fulvum* showed regions of homology and each contained one additional putative ORF without significant similarity to known sequences. The distribution of the two mating-type genes in a world-wide collection of 86 *C. fulvum* strains showed a departure from a 1:1 ratio ($\chi^2 = 4.81$, $df = 1$). AFLP analysis revealed a high level of genotypic diversity, while strains of the fungus were identified with similar virulence spectra but distinct AFLP patterns and opposite mating-types. These features could suggest the occurrence of recombination in *C. fulvum*.

Keywords: AFLP; Ascomycete; Asexual; Genotypic diversity; Mating-type genes; Polymorphism; Population differentiation; Race; Recombination

---

1. Introduction

Cladosporium fulvum [syn *Passalora fulva* (Braun et al., 2003)] is a non-obligate biotrophic fungus that causes leaf mold on tomato plants (*Lycopersicon esculentum*). It is an asexual hyphomycetous member of the Mycosphaerellaceae (*Capnodiales*), suggesting that if a teleomorph state were to be found for this fungus, it would be a species of *Mycosphaerella* (Braun et al., 2003; Goodwin et al., 2001). Typical disease symptoms on tomato plants are patches of white mold on the abaxial leaf surface that turn brown when the fungus starts to sporulate (Thomma et al., 2005). The disease is thought to have originated from South America, the centre of origin of tomato and other wild *Lycopersicon* species (Cooke, 1906), but to date it has an almost world-wide distribution as tomatoes are globally produced outdoors and in glasshouses, under cultivation practices that are often conducive to *C. fulvum* infections.

Cladosporium fulvum used to be an economically important disease that caused considerable yield losses. However, the introduction during the last 50 years of *Cf*-resistance (for *C. fulvum*) genes into cultivated tomato from wild *Lycopersicon* species, successfully contained the disease in most cultivation areas (Joosten and De Wit, 1999; Rivas and Thomas, 2005). Over the last few decades, the pathosystem *C. fulvum*-tomato has been intensively studied, and has become a model for the study of gene-for-gene interactions (De Wit et al., 2002). In that respect, *C. fulvum* was the first pathogen from which fungal avirulence (*Avr*) genes were cloned and were shown to induce *Cf*-mediated resistance responses in tomato. In a similar way, many cognate *Cf*-resistance genes have been cloned from wild *Lycopersicon* species that are resistant to this pathogen

---

[*] Corresponding author. Fax: +31 317483412.
E-mail address: Pierre.deWit@wur.nl (P.J.G.M. De Wit).

[1] Present address: De Ruiter Seeds, P.O. Box 1050, 2660 BB Bergschenhoek, The Netherlands.

1087-1845/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.fgb.2006.11.004

(Rivas and Thomas, 2005). However, in many cases resistance based on *Cf* genes has been rapidly overcome after their deployment into commercial tomato lines by the appearance of new races of the fungus (Westerink et al., 2004b). Such "boom-and-bust" cycles (Stakman, 1957) have been described for many gene-for-gene-based pathosystems, and are thought to have major impacts on pathogen evolution and population structure (McDonald and Linde, 2002). To date many races of *C. fulvum* exist that are able to evade recognition from several combinations of *Cf*-resistance genes present in cultivated tomato lines. It is believed that such specific race configurations of the fungus have arisen from a few clonal lineages by the consecutive accumulation of mutations in the different *Avr* genes (Joosten and De Wit, 1999; Westerink et al., 2004a).

Sexual reproduction in fungi is controlled by mating-type genes, which have been characterized for several species of ascomycetes (Arie et al., 2000; Coppin et al., 1997; Kronstad and Staben, 1997; Poggeler, 2001). These include species of Mycosphaerellaceae, such as *Mycosphaerella graminicola* (Waalwijk et al., 2002), *Septoria passerinii* (Goodwin et al., 2003), and several *Cercospora* species (Groenewald et al., 2006). Heterothallic fungi can only reproduce sexually when two individuals of opposite mating-type are present. In most heterothallic filamentous ascomycetous fungi mating is controlled by a single mating-type (MAT) locus, which is represented by two idiomorphs known as MAT1-1 and MAT1-2. Although the two idiomorphs are surrounded by identical flanking regions, they are otherwise completely dissimilar in their structural organization, as they encode proteins that differ in number and function (Metzenberg and Glass, 1990; Turgeon, 1998). Members of Loculoascomycetes (Mycosphaerellaceae) exhibit a similar organizational structure in their mating-type locus; each MAT idiomorph contains a single gene encoding a protein with an alpha-domain (MAT1-1-1) or a protein with a DNA-binding domain of the high-mobility group (HMG) (MAT1-2-1) (Poggeler, 2001; Turgeon and Yoder, 2000). Regions with high similarities can be found in the alpha-domain as well as HMG-domain of different species (Turgeon, 1998), and such homologous regions have been extensively explored in PCR-based approaches for cloning of mating-type genes from various fungi (Arie et al., 1997; Poggeler, 2001).

Cladosporium fulvum is thought to be a strictly asexual fungus, since its teleomorph has never been found. However, failure to detect sexual structures, does not necessarily exclude that genetic recombination occurs in fungal populations. With the availability of novel molecular genetic tools in recent years and significant advances in molecular markers technology, it is now possible to test for evidence of recombination in the absence of a known sexual stage (Milgroom, 1996; Tibayrenc et al., 1991). As a result, several studies have revealed an ever growing number of fungi that were previously thought to reproduce strictly asexually, but which in fact undergo cryptic sex in nature (Dodgson et al., 2005; Litvintseva et al., 2003; Taylor et al., 1999). The presence of regular out-crossing in a fungal population constantly creates new genotypes that result in higher levels of genotypic diversity. This type of genetic structure is seen for example in most populations of *M. graminicola* and *Phaeosphaeria nodorum* as well as for other pathogens that appear to be randomly mating (Keller et al., 1997; Linde et al., 2002; Zhan and McDonald, 2004; Zhan et al., 2003). The occurrence and frequency distribution of *MAT* genes in a population may also be indicative of the reproductive behavior of a pathogen. Thus, in populations of sexually reproducing pathogens the two *MAT* genes occur in approximately equal frequencies, whereas skewed ratios are indicative for asexual populations (Milgroom, 1996). However, the presence of the mating-type idiomorphs in a given species alone is insufficient to prove the existence of a sexual stage, as has been demonstrated for the filamentous ascomycetes *Alternaria alternata* and *Fusarium oxysporum* (Arie et al., 2000).

In this study, we describe the cloning, characterization and population distribution of the mating-type idiomorphs from *C. fulvum*. It is presently accepted that this pathogen only reproduces asexually, but here we show that strains of the fungus contain *MAT1-1-1* or *MAT1-2-1* genes that show high similarity to homologous genes from other filamentous ascomycetous fungi. In addition, by using amplified fragment length polymorphism (AFLP) multilocus fingerprints (Vos et al., 1995), we explored the genetic variation of a worldwide collection of strains of this fungus. AFLP analysis revealed a level of genotypic diversity that is too high for a fungus that is expected to reproduce solely asexually, and which contrasts the idea of the dispersal of a few clonal lineages of the pathogen around the world. Therefore, we suggest that sexual recombination might occur in *C. fulvum*.

2. Materials and methods

2.1. Fungal material and culture conditions

Eighty-six *C. fulvum* strains were isolated over a period of 50 years from commercially cultivated tomato lines in different parts of the world (Table 1) and stored at −80 °C at the laboratory of Phytopathology, Wageningen University, The Netherlands. Strains were collected from different geographical regions and were grouped according to the continent from which they were collected. As the strains used in this study were collected over long distances and over a period of several decades and from often previously resistant tomato plants, they represent a collection of strains that could be biased rather than a random population. The collection from Europe contained 50 strains originating from The Netherlands ($n = 22$), France ($n = 13$), Belgium ($n = 4$), Bulgaria ($n = 2$), UK ($n = 5$), Italy ($n = 1$), and Poland ($n = 3$). The collection from the Americas contained 15 strains originating from Canada ($n = 9$), USA ($n = 2$), Argentina ($n = 1$), Brazil ($n = 1$), and other South American regions ($n = 2$). Additional but substantially smaller collections originated from Japan ($n = 12$), former USSR ($n = 2$), Turkey ($n = 4$), New Zealand ($n = 2$), and one from the African continent, namely Zimbabwe ($n = 1$). Most strains were isolated from tomato plants grown

Table 1
Strains of *C. fulvum* used in this study

Strain (#)	Code	Origin and year of isolation	MAT-type	Strain (#)	Code	Origin and year of isolation	MAT-type
1	0	Netherlands	MAT1-1	51	IMI Day2 054978	UK	MAT1-1
2	2	Netherlands	MAT1-2	52	IMI Day5 054977	UK	MAT1-2
3	4E	Netherlands, 1968	MAT1-2	53	IMI Day8 054979	UK	MAT1-2
4	2 4	Netherlands, 1971	MAT1-1	54	IMI Day9 054980	UK	MAT1-2
5	2 4 11	Poland	MAT1-1	55	IMI Zimba 050487	Zimbabwe	MAT1-2
6	2 4 5	Netherlands, 1977	MAT1-1	57	IPO 2459 (30787)	Netherlands, 1981	MAT1-2
7	2 4 5 11	Netherlands	MAT1-2	58	IPO 2459 (50381)	Netherlands, 1987	MAT1-1
8	2 4 5 7	Netherlands	MAT1-1	59	IPO 2459 (60787)	Netherlands, 1987	MAT1-2
9	2 4 5 9	Netherlands, 1980	MAT1-1	60	IPO 248911 Polen	Poland, 1987	MAT1-1
10	2 4 5 9 11 IPO	Netherlands	MAT1-2	61	IPO 249 France	France	MAT1-1
11	2 4 8 11	Netherlands	MAT1-1	62	IPO 2679 SECRET	New Zealand	MAT1-2
12	2 4 9 11	Poland	MAT1-1	63	IPO 5 (104)	Netherlands	MAT1-2
13	2 5	Bulgaria	MAT1-1	64	IPO 5 (116)	Netherlands	MAT1-2
15	2 5 9	France, 1987	MAT1-1	65	IPO 5 (15104)	Netherlands	MAT1-2
16	4	Netherlands, 1971	MAT1-1	66	IPO 80379	Netherlands	MAT1-1
17	4 (2)	Netherlands	MAT1-1	67	Jap 12	Japan	MAT1-1
18	5	France, 1979	MAT1-2	68	Jap 14	Japan	MAT1-1
19	5 Kim	France, 1979	MAT1-2	69	Jap 15	Japan	MAT1-1
20	5 Marmeisee	France, 1979	MAT1-2	70	Jap 16	Japan	MAT1-1
21	5.1	France, 1979	MAT1-2	71	Jap Cf32	Japan	MAT1-1
22	Alenya B	France, 1979	MAT1-1	72	Jap Cf44	Japan	MAT1-1
23	AP 0	Netherlands	MAT1-1	73	Jap Cf5	Japan	MAT1-1
24	Brest 84	France	MAT1-1	74	Jap Cf56	Japan	MAT1-1
25	Brest Rianto 85	France, 1986	MAT1-1	75	Jap Cf9	Japan	MAT1-2
26	Bul 20	Bulgaria	MAT1-1	76	La Maxe 2	France, 1978	MAT1-1
30	Can Brasil	Brazil, 1989	MAT1-2	78	MUCL723	Belgium, 1959	MAT1-1
31	Can USA Amherst	USA	MAT1-1	79	MUCL724	Belgium, 1959	MAT1-1
32	Can 35	Canada, 1980-1983	MAT1-2	80	MUCL725	Belgium, 1959	MAT1-1
34	Can 38	USA, 1962	MAT1-1	81	MUCL726	Belgium, 1959	MAT1-1
35	Can 43	Canada, 1969	MAT1-2	82	Nantes 89	France	MAT1-1
36	Can 47	Canada, pre-1965	MAT1-2	83	NZ	New Zealand	MAT1-1
37	Can 48	Canada, 1980-1983	MAT1-1	84	Pons 89	Netherlands	MAT1-1
38	Can 56	Canada, 1980-1983	MAT1-2	85	Sarrians 86	France, 1978	MAT1-1
39	Can 57	Canada, 1980	MAT1-1	86	Sicile 93	Italy	MAT1-1
40	Can 62	Canada, 1980	MAT1-2	87	T Hijwegen	Netherlands	MAT1-2
41	Can 69	Canada, 1980-1983	MAT1-1	110	VKM 1392	Former USSR	MAT1-2
42	Can 84	Canada, 1982	MAT1-1	111	VKM 1437	Former USSR	MAT1-2
44	Gattieres Furon 90	France	MAT1-2	112	Z. Am 1	South America	MAT1-2
46	IFO 31254	Japan	MAT1-1	113	Zuid Amerika 1336	South America	MAT1-2
47	IFO 8419	Japan	MAT1-1	117	Turk 1a	Turkey, 2005	MAT1-2
48	IFO 9759	Japan	MAT1-2	119	Turk 3a	Turkey, 2005	MAT1-1
49	IMI Argent 358077	Argentina, 1991	MAT1-2	121	Turk 3b	Turkey, 2005	MAT1-2
50	IMI Day? 054976	UK	MAT1-1	122	Turk 3c	Turkey, 2005	MAT1-1

in glasshouses, while a few were collected from outdoor grown tomatoes. Unfortunately, records on the year of isolation of many of these strains were not available. Strains were cultured on half potato-dextrose agar (PDA 19.5 g/L, agar technical 15 g/L; Oxoid Ltd., Hampshire, England) at 22 °C. Conidia were harvested from 15-day-old cultures and freeze-dried prior to DNA extraction. Genomic DNA isolations were performed using the DNeasy® Plant Mini Kit (Qiagen Benelux bv, Venlo, The Netherlands) according to the manufacturer's instructions.

2.2. Cloning and characterization of the mating-type genes and idiomorphs

Two degenerate primer sets, MgMfSpMAT1-1 and MgMfSpMAT1-2 (Table 2) that were previously designed to amplify a region within *MAT1-1-1* and *MAT1-2-1*, respectively, of different *Mycosphaerella* species (Groenewald et al., 2006), were used to screen nine *C. fulvum* strains for the presence of mating-type genes. These included strains from The Netherlands (#23, #66), France (#22, #82), Belgium (#79, #80), the UK (#54), Japan (#46), and New Zealand (#83). PCR conditions as described by Groenewald et al. (2006) were used for the amplification of both gene regions.

Internal walking primers (Table 2) were designed based on the partial *C. fulvum MAT1-1-1* and *MAT1-2-1* sequences obtained. These primers in combination with primers from the DNA walking kit (Seegene Inc., Rockville, Maryland), were used to amplify the full length sequences of *MAT1-1-1* and *MAT1-2-1* as well as for sequencing of the complete MAT idiomorphs from strains #22 (MAT1-1) and #54 (MAT1-2). In all cases,

Table 2
MAT1-1- and MAT1-2-related primers used in this study

MAT1-1		MAT1-2	
Primer	Sequence 5′–3′	Primer	Sequence 5′–3′
Degenerate[a]			
MgMfSpMAT1-1f1	Groenewald et al. (2006)	MgMfSpMAT1-2f2	Groenewald et al. (2006)
MgMfSpMAT1-1r2	Groenewald et al. (2006)	MgMfSpMAT1-2r1	Groenewald et al. (2006)
Gene-walking			
CfMat1-CW1	CATTCATCCTCATGTGCTAAC	CfMat2-CW1	CTGTCAAAGACGAGTACAAGC
CfMat1-CW2	CTTCACCTCAAACCTCGACAC	CfMat2-CW2	TGAGGTCGGTCTTCATCTTCC
CfMat1-CW3	GACCTGGTCAACCACTGCTAC	CfMat2-CW3	GTGACTGACATCTCGCAGGAC
CfMat1-CW4	GACACGATGTGTCTTCCAG	CfMat2-CW4	CATGAGTGTCAGTGAGGATG
CfMat1-CW5	GAAGGTTCGGAAATCGTCTG	CfMat2-CW5	TGAGGATGCTCAGTAGCATGG
CfMat1-CW6	AAATCGTCTGCCATTGTGTTG	CfMat2-CW6	TGTTATGCATTCCAGGGTACG
CfMat1-CW7	GTTGATGGCACAGAATGAGG	CfMat2-CW7	CAACATAGCCTTGATGATCG
CfMat1-CW8	TGGCACAGAATGAGGAAGG	CfMat2-CW8	AGCCCTCCTCCAACTTCTCC
CfMat1-CW9	CTGGGAGGACTTCATCAACG	CfMat2-CW9	TCATTGATGACGATGCTTGC
CfMat1-CW10	TATGTGATGATCGAACTTGC	CfMat2-CW10	CACTCGTGTGGTTCTTGTGC
CfMat1-CW11	TAGTGCAGTGCGACGATGAC	CfMat2-CW11	GGTTCTTGTGCTTGCAGTGG
CfMat1-CW12	AAGTTTCGCAACGGCCTATC	CfMat2-CW12	AAAGCAGAACTGGCAGAAGG
CfMat1-CW13	TGACTTTTCTTGATGTAGATGC	CfMat2-CW13	CAGTGTCTCAGACGATAGACC
CfMat1-CW14	GGACTCATCTTCGTCTGTGTCC	CfMat2-CW14	TTGTCTGAACCGCTGTCTAATG
CfMat1-CW15	CAGCTTGAGGTCGAGTGAGG	CfMat2-CW15	TACCAACGGAAGGATTTTAGCC
CfMat1-CW16	GAGTCTTCAGCGTGAGAGG	CfMat2-CW16	GAGTCTTCAGCGTGAGAGG
CfMat1-CW17	GAGAGTGGAACAAGGCTTCG	CfMat2-CW17	GAGAGTGGAACAAGGCTTCG
CfMat1-CW18	TGATGTTTCTGTTGTGATGTGC	CfMat2-CW18	TGATGTTTCTGTTGTGATGTGC
PCR amplifications			
MAT1-1_P1F	CTTCACCACACCCAAAC	MAT1-2_P1F	CTGCCAGTTCTGCTTTG
MAT1-1_P4R	TGTTCGGTGTCGTGATG	MAT1-2_P4R	TCCACGTCGAAGTAGAG
Sequencing			
MAT1-1_P3F	AATGCTCAGAGGACACAC	MAT1-2_P3F	ATCTACCGTCTCAACCAC
MAT1-1_P6F	ACACACATGACATCTTTC	MAT1-2_P6F	CCTTACCAGAACAACAC

[a] Groenewald et al. (2006).

mating-type genes and idiomorphs were specified according to the nomenclature proposed by Turgeon and Yoder (2000). Primer design and amplification conditions were according to the manufacturer's instructions. The amplified products were sequenced and analyzed as described above. Blastx and Blastp (Altschul et al., 1997) were used to compare the sequences obtained from *C. fulvum* with nucleotide or protein sequences present in the NCBI non-redundant database. Open reading frames (ORFs) were predicted by comparing the *C. fulvum* mating-type sequences to known *MAT* sequences of other filamentous fungi as well as by predictions using the "geneid v1.2 web server" software package (http://www1.imim.es/geneid.html; © Genome Bioinformtics Research laboratory, Barcelona, Spain) and the FEX (Solovyev et al., 1994) and FGENESH (Salamov and Solovyev, 2000) programs from the MOLQUEST software package (Softberry Inc. NY, USA) available at (http://sun1.softberry.com/berry.phtml). In all cases intron/exon boundaries were predicted using the genetic code of *Fusarium graminearum* as a model. FGENESH has been described as the most accurate gene finding program (Yu et al., 2002). However, the validity of these programs in identifying potential intron/exon boundaries was examined by analyzing first *MAT* sequences from other fungal species.

2.3. Mating-type determination and characterization of polymorphisms

The presence of *MAT1-1-1* and/or *MAT1-2-1* in the collection of 86 fungal strains was examined by PCR amplification using gene-specific primers (Table 2). *MAT1-1-1*-specific primers were MAT1-1_P1F (forward), located 39 bp before the predicted translation start of *MAT1-1-1* and MAT1-1_P4R (reverse), located 31 bp after the predicted stop-codon of this gene. *MAT1-2-1* specific primers were MAT1-2_P1F (forward), located 113 bp before the predicted translation start of *MAT1-2-1* and MAT1-2_P4R (reverse), located 148 bp after the predicted stop-codon of this gene. PCR-reaction mixes included 5.0 µl of 10× SuperTaq PCR-reaction buffer, 10 mM of each dNTP (Promega Benelux bv, Leiden, The Netherlands), 20 µM of each primer (Biolegio bv, Nijmegen, The Netherlands), 1 U of SuperTaq DNA polymerase (HT Biotechnology Ltd., Cambridge, England) and approximately 100 ng genomic DNA. The final reaction volume was adjusted to 50 µl with sterile H_2O. The PCR program consisted of an initial 5 min denaturation step at 94 °C, followed by 35 cycles of denaturation at 94 °C (30 s), annealing at 54 °C (90 s) and extension at 68 °C (30 s). A final extension step at 68 °C (7 min) concluded the reaction.

Following amplifications, the full-length *MAT1-1-1* and *MAT1-2-1* genes were sequenced from 21 and 19 *C. fulvum* strains, respectively, in order to determine possible sequence variation among the genes. PCR products were excised from 0.8% agarose gels and purified using the GFX^{TM} PCR DNA and Gel Band Purification Kit (Amersham Biosciences UK limited, Buckinghamshire, England). *MAT1-1-1*-specific fragments were sequenced using primers MAT1-1_P1F and MAT1-1_P4R as well as two internal forward primers located 308 bp (MAT1-1_P3F) and 881 bp (MAT1-1_P6F) after the predicted translation–initiation codon, respectively (Table 2). In a similar way, *MAT1-2-1*-specific fragments were sequenced using primers MAT1-2_P1F and MAT1-2_P4R as well as two internal forward primers located 399 bp (MAT1-2_P3F) and 839 bp (MAT1-2_P6F) after the predicted translation–initiation codon, respectively. Sequencing was performed at Macrogen Inc. (Seoul, South Korea) directly on the purified PCR products and generated chromatographs were analyzed using the Vector NTI Suite 8 (InforMax Inc., Europe Headquarters, Oxford, UK).

2.4. AFLP analysis

The intra-specific diversity among 67 *C. fulvum* strains from the world-wide collection was analyzed by AFLP fingerprinting. These included strains from Europe ($n = 39$), the Americas ($n = 13$), Japan ($n = 10$), the former USSR ($n = 2$), New Zealand ($n = 2$), and Africa ($n = 1$). AFLP analysis was performed according to Vos et al. (1995) with minor modifications as described by Zhao et al. (2005). Genomic DNA (350 ng) from 67 the *C. fulvum* strains was digested with the restriction enzymes *Eco*RI (E) and *Mse*I (M) (New England Biolabs Inc., Ipswich, Massachusetts) and ligated to the corresponding adaptors. Pre-amplifications were performed using the non-selective primers E00 and M00. Selective amplifications were carried out with primers that contained two selective nucleotides for *Eco*RI primers and one selective nucleotide for *Mse*I primers. In preliminary experiments, 104 E + 2/M + 2 and E + 2/M + 1 primer-pairs were tested on 10 *C. fulvum* strains and the produced AFLP fingerprints were evaluated for overall quality, and the number of polymorphic fragments generated (data not shown). From the set of 104 tested primer-pairs, five E + 2/M + 1 primer combinations, namely E15/M02, E18/M02, E18/M03, E20/M04, and E23/M02 were selected and used for the final analysis (Table 3). The *Eco*RI primers were fluorescently labeled with either IRD700 (E15, E23) or IRD800 (E18, E20) at their 5′-end (Biolegio bv, Nijmegen, The Netherlands). AFLP fingerprints were analyzed using the AFLP-QUANTAR^{TM} 1.0 fingerprint analysis software package (KeyGene Products bv, Wageningen, The Netherlands).

AFLP bands were scored manually as binary characters and bands at the same migration height were treated as putative unique AFLP loci with absence or presence of amplification products as putative alleles. A binary matrix was constructed containing all AFLP amplified fragments and all strains. In subsequent analyses, marker data were combined to haplotype data. Genetic similarities were calculated with Jaccard's similarity coefficient by NTSYS-pc version 2.02j (Rohlf, 1997). Jaccard's similarity coefficient only takes the presence of bands into account, while absence of bands is not interpreted as a similar character between strains. The similarity matrix was used to construct a dendrogram by the UPGMA cluster method. Bootstrap values were calculated for 1000 replicates with SplitsTree version 4 (Huson, 1998). Branches with at least 70% bootstrap support were considered as informative. The indices of genotypic diversity were calculated using Nei's (1987) diversity index corrected for sample size using GENODIVE (Meirmans and Van Tienderen, 2004). TFPGA version 1.3 (Miller, 1997) was used to calculate Nei's unbiased measure of genetic identity between geographically diverse collections (Nei, 1978) as well as Wright's geometric average modification on Rogers' genetic distance (Rogers, 1972; Wright, 1978). TFPGA was also used to quantify collection subdivision using hierarchical *F*-statistics by calculating Weir and Cockerham's (1984) theta (θ), the equivalent of Wright's F_{st}. We interpreted the resultant $\theta(F_{st})$ values based on Wright's (1978) suggested qualitative guidelines of $\theta(F_{st})$ values. In that respect, $\theta(F_{st}) = 0$–0.05 indicates no or little collection differentiation, 0.05–0.15 indicates moderate differentiation, 0.15–0.25 indicates great differentiation, and >0.25 indicates very great differentiation. The 95% confidence level of $\theta(F_{st})$ was generated using 10,000 bootstrap replicates. Confidence limits around θ that did not overlap with 0 were taken as evidence for significant genetic differentiation of collections. The multi-loci statistic of Fisher's combined probability test of genetic differentiation was estimated using Genepop DOS version 3.4 (Raymond and Rousset, 1995). The following settings were used: dememorisation number = 1000, number of batches = 1000, number of iterations = 10,000. The null hypothesis for genetic differentiation was H_0: 'the allele distribution of AFLP loci is identical across different geographic collections.

Table 3
Primers used for the AFLP analysis

Primers	Sequence (5′–3′)
E00	GACTGCGTACCAATTC
E15	E00 + CA
E18	E00 + CT
E20	E00 + GC
E23	E00 + TA
M00	GATGAGTCCTGAGTAA
M02	M00 + C
M03	M00 + G
M04	M00 + T

3. Results

3.1. Cloning and characterization of the mating-type idiomorphs of C. fulvum

Using the degenerate primers MgMfSpMAT1-1f1 and MgMfSpMAT1-1r2 a 912 bp PCR fragment was amplified from eight of the *C. fulvum* strains examined. This fragment showed highest similarity to the alpha-domain of MAT1-1-1 from *M. graminicola* and other filamentous ascomycetous fungi. Subsequent chromosome-walking steps, in both upstream and downstream directions, generated a 5.433 kb fragment that contained the entire MAT1-1 idiomorph along with 661 and 611 bp of 5′- and 3′-flanking sequences, respectively. The MAT1-1 idiomorph is 4.161 kb long and contains at least a putative *MAT1-1-1* ORF flanked by 1.509 and 1.349 kb of 5′–3′ idiomorph-sequences, respectively (Fig. 1A). The predicted *MAT1-1-1* ORF from *C. fulvum* is 1.170 kb and encodes a protein of 389 amino acids. The ORF is interrupted by three putative introns of 48, 48, and 53 bp, respectively (Fig. 1B). Perfect lariat sequences (*R*CT*R*AC) could be found within the nucleotide sequences of all three introns. Alignment of the *C. fulvum* MAT1-1-1 protein with similar proteins from other fungal species showed that the first two putative introns are located in the alpha-domain of MAT1-1-1 at the same positions (S83 and W114, respectively) as introns found in related fungal species, such as *M. graminicola* (Waalwijk et al., 2002), *S. passerinii* (Goodwin et al., 2003), and others (Fig. 2). Recently, the presence of an additional third intron downstream of the alpha-domain region was suggested to be present in *MAT1-1-1* sequences of several *Cercopsora* species (Groenewald et al., 2006). The positioning of the third putative intron present in *MAT1-1-1* of *C. fulvum* is in perfect synteny with the third intron suggested for the *Cercopsora* species (Fig. 2). Blastp analysis showed that MAT1-1-1 from *C. fulvum* exhibits highest similarity to the MAT1-1-1 proteins from *Cercospora beticola* (47% identity, 60% similarity), *M. graminicola* (49% identity, 62% similarity), *S. passerinii* (40% identity, 52% similarity), *Aspergillus fumigatus* (40% identity, 55% similarity), *Rhynchosporium secalis* (39% identity, 55% similarity), and several other ascomycetous fungi. High similarity was found within the alpha-box domains but only limited similarity was present outside this domain. Sequence analysis revealed the presence of an additional putative ORF within the MAT1-1 idiomorph of *C. fulvum*. This ORF is located on the opposite DNA strand 350 bp upstream of the *MAT1-1-1* gene and it has been designated as *ORF1-1-2* (Fig. 1A). *ORF1-1-2* is 1.074 kb long and is interrupted by a putative intron of 50 bp, encoding a putative protein of 357 amino acids. Blast analysis showed no significant similarity between the predicted protein product of *ORF1-1-2* and any other proteins currently present in the NCBI GenBank database.

Using the degenerate primers MgMfSpMAT1-2f2 and MgMfSpMAT1-2r1 a 236 bp fragment was amplified from all strains screened as well as a 333 bp fragment, which was found only in the *C. fulvum* strain of the test panel that did not generate a PCR product using the degenerate *MAT1-1-1* primers. Sequencing revealed that the 236 bp fragment did not show similarity to any protein sequence present in the database. However, the translated product of the 333 bp fragment showed highest similarity to the HMG-domain present in the MAT1-2-1 proteins of *S. passerinii* and *M. graminicola*, respectively. Subsequent chromosome-walking steps, in

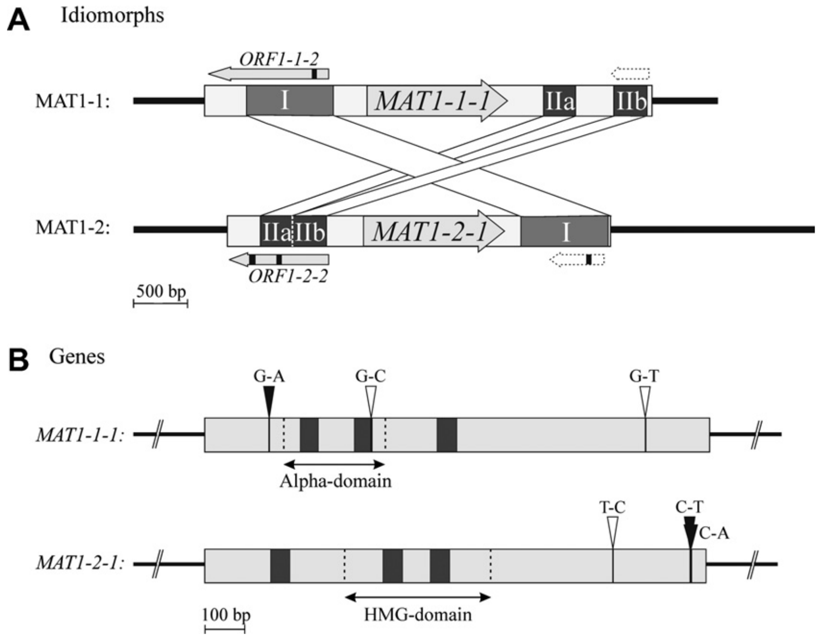


Fig. 1. In scale physical map of the mating-type idiomorphs (A) and the mating-type genes (B) of *C. fulvum*. (A) Idiomorphs are presented as boxes and their flanking regions as solid black lines. The positioning and transcriptional direction of the mating-type genes in each idiomorph is indicated by an arrow. “Islands” of high homology between the two idiomorphs are shown as shaded-grey boxes (I, IIa, IIb). The additional putative ORFs (*ORF1-1-2* and *ORF1-2-2*) identified in each idiomorph are indicated by arrows and putative introns are shown in black. Segments of these ORFs that are only partially present in the opposite idiomorphs are indicated as transparent arrows. (B) Open reading frames (ORFs) are indicated as grey-filled boxes. Introns are presented as dark-grey boxes. The relative position of the alpha-domain of MAT1-1-1 and the HMG-domain of MAT1-2-1 is indicated below the ORFs by double-headed arrows. Identified polymorphisms within *MAT1-1-1* and *MAT1-2-1* are shown as black arrow-heads whenever they are predicted to cause a mutation in the produced protein, or as white arrow-heads when there is no predicted effect on the protein.

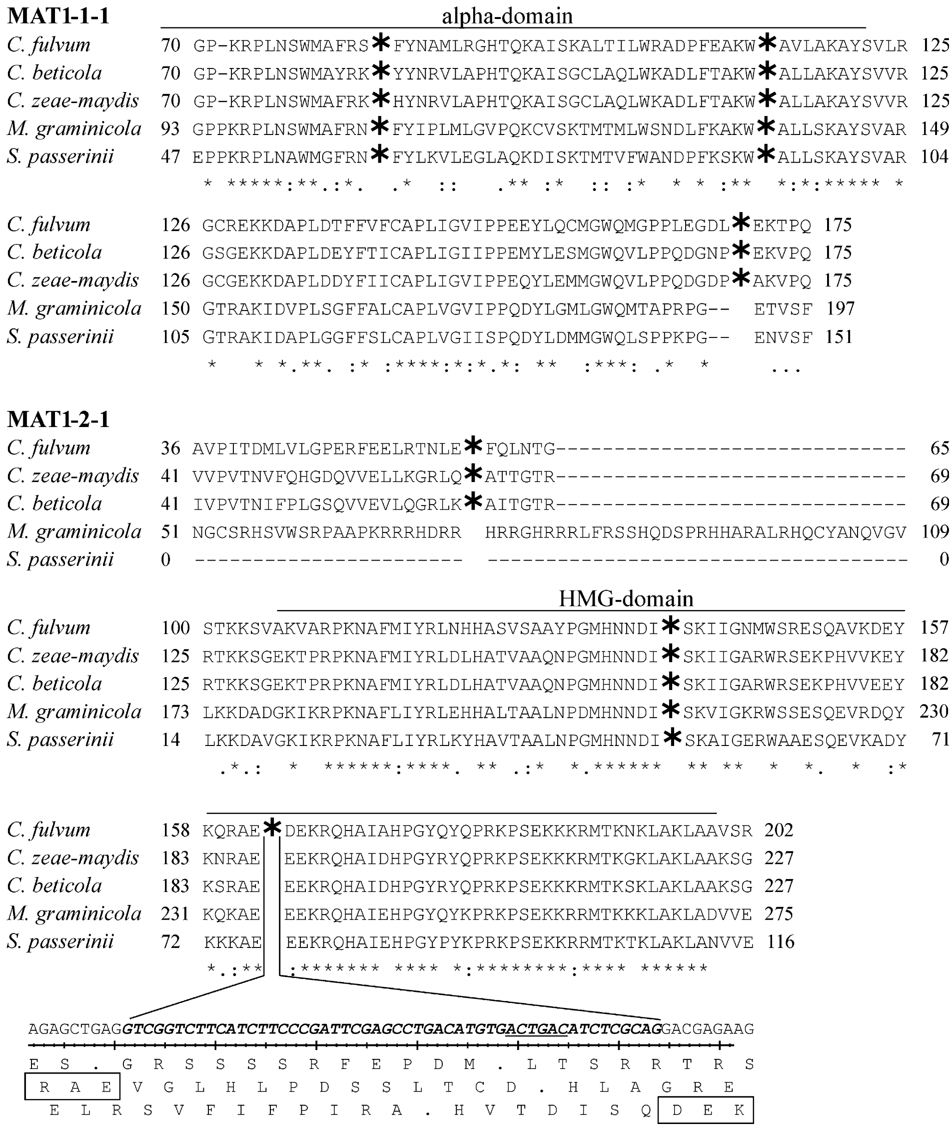


Fig. 2. Sequence alignments of MAT proteins from selected species of *Mycosphaerella* indicating the conserved positions of introns (∗) disrupting the corresponding DNA sequences. Alpha- and HMG domains are indicated as black lines above the alignment. The third intron predicted in *MAT1-2-1* of *C. fulvum* is described in detail. The DNA sequence of this intron is given in bold-italics and a putative lariat sequence is underlined. The three reading frames of the DNA sequence are depicted and amino acids of the predicted MAT1-2-1 protein are boxed. MAT1-1-1 and MAT1-2-1 protein sequences used in these alignments were those of *C. fulvum* (ABG45907, ABG49507), *Cercospora zeae-maydis* (DQ264747, DQ264761), *Cercospora beticola* (DQ192581, DQ192582), *Mycosphaerella graminicola* (AAL30838, AAL30836), and *Septoria passerinii* (AAO49357, AAO49358).

both upstream and downstream directions of this 333 bp fragment, generated a 6.344 kb fragment that contained the full MAT1-2 idiomorph along with 881 and 1910 bp of 5′- and 3′-flanking sequences, respectively. The MAT1-2 idiomorph is 3.553 kb long and contains at least a putative *MAT1-2-1* ORF flanked by 1.259 kb of 5′-sequences and 985 bp of 3′ idiomorph-sequences (Fig. 1A). The predicted 1.155 kb *MAT1-2-1* ORF is interrupted by three putative introns of 51, 52, and 52 bp, respectively, and encodes a protein of 384 amino acids (Fig. 1B). Two of the predicted introns disrupt the HMG-domain of *MAT1-2-1* and to our knowledge this is the first time that more than one intron is identified in this domain of *MAT1-2-1*. The predicted position of the first intron (Ile138) disrupting the HMG-domain, corresponds to the position of the intron present in the same region of other fungal species (Fig. 2). No expression data are available that could confirm the existence of the additional predicted intron. However, when this intron is not spliced this would result in a premature stop codon for the protein (Fig. 2). Blastp analysis showed that the MAT1-2-1 protein of *C. fulvum* shows highest similarity to MAT1-2-1 proteins from *C. beticola* (46% identity, 55% similarity), *M. graminicola* (55% identity, 65% similarity) *S. passerinii* (51% identical, 61% similarity), *A. fumigatus* (26% identity, 43% similarity), and other ascomycetous fungi. Sequence similarity was particularly high within the HMG-domains of these proteins. Sequence analysis revealed the presence of an additional putative ORF within the MAT1-2 idiomorph of *C. fulvum*. The ORF is located on the opposite DNA strand 323 bp upstream of the *MAT1-2-1* gene and it has been designated *ORF1-2-2*. The putative *ORF1-2-2* is 816 bp long and contains two predicted introns of 54 and 64 bp, respectively, encoding a putative protein of 271 amino acids. However, Blast search showed no significant similarity between the putative ORF1-2-2 protein and other proteins present in the NCBI database.

Pairwise sequence alignment showed that the 616 and 600 bp of 5′- and 3′-sequences flanking the two MAT idiomorphs of *C. fulvum* share 97 and 99% identity, respectively. No significant similarities were found between the flanking sequences of the two idiomorphs and other sequences currently present at the NCBI database ($P < 10^{-5}$). Besides the identity in the flanking regions, regions of high homology between MAT1-1 and MAT1-2 were identified that are not part of their flanking regions. The first of these regions is 806 bp long and exhibits 90% identity between the two idiomorphs (Fig. 1A). This region encompasses almost the entire sequence of *ORF1-1-2* in MAT1-1, whereas a similar ORF is only partially present in MAT1-2 as it is interrupted by several stop codons. The second DNA region of high homology between the two idiomorphs is 613 bp long and in MAT1-2 is included entirely within *ORF1-2-2*. However, in MAT1-1 this region is split into a segment of 301 bp with 75% identity to its homologous MAT1-2 counterpart and a segment of 312 bp with 88% indentity to its MAT1-2 counterpart, separated by an insertion of 349 bp (Fig. 1A).

The genomic sequences of the two MAT idiomorphs have been deposited in the NCBI GenBank under the Accession Nos. DQ659350 (MAT1-1) and DQ659351 (MAT1-2).

3.2. Continental distribution of the mating-type genes

The geographic distribution of both mating-type genes of *C. fulvum* was examined in a world-wide collection of 86 strains (Table 1). None of the 86 strains contained both *MAT* genes or lacked one of these two genes. *MAT1-1-1* and *MAT1-2-1* were identified in strains collected from all continents that were examined, except the ones that were represented by too small sample-sizes. In that respect, binomial χ^2 "goodness-of-fit" tests were performed only for the overall collection ($n = 87$) and the European collection ($n = 50$) of strains. The sample-sizes of the other collections were too small for reliable statistical analyses (Table 4). In both collections, the frequency distribution of *MAT* genes deviated significantly from a 1:1 ratio, thus suggesting a potential unbalanced distribution of the two mating-type genes. Indeed, *MAT1-1-1* was observed at a higher frequency than *MAT1-2-1* in most of the collections examined, except for the American collection of strains. Similar results were also obtained when the different collections were corrected to include haplotypes only (Section 3.5).

Table 4
Frequency distribution of *MAT1-1-1* and *MAT1-2-1* of *C. fulvum* in a geographically diverse collection of 86 strains

Collection	N_{strains} ($N_{\text{genot.}}$)[a]	MAT-type		Frequencies		χ^2 values[b]
		MAT1-1-1	*MAT1-2-1*	*MAT1-1-1*	*MAT1-2-1*	
Overall	86 (75)	51 (47)	35 (28)	0.61 (0.63)	0.39 (0.37)	2.98 (**4.81**[c])
Europe	50 (41)	32 (29)	18 (12)	0.64 (0.70)	0.36 (0.30)	**3.92** (**7.05**)
Americas	15 (15)	6 (6)	9 (9)	0.40 (0.40)	0.60 (0.60)	n.d.[d]
Japan	12 (11)	10 (9)	2 (2)	0.83 (0.82)	0.17 (0.18)	n.d.
Turkey	4 (4)	2 (2)	2 (2)	0.5 (0.5)	0.5 (0.5)	n.d.
Former USSR	2 (0)	0 (0)	2 (0)	0.0 (0.0)	1.0 (1.0)	n.d.
New Zealand	2 (2)	1 (1)	1 (1)	0.5 (0.5)	0.5 (0.5)	n.d.
Africa	1 (1)	0 (0)	1 (1)	0.0 (0.0)	1.0 (1.0)	n.d.

[a] Numbers refer to the actual number of strains. Numbers inside the parenthesis refer to the data as clone-corrected for haplotypes only.

[b] χ^2 "goodness-of-fit" tests. χ^2 values calculated for a 1:1 ratio with one degree of freedom. Tests were performed only for the Overall and European collection of strains.

[c] Values in bold indicate frequencies that are statistically significantly different at the $P < 0.05$ level.

[d] Frequencies were not determined (n.d.) due to small sample-sizes.

Table 5
Sequence variation in the *MAT1-1-1* and *MAT1-2-1* genes of *C. fulvum* at the nucleotide and amino acid level

Nucleotide substitutions	Amino acid substitutions	Strains containing the substitutions
MAT1-1-1		
G > A 159 bp[a]	Gly52[b] > Lys	#31, #41, #42, #51, #74, #78, #85
G > C 435 bp	—	#31, #41, #42
C > T 1856 bp	Ser334 > Ser (silent)	#31, #41, #42
MAT1-2-1		
T > C 1067 bp	Pro304 > Pro (silent)	#30
C > T 1270 bp	Pro372 > Leu	#30
C > A 1271 bp	Pro372 > Leu	#30

[a] Indicates position of the substitution relative to the A nucleotide (+1 bp) of the ATG start codon.

[b] Indicates the amino acid affected relatively to the start of the protein (Met is +1 amino acid).

3.3. Sequence variation in the MAT genes

The full length *MAT1-1-1* sequence was determined from 21 *C. fulvum* strains originating from Europe (#1, #4, #11, #12, #15, #16, #25, #26, #51, #58, #60, #78, #85), the Americas (#31, #41, #42), Japan (#46, #67, #69, #74), and Turkey (#119). Sequence variation within the *MAT1-1-1* gene was very limited (Table 5 and Fig. 1B). One nucleotide substitution (G > A at 159 bp), predicted to result in an amino acid substitution (Gly52 > Lys) was detected in seven strains originating from Europe (#85, #51, #78), the Americas (#31, #41, #42), and Japan (#74). Furthermore, the strains originating from the Americas (#31, #41, #42) showed the presence of two additional nucleotide substitutions (G > C and C > T at 435 and 1856 bp, respectively) but these substitutions are not predicted to affect the amino acid composition of the produced protein as the G > C substitution is located inside the second putative intron of *MAT1-1-1* and the C > T substitution is silent.

Among the 19 strains of *C. fulvum* analyzed, only three nucleotide substitutions were observed, all present in the *MAT1-2-1* gene of the Brazilian strain (#30). These were a T > C at 1067 bp, C > T at 1270 bp, and C > A at 1271 bp nucleotide substitutions, predicted to cause a silent (T > C) or a Pro372 > Leu amino acid substitution (C > T and C > A combined). All other strains originating from Europe (#2, #7, #18, #44, #53, #57, #63, #87, #117), the Americas (#32, #49, #35, #36), Japan (#75), Turkey (#117, #121), former USSR (#111), New Zealand (#62), and Africa (#55) showed no nucleotide substitutions.

3.4. AFLP analysis

Each of the five primer-pairs used for the AFLP analysis, produced evenly distributed AFLP fragments between 100 and 700 bp. However, the number of AFLP fragments produced by each primer-pair differed significantly. For example primer-pair E17/M25 generated 21 clearly visible fragments, while primer-pair E19/M25 resulted in 38 clear fragments (data not shown). In general, good results were obtained with E + 2/M + 1 primer-pairs, which produced between 50 and 60 clearly distinguishable fragments per primer-pair and of which almost one third were polymorphic. Therefore, five E + 2/M + 1 primer-pairs, i.e. E15/M02, E18/M02, E18/M03, E23/M02, and E20/M04, were selected in order to determine the intra-specific diversity in the collection of 67 *C. fulvum* strains (Table 3).

In total 255 AFLP fragments between 100 and 700 bp were generated using the five selected primer combinations, of which 72 (28.2%) were polymorphic among the overall collection of *C. fulvum* strains analyzed. Of the 72 polymorphic fragments, 55 (76.4%) showed different alleles in more than 5% of the strains, while the remaining 17 AFLP loci (23.6%) showed different alleles at a frequency of 5% or less, indicating possible rare alleles (Hartl and Clark, 1997). No considerable differences were observed among the different primer-pairs with respect to the number of polymorphic fragments generated within each geographic collection of strains. However, when data from all five primer-pairs were combined, then higher levels of polymorphic fragments were observed within the American (24.3%) as compared to the European (18.4%) and Japanese (18.8%) collection of strains.

3.5. Haplotypic diversity

Among the 67 strains of *C. fulvum*, 55 different multilocus AFLP haplotypes were identified (Table 6). Six haplotypes were detected more than once. The most frequent haplotype was detected five times and included four Dutch strains (#3, #23, #58, #66) and a French strain (#61),

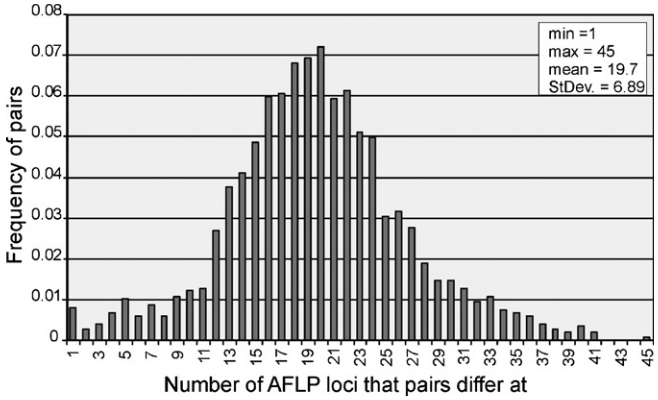


Fig. 3. Pair-wise comparison of 55 unique AFLP haplotypes showing the frequency of haplotype-pairs (y-axis) differing in one or more AFLP fragments (x-axis).

Table 6
Haplotypic diversity within the geographical collections of *C. fulvum* strains based on 255 AFLP fragments

Collection	Number of strains	Distinct haplotypes	Maximum frequency[a]	% Different haplotypes[b]	Haplotypic diversity[c]
Overall	67	55	5	82.1%	0.990
Europe	39	30	5	76.9%	0.977
Americas	13	13	1	100%	1.000
Japan	10	9	2	90%	0.978
New Zealand	2	2	1	—	—
Former USSR	2	2	1	—	—
Africa	1	1	1	—	—

[a] Frequency of the most frequent haplotype.

[b] G_{max} (100 × number of distinct haplotypes/number of strains).

[c] Nei's (1987) diversity index corrected for sample size.

whereas another haplotype was detected four times and included three French strains (#19, #20, #21) and one strain from the former USSR (#111). One haplotype was identified three times and included only Dutch strains (#10, #57, #59), while three additional haplotypes were detected twice and included pairs of Dutch (#64, #65) and Japanese strains (#68, #71), and a pair of a French (#18) strain together with a strain from the former USSR (#110). Pairwise comparisons of the 55 unique haplotypes showed that they differ between one and 45 AFLP fragments, following a normal distribution within this range of fragments. On average haplotypes differed in 20 AFLP fragments out of the 255 scored on the fingerprints (Fig. 3). In total, 97% of the haplotypes varied in more than five fragments, while only 3% of the haplotypes differed in five or less fragments. Nei's (1987) genotypic diversity corrected for sample size

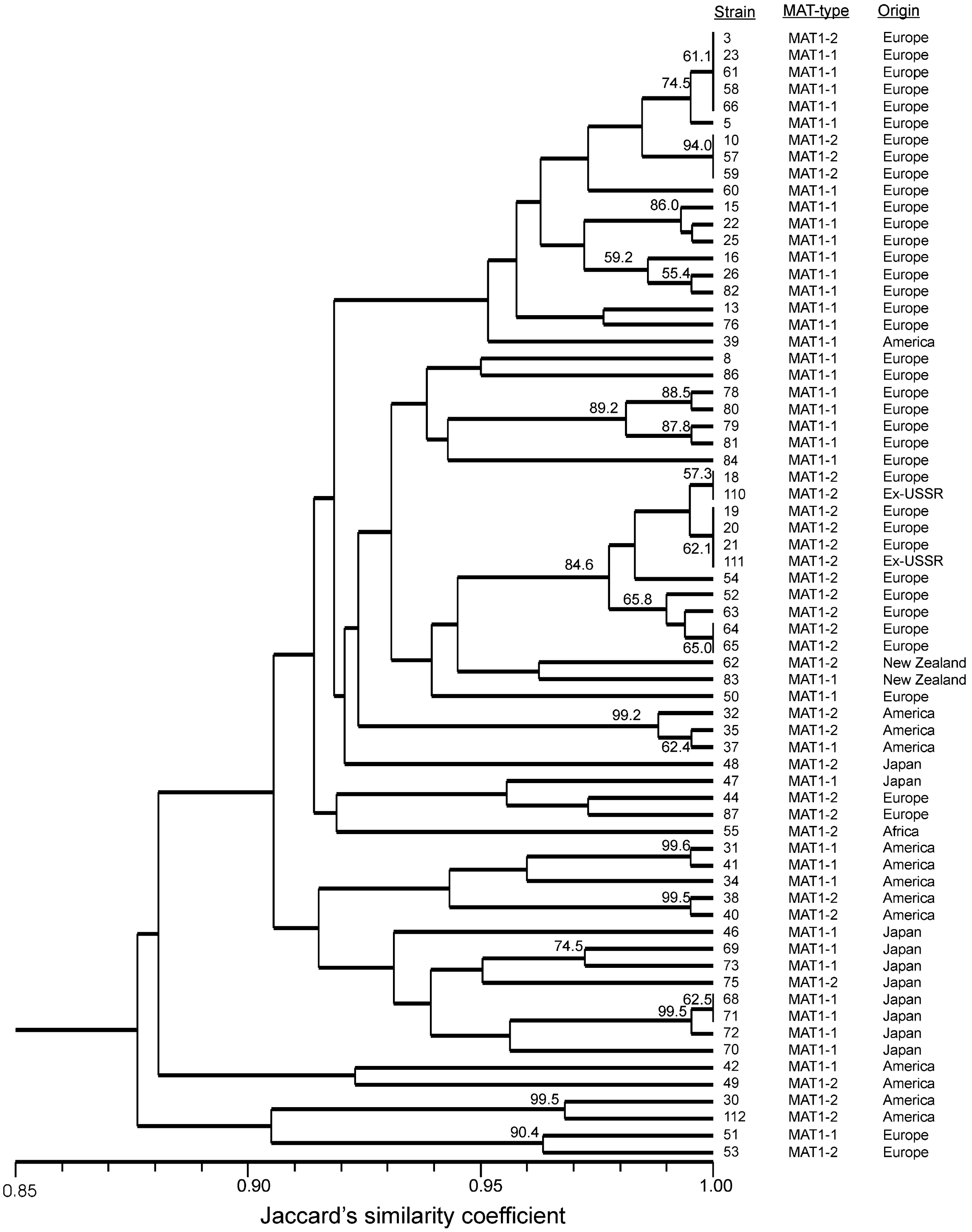


Fig. 4. UPGMA clustering of 67 strains of *C. fulvum* based on Jaccard's correlation coefficient, calculated from 255 AFLP fragments. Bootstrap values higher than 50% are shown above each branch. Mating-type as well as macro-geographic origin of the strains are also indicated.

was 0.99 for the overall collection, thus indicating that almost every strain represented a unique haplotype. Indices of haplotypic diversity were also almost maximal for the different continental collections. Nei's genotypic diversity corrected for sample size was 0.97, 0.98, and 1.0 for the European, American, and Japanese collections, respectively. Estimates of haplotypic evenness were quite high and were estimated at 0.69 and 0.93 for the European and Japanese collections, respectively. Collections from the former USSR, New Zealand, and Africa, are too small to draw any reliable conclusions.

3.6. Genetic distances and clustering of the strains

Jaccard's similarity coefficient was used to evaluate the genetic relatedness among the different strains of *C. fulvum*, using the combined AFLP data of all five primer combinations. These data were subsequently used to construct a UPGMA dendrogram of the 67 *C. fulvum* strains analyzed. Significant clustering was detected by bootstrap analysis (Fig. 4). Similarity coefficient values ranged between 0.87 and 1.0. Bootstrap analyses showed 14 nodes with support values higher than 70%, of which seven supported clusters with more than two strains. Most of the supported nodes contained strains of the same mating-type. One cluster with 84.6% of bootstrap support contained eleven strains that originated from Europe and the former USSR and were all of MAT1-2-type. A second cluster with 89.2% of bootstrap support consisted of four Belgian strains that were collected in 1959 and were MAT1-1-type. However, since the deepest nodes of the cladogram were not highly supported by bootstrap no reliable grouping of the strains could be made based on their geographic origin or mating-type.

3.7. Genetic differentiation

Genetic differentiation among the different geographical collections of strains was evaluated using Wright's *F*-statistics as estimated by theta (θ) (Cockerham and Weir, 1993) and Fisher's combined probability tests (Fisher, 1954) (Table 7). Collections from New Zealand, former USSR, and Africa were excluded from the analysis due to their small sample-sizes. Pair-wise comparisons at the 95% confidence interval level showed that the European collection was significantly differentiated from the American ($\theta = 0.213$) and Japanese ($\theta = 0.235$) collections, whereas the latter two collections were only moderately differentiated ($\theta = 0.133$) (Weir and Cockerham, 1984). Fisher's combined probability test further provided additional support for these results. Nei's (1972) genetic distance as well as Wright's (1978) geometric average modification on Rogers' distance (1972) was lowest for the pair of America and Japan (0.0207 and 0.138, respectively) as compared to pairs of Europe and America (0.024 and 0.150, respectively) and Europe and Japan (0.026 and 0.156, respectively). Bootstrap analysis and a UPGMA dendrogram produced based on Nei's (1972) genetic distances supported (83.9%) the clustering between the American and Japanese collections (Fig. 5).

4. Discussion

Here, we report on the cloning of mating-type idiomorphs from *C. fulvum*, a pathogen that until now was considered to be strictly asexual. However, the presence of opposite mating-type genes and the high levels of genotypic diversity observed in this pathogen suggest the occurrence of recombination or other sources of genetic variation.

The cloning and characterization of the mating-type genes from the tomato pathogen *C. fulvum* was performed using an approach that has been successfully applied in the past for the cloning of mating-type genes from other ascomycetous fungi (Arie et al., 1997; Groenewald et al., 2006). All *C. fulvum* strains analyzed thus far have either the *MAT1-1-1* or *MAT1-2-1* gene present in their genome, thus indicating that if a sexual cycle were to be found for *C. fulvum* then the fungus would be heterothallic. The mating-type genes of *C. fulvum* showed highest similarity to the *MAT* genes of two phylogenetically closely related species, namely *M. graminicola* and *S. passerinii* (Crous et al., 2001; Goodwin et al., 2001). Both fungal species exhibit a similar

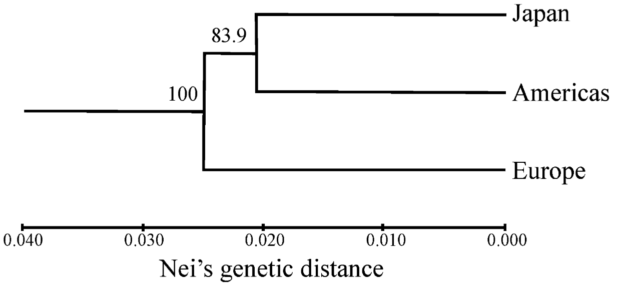


Fig. 5. UPGMA dendrogram generated based on Nei's (1972) genetic distances among the different *C. fulvum* geographic collections. Bootstrap support was obtained after 10,000 permutations over 255 AFLP fragments.

Table 7
Genetic differentiation among three *C. fulvum* collections, inferred from Wright's F_{st} statistics as estimated by theta (θ) (above diagonals) and Fishers' combined probability test (below diagonals)

	Europe	Americas	Japan
Europe	***	$\theta = 0.213^a$ (0.293-0.139^b)	$\theta = 0.235^a$ (0.347–0.130)
Americas	$P < 0.0001$	***	$\theta = 0.133$ (0.194–0.071)
Japan	$P < 0.0001$	$P = 0.056$	***

[a] Significant at the $P < 0.05$ level.

[b] Confidence intervals (CI) derived after bootstrapping with 10,000 permutations.

organizational structure in their mating-type locus; each *MAT* idiomorph contains a single gene encoding a protein with an alpha-domain (MAT1-1-1) or an HMG-domain (MAT1-2-1). This organization is commonly present in heterothallic members of the *Dothideomycetes* to which *M. graminicola*, *S. passerinii* and *C. fulvum* belong (Braun et al., 2003; Crous et al., 2001; Goodwin et al., 2001; Groenewald et al., 2006; Poggeler, 2001; Schoch et al., 2006). However, although the individual *MAT* genes of *C. fulvum* are highly similar to the *MAT* genes from the *Dothiomycetes*, the overall organization of the MAT idiomorphs in *C. fulvum* seems to be deviating. In this study the presence of an additional ORF in each of the two idiomorphs of *C. fulvum* was observed. It is currently unknown whether the ORFs are transcribed into functional proteins and it is difficult to speculate on their putative function since they do not exhibit significant similarity to other sequences currently present in the database. Moreover, two highly homologous "islands" of identity between the otherwise largely dissimilar idiomorphs were identified. Although these homologous regions are part either of *ORF1-1-2* or *ORF1-2-2* in MAT1-1 and MAT1-2, respectively, *ORF1-1-2* and *ORF1-2*-2 are only partially present in the opposite idiomorphs. Similar "islands" of identity, but only containing eight to nine bps, have been reported to be present in heterothallic *Cochliobolu*s species where they might function as putative sites for rare homologous recombination events, and may in this way be involved in the evolution of homothallic fungi from heterothallic progenitors (Yun et al., 1999).

The number of introns predicted to be present in the *MAT1-1-1* and *MAT1-2-1* gene of *C. fulvum* is higher than observed in other fungal species. Three introns were predicted for *MAT1-1-1* and they display a positional conservation to introns predicted in this gene of several *Cercospora* species, suggesting a close phylogenetic relation among these species (Goodwin et al., 2001; Groenewald et al., 2006). For *MAT1-2-1* an additional intron is predicted to be located in the HMG-box domain, which has not been observed in other fungal species. Although a growing number of studies indicate introns that are present at specific positions in one species but are absent in closely related taxa, the biological significance and mechanisms of intron gain are not yet clear (Logsdon, 1998; Logsdon et al., 1998; Lynch and Richardson, 2002). It has been postulated that introns can be gained and lost in different genomes in response to strong selective forces (Belshaw and Bensasson, 2006) and as such could constitute a significant driving force in the evolution of fungal genes (Nielsen et al., 2004). Introns of orthologous genes aligning at the same position are thought to have been inherited from a common ancestor, whereas lineage-specific introns mostly reflect gain events at later stages of evolution (Fedorov et al., 2002; Rogozin et al., 2003; Sverdlov et al., 2005). Therefore, the presence of the additional predicted introns in the *MAT* genes of *C. fulvum* might suggest recent evolutionary divergence of these genes from similar genes present in closely related species, such as *M. graminicola* and *S. passerinii.*

It is tempting to speculate on the functionality of the mating-type genes in the absence of a known sexual stage, as in the case of *C. fulvum*. However, heterologous expression and functionality of mating-type genes from supposedly asexual fungi in the genetic background of close sexual relatives has been demonstrated for *A. alternata* (Arie et al., 2000). In this case, absence of a sexual stage in this pathogen has been attributed to the lack or failure of some other important components of the mating signal-transduction pathway, and not to disfunctionality of *MAT* genes (Arie et al., 2000; Yun et al., 2000). Despite the fact that the functionality of *MAT1-1-1* and *MAT1-2-1* of *C. fulvum* has not been investigated yet, their high similarity to mating-type genes from other sexually active members of *Mycosphaerella* and the presence in their coding regions of only limited polymorphisms, suggests that they are still functional. Therefore, heterologous expression of *MAT1-1-1* and *MAT1-2-1* from *C. fulvum* in *MAT*$^-$ mutants of a closely related and sexually highly active species, such as *M. graminicola*, could confirm the functionality of the mating-type genes of *C. fulvum* as well.

Mating-type genes are frequently used in population studies as their presence, relative frequency and distribution within a population could be indicative of the reproductive mode of a fungus (Groenewald et al., 2006; Tredway et al., 2003; Zhan et al., 2002). In a sexual population, negative frequency-dependent selection is expected to retain an equilibrium in the two mating-type idiomorphs over several spatial scales, whereas in asexual populations this ratio would be skewed (Goodwin et al., 2003; Richman, 2000). A deviation from 1:1 ratios was observed for all of the *C. fulvum* genotype-corrected collections analyzed, therefore suggesting that asexual propagation is predominant in the epidemiology of this pathogen. However, both mating-types were present in almost all collections and none of them seemed to be in fixation in a particular collection, suggesting that the potential for sexual reproduction is at least present in the collections. Skewed mating-type ratios may also be caused by factors that are unrelated to the reproduction mode of a fungus (Milgroom, 1996). For instance, it has been reported that mating-type genes may also function in the maintenance of cell wall integrity, virulence and other cellular processes (Kunz and Haynes, 1981; Kwon-Chung et al., 1992; Verna and Ballester, 1999). In these cases, selection pressure acting on a mating-type or a closely linked locus due to for example fungicide applications or a resistant cultivar, might favor the propagation of one of the two mating-type idiomorphs in a population. Gene-for-gene systems can be particularly influenced by epistatic selection of particular avirulence genes, based on the resistance genes employed in host crop plants (Kolmer, 1992; Wolfe and McDermott, 1994). Such selection has also been imposed on the *C. fulvum* avirulence (*Avr*) genes following the introduction of the *Cf*-resistance genes into commercially grown tomato plants (Westerink et al., 2004b). It is possible that the major part of the collection of *C. fulvum* strains used in this study has been sampled from resistant plants that had become susceptible to newly arisen virulent races of the fungus. This means that the collection of strains is not a random, but a skewed sample, as it might have been strongly affected by the employment of *Cf*-genes, which could have influenced the spatial distribution of the two mating-type genes. Unfortunately, for the major part of the collection it is not known from which commercial cultivars the strains were collected, while conclusions drawn from small sample-sizes are only indicative. Therefore, the presence of the two mating-type genes alone does not allow us to draw any firm conclusion on frequency and occurrence of recombination in *C. fulvum*, unless supported by additional genetic data.

In *C. fulvum*, AFLP analysis distinguished 55 haplotypes among the 67 strains analyzed in our collection, thereby revealing the overall high genotypic diversity present in this collection. On average, most haplotypes differed from each other at 20 AFLP loci out of the 255 amplified fragments, indicating that haplotypes were unambiguously identified. The high levels of genotypic diversity and the large number of loci in which *C. fulvum* strains differ are not typical for a strictly asexual fungus, but suggest the occurrence of recombination in this pathogen. This could also explain the fact that strains of the fungus were identified that shared the same virulence spectrum but were of opposite mating-type. Several mutations have been identified in *C. fulvum Avr* genes that determine the virulence spectrum of the different races of the fungus (Westerink et al., 2004b), while specific complex virulence spectra were thought to have arisen in a few clonal lineages by successive accumulation of mutations in the different *Avr* genes (Joosten and De Wit, 1999; Westerink et al., 2004a). We found evidence to partially reject this hypothesis as strains of the fungus with the same complex virulence spectrum but with opposite mating-types were identified. For example, the Dutch strains IPO2459 (50381) and IPO2459 (30787) are both races 2.4.5.9 and overcome the resistance genes *Cf-2*, *Cf-4*, *Cf-5*, and *Cf-9* (Boukema, 1981; Lindhout et al., 1989), but have opposite mating-types and share distinct AFLP patterns. Therefore, clonal propagation and the dispersal of a clonal lineage around the world can not account for the occurrence of strains with an identical virulence spectrum, but opposite mating-types. In this case, the virulence spectrum of such strains would either have to be defined by different mutations in the respective *Avr* genes, or otherwise a chromosomal exchange containing the mating-type locus must have taken place.

Despite the fact that recombination could explain a number of features revealed in the collection of *C. fulvum* strains analyzed, it should also be taken into account that events, such as high mutation rates, highly active transposons, mitotic recombination, or the occurrence of a parasexual cycle (Fierro and Martin, 1999) could also act as a source of genetic variation. As mentioned above, the successive introduction of *Cf*-resistance genes into commercial tomato cultivars since the early 1940s has imposed an enormous selection pressure on *C. fulvum*, which has generated races with complex virulence spectra, some of which can overcome as many as five different *Cf* genes (Lindhout et al., 1989). This transition from avirulence to virulence is generally associated with DNA modifications in the *Avr* genes of the fungus that code for race-specific elicitors. Such modifications include point and frameshift mutations, insertions of transposon-like elements, or even deletion of an entire ORF (Westerink et al., 2004b). Moreover, pulse-field gel electrophoresis revealed chromosome length polymorphism including large deletions in different races of *C. fulvum* (Talbot et al., 1991). Chromosome polymorphisms have been frequently observed in natural strains of many fungal species and this phenomenon seems to occur more frequently in asexual than sexual pathogens (Fierro and Martin, 1999). In addition, a high content of repetitive DNA sequences and retro-transposons has been identified in many chromosomes of *C. fulvum* (Talbot et al., 1991), which can trigger extensive chromosome rearrangements through various molecular processes (McHale et al., 1992). Such phenomena have been reported to occur frequently in the rice pathogen *Magnaporthe grisea* (Skinner et al., 1993). Lastly, it has been shown that during an induced parasexual cycle in *C. fulvum*, mitotic recombination can lead to a high degree of sequence rearrangement in this pathogen (Arnau et al., 1994; Arnau and Oliver, 1993). In conclusion, in addition to sexual recombination the later phenomena could also contribute to the genetic variability observed in *C. fulvum*.

Acknowledgments

We kindly acknowledge Petra van de Berg (Laboratory of Plant Breeding, Wageningen University) for technical assistance with the AFLP analysis. We are thankful to Prof. Dr Verna Higgins and Alice Cheung (University of Toronto Canada) for providing the Canadian strains of this collection and to Dr. Emel Gakir (Plant Protection Central Research Institute, Ankara, Turkey) for the Turkish strains in the collection. Dr Ioannis Stergiopoulos was financially supported by a Research Training Network (RTN) Grant of the European Commission (ACE; Contact No. HPRN-CT-2002-00249). Marizeth Groenewald was financially supported by CBS-Odo van Vloten Stichting and the Royal Netherlands Academy of Arts and Sciences.

References

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

Arie, T., Christiansen, S.K., Yoder, O.C., Turgeon, B.G., 1997. Efficient cloning of ascomycete mating type genes by PCR amplification of the conserved MAT HMG box. Fungal Genet. Biol. 21, 118–130.

Arie, T., Kaneko, I., Yoshida, T., Noguchi, M., Nomura, Y., Yamaguchi, I., 2000. Mating-type genes from asexual phytopathogenic ascomycetes

Fusarium oxysporum and *Alternaria alternata*. Mol. Plant. Microbe Interact. 13, 1330–1339.

Arnau, J., Oliver, R.P., 1993. Inheritance and alteration of transforming DNA during an induced parasexual cycle in the imperfect fungus *Cladosporium fulvum*. Curr. Genet. 23, 508–511.

Arnau, J., Housego, A.P., Oliver, R.P., 1994. The use of RAPD markers in the genetic-analysis of the plant-pathogenic fungus *Cladosporium fulvum*. Curr. Genet. 25, 438–444.

Belshaw, R., Bensasson, D., 2006. The rise and falls of introns. Heredity 96, 208–213.

Boukema, I.W., 1981. Races of *Cladosporium fulvum* (*Fulvia fulva*) and genes of resistance in the tomato (Lycopersicon Mill). Proceedings of the Meeting of the Eucarpia Tomato Working Group, Avignon, 287–292.

Braun, U., Crous, P.W., Dugan, F., Groenewald, J., Sybren De Hoog, G., 2003. Phylogeny and taxonomy of *Cladosporium*-like hyphomycetes, including *Davidiella*, the teleomorph of *Cladosporium*. Mycol. Prog. 2, 3–18.

Cockerham, C.C., Weir, B.S., 1993. Estimation of gene flow from *F*-statistics. Evolution 47, 855–863.

Cooke, M.C., 1906. In: Fungoid Pests of Cultivated Plants.

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

Crous, P.W., Kang, J.C., Braun, U., 2001. A phylogenetic redefinition of anamorph genera in *Mycosphaerella* based on ITS rDNA sequence and morphology. Mycologia 93, 1081–1101.

De Wit, P.J.G.M., Brandwagt, B.F., van den Burg, H.A., Cai, X., van der Hoorn, R.A.L., De Jong, C.F., van't Klooster, J., De Kock, M.J.D., Kruijt, M., Lindhout, W.H., Luderer, R., Takken, F.L.W., Westerink, N., Vervoort, J.J.M., Joosten, M.H.A.J., 2002. The molecular basis of co-evolution between *Cladosporium fulvum* and tomato. Anton. Leeuw. Int. J.G. 81, 409–412.

Dodgson, A.R., Pujol, C., Pfaller, M.A., Denning, D.W., Soll, D.R., 2005. Evidence for recombination in *Candida glabrata*. Fungal Genet. Biol. 42, 233–243.

Fedorov, A., Merican, A.F., Gilbert, W., 2002. Large-scale comparison of intron positions among animal, plant, and fungal genes. Proc. Natl. Acad. Sci. USA 99, 16128–16133.

Fierro, F., Martin, J.F., 1999. Molecular mechanisms of chromosomal rearrangement in fungi. Crit. Rev. Microbiol. 25, 1–17.

Fisher, R.A., 1954. Statistical Methods for Research Workers. Oliver and Boyd, Edimburgh.

Goodwin, S.B., Dunkle, L.D., Zismann, V.L., 2001. Phylogenetic analysis of *Cercospora* and *Mycosphaerella* based on the internal transcribed spacer region of ribosomal DNA. Phytopathology 91, 648–658.

Goodwin, S.B., Waalwijk, C., Kema, G.H.J., Cavaletto, J.R., Zhang, G., 2003. Cloning and analysis of the mating-type idiomorphs from the barley pathogen *Septoria passerinii*. Mol. Genet. Genomics 269, 1–12.

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

Hartl, D.L., Clark, A.G., 1997. Genetic and Phenetic Variation. Sinauer Associates Inc., Sunderland, MA.

Huson, D.H., 1998. SplitsTree: analyzing and visualizing evolutionary data. Bioinformatics 14, 68–73.

Joosten, M.H.A.J., De Wit, P.J.G.M., 1999. The tomato-*Cladosporium fulvum* interaction: a versatile experimental system to study plant–pathogen interactions. Annu. Rev. Phytopathol. 37, 335–367.

Keller, S.M., Wolfe, M.S., McDermott, J.M., McDonald, B.A., 1997. High genetic similarity among populations of *Phaeosphaeria nodorum* across wheat cultivars and regions in Switzerland. Phytopathology 87, 1134–1139.

Kolmer, J.A., 1992. Diversity of virulence phenotypes and effect of host sampling between and within populations of *Puccinia recondita* f.sp tritici in Canada. Plant Dis. 76, 618–621.

Kronstad, J.W., Staben, C., 1997. Mating type in filamentous fungi. Annu. Rev. Genet. 31, 245–276.

Kunz, B.A., Haynes, R.H., 1981. Phenomenology and genetic-control of mitotic recombination in yeast. Annu. Rev. Genet. 15, 57–89.

Kwon-Chung, K.J., Edman, J.C., Wickes, B.L., 1992. Genetic association of mating types and virulence in *Cryptococcus* neoformans. Infect. Immun. 60, 602–605.

Linde, C.C., Zhan, J., McDonald, B.A., 2002. Population structure of *Mycosphaerella graminicola*: from lesions to continents. Phytopathology 92, 946–955.

Lindhout, P., Korta, W., Cislik, M., Vos, I., Gerlagh, T., 1989. Further Identification of races of *Cladosporium fulvum* syn. *Fulvia fulva* on tomato originating from the Netherlands, France and Poland. Neth. J. Plant Pathol. 95, 143–148.

Litvintseva, A.P., Marra, R.E., Nielsen, K., Heitman, J., Vilgalys, R., Mitchell, T.G., 2003. Evidence of sexual recombination among *Cryptococcus* neoformans serotype A isolates in sub-Saharan Africa. Eukaryot. Cell. 2, 1162–1168.

Logsdon, J.M., 1998. The recent origins of spliceosomal introns revisited. Curr. Opin. Genet. Dev. 8, 637–648.

Logsdon, J.M., Stoltzfus, A., Doolittle, W.F., 1998. Molecular evolution: Recent cases of spliceosomal intron gain? Curr. Biol. 8, R560–R563.

Lynch, M., Richardson, A.O., 2002. The evolution of spliceosomal introns. Curr. Opin. Genet. Dev. 12, 701–710.

McDonald, B.A., Linde, C., 2002. Pathogen population genetics, evolutionary potential, and durable resistance. Annu. Rev. Phytopathol. 40, 349–379.

McHale, M.T., Roberts, I.N., Noble, S.M., Beaumont, C., Whitehead, M.P., Seth, D., Oliver, R.P., 1992. Cft-I - an Ltr-retrotransposon in *Cladosporium fulvum*, a fungal pathogen of tomato. Mol. Gen. Genet. 233, 337–347.

Meirmans, P.G., Van Tienderen, P.H., 2004. GENOTYPE and GENODIVE: two programs for the analysis of genetic diversity of asexual organisms. Mol. Ecol. Notes 4, 792–794.

Metzenberg, R.L., Glass, N.L., 1990. Mating type and mating strategies in neurospora. Bioessays 12, 53–59.

Milgroom, M.G., 1996. Recombination and the multilocus structure of fungal populations. Annu. Rev. Phytopathol. 34, 457–477.

Miller, M., Tools for population genetic analyses (TFPGA) 1.3: A windows program for the analysis of allozyme and molecular population genetic data. Computer software distributed by author. 1997.

Nei, M., 1972. Genetic distance between populations. Am. Nat. 106, 283.

Nei, M., 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics 89, 583–590.

Nei, M., 1987. Molecular evolutionary genetics. New York, NY, USA.

Nielsen, C.B., Friedman, B., Birren, B., Burge, C.B., Galagan, J.E., 2004. Patterns of intron gain and loss in fungi. PLoS. Biol. 2, 2234–2242.

Poggeler, S., 2001. Mating-type genes for classical strain improvements of ascomycetes. Appl. Microbiol. Biotechnol. 56, 589–601.

Raymond, M., Rousset, F., 1995. Genepop (Version-1.2)—population-genetics software for exact tests and ecumenicism. J. Hered. 86, 248–249.

Richman, A., 2000. Evolution of balanced genetic polymorphism. Mol. Ecol. 9, 1953–1963.

Rivas, S., Thomas, C.M., 2005. Molecular interactions between tomato and the leaf mold pathogen *Cladosporium fulvum*. Annu. Rev. Phytopathol. 43, 395–436.

Rogers, J.S., 1972. Measures of Genetic Similarity and Genetic Distance. University of Texas Publication no. 7213, Austin.

Rogozin, I.B., Wolf, Y.I., Sorokin, A.V., Mirkin, B.G., Koonin, E.V., 2003. Remarkable interkingdom conservation of intron positions and massive, lineage-specific intron loss and gain in eukaryotic evolution. Curr. Biol. 13, 1512–1517.

Rohlf, F.J., 1997. NTSYSpc: Numerical Taxonomy and Multivariate Analysis System, version 201. Department of Ecology and Evolution, State University of New York.

Salamov, A.A., Solovyev, V.V., 2000. Ab initio gene finding in *Drosophila* genomic DNA. Genome Res. 10, 516–522.

Schoch, C., Shoemaker, R.A., Seifert, K.A., Hambleton, S., Spatafora, J.W., Crous, P.W., 2006. A multigene phylogeny of the Dothideomycetes using nuclear loci. Mycologia. In press.

Skinner, D.Z., Budde, A.D., Farman, M.L., Smith, J.R., Leung, H., Leong, S.A., 1993. Genome organization of *Magnaporthe grisea*—genetic map, electrophoretic karyotype, and occurrence of repeated DNAs. Theor. Appl. Genet. 87, 545–557.

Solovyev, V.V., Salamov, A.A., Lawrence, C.B., 1994. Predicting internal exons by oligonucleotide composition and discriminant analysis of spliceable open reading frames. Nucleic Acids Res. 22, 5156–5163.

Stakman, E.C., 1957. Problems in preventing plant disease epidemics. Am. J. Bot. 44, 259–267.

Sverdlov, A.V., Rogozin, I.B., Babenko, V.N., Koonin, E.V., 2005. Conservation versus parallel gains in intron evolution. Nucleic Acids Res. 33, 1741–1748.

Talbot, N.J., Oliver, R.P., Coddington, A., 1991. Pulsed field gel-electrophoresis reveals chromosome length differences between strains of *Cladosporium fulvum* (syn *Fulvia fulva*). Mol. Gen. Genet. 229, 267–272.

Taylor, J.W., Jacobson, D.J., Fisher, M.C., 1999. The evolution of asexual fungi: reproduction, speciation and classification. Annu. Rev. Phytopathol. 37, 197–246.

Thomma, B.P.H.J., Van Esse, H.P., Crous, P.W., De Wit, P.J.G.M., 2005. *Cladosporium fulvum* (syn. *Passalora fulva*), a highly specialized plant pathogen as a model for functional studies on plant pathogenic *Mycosphaerellaceae*. Mol. Plant Pathol. 6, 379–393.

Tibayrenc, M., Kjellberg, F., Arnaud, J., Oury, B., Breniere, S.F., Darde, M.L., Ayala, F.J., 1991. Are eukaryotic microorganisms clonal or sexual—a population-genetics vantage. Proc. Natl. Acad. Sci. USA 88, 5129–5133.

Tredway, L.P., Stevenson, K.L., Burpee, L.L., 2003. Mating type distribution and fertility status in *Magnaporthe grisea* populations from turfgrasses in Georgia. Plant Dis. 87, 435–441.

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

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

Verna, J., Ballester, R., 1999. A novel role for the mating type (MAT) locus in the maintenance of cell wall integrity in *Saccharomyces cerevisiae*. Mol. Gen. Genet. 261, 681–689.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., Vandelee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M., Zabeau, M., 1995. AFLP—a new technique for DNA-fingerprinting. Nucleic Acids Res. 23, 4407–4414.

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

Weir, B.S., Cockerham, C.C., 1984. Estimating *F*-statistics for the analysis of population-structure. Evolution 38, 1358–1370.

Westerink, N., Brandwagt, B.F., De Wit, P.J.G.M., Joosten, M.H.A.J., 2004a. *Cladosporium fulvum* circumvents the second functional resistance gene homologue at the Cf-4 locus (Hcr9-4E) by secretion of a stable avr4E isoform. Mol. Microbiol. 54, 533–545.

Westerink, N., Joosten, M.H.A.J., De Wit, P.J.G.M., 2004b. Fungal (a)virulence factors at the crossroads of disease susceptibility and resistance. In: Punja, Z.K. (Ed.), Fungal Disease Resistance in Plants: Biochemistry, Molecular Biology, and Genetic Engineering. Food Products Press, Binghamton, USA, pp. 93–137.

Wolfe, M.S., McDermott, J.M., 1994. Population-genetics of plant pathogen interactions—the example of *Erysiphe graminis–Hordeum vulgare* pathosystem. Annu. Rev. Phytopathol. 32, 89–113.

Wright, S., 1978. Variability within and among Natural Populations. University of Chicago Press, Chicago.

Yu, J., Hu, S., Wang, J., Wong, G.K.-S., Li, S., Liu, B., Deng, Y., Dai, L., Zhou, Y., Zhang, X., Cao, M., Liu, J., Sun, J., Tang, J., Chen, Y., Huang, X., Lin, W., Ye, C., Tong, W., Cong, L., Geng, J., Han, Y., Li, L., Li, W., Hu, G., Huang, X., Li, W., Li, J., Liu, Z., Li, L., Liu, J., Qi, Q., Liu, J., Li, L., Li, T., Wang, X., Lu, H., Wu, T., Zhu, M., Ni, P., Han, H., Dong, W., Ren, X., Feng, X., Cui, P., Li, X., Wang, H., Xu, X., Zhai, W., Xu, Z., Zhang, J., He, S., Zhang, J., Xu, J., Zhang, K., Zheng, X., Dong, J., Zeng, W., Tao, L., Ye, J., Tan, J., Ren, X., Chen, X., He, J., Liu, D., Tian, W., Tian, C., Xia, H., Bao, Q., Li, G., Gao, H., Cao, T., Wang, J., Zhao, W., Li, P., Chen, W., Wang, X., Zhang, Y., Hu, J., Wang, J., Liu, S., Yang, J., Zhang, G., Xiong, Y., Li, Z., Mao, L., Zhou, C., Zhu, Z., Chen, R., Hao, B., Zheng, W., Chen, S., Guo, W., Li, G., Liu, S., Tao, M., Wang, J., Zhu, L., Yuan, L., Yang, H., 2002. A draft sequence of the rice genome (*Oryza sativa* L. ssp. indica). Science 296, 79–92.

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

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

Zhan, J., McDonald, B.A., 2004. The interaction among evolutionary forces in the pathogenic fungus *Mycosphaerella graminicola*. Fungal Genet. Biol. 41, 590–599.

Zhan, J., Kema, G.H.J., Waalwijk, C., McDonald, B.A., 2002. Distribution of mating type alleles in the wheat pathogen *Mycosphaerella graminicola* over spatial scales from lesions to continents. Fungal Genet. Biol. 36, 128–136.

Zhan, J., Pettway, R.E., McDonald, B.A., 2003. The global genetic structure of the wheat pathogen *Mycosphaerella graminicola* is characterized by high nuclear diversity, low mitochondrial diversity, regular recombination, and gene flow. Fungal Genet. Biol. 38, 286–297.

Zhao, J.J., Wang, X.W., Deng, B., Lou, P., Wu, J., Sun, R.F., Xu, Z.Y., Vromans, J., Koornneef, M., Bonnema, G., 2005. Genetic relationships within Brassica rapa as inferred from AFLP fingerprints. Theor. Appl. Genet. 110, 1301–1314.

Mycol. Res. **108** (11): 1271–1282 (November 2004). © The British Mycological Society
DOI: 10.1017/S0953756204001054 *Printed in the United Kingdom.*

Mycosphaerella punctiformis revisited: morphology, phylogeny, and epitypification of the type species of the genus *Mycosphaerella* (*Dothideales*, *Ascomycota*)

Gerard J. M. VERKLEY[1], Pedro W. CROUS[1], J. Z. (Ewald) GROENEWALD[1], Uwe BRAUN[2] and André APTROOT[1]

[1] *Centraalbureau voor Schimmelcultures, P.O. Box 85167, NL-3508 AD Utrecht, The Netherlands.*
[2] *F.B. Biologie, Institut für Geobotanik und Botanischer Garten, Martin-Luther-Universität, Neuwerk 21, D-06099 Halle (Saale), Germany.*
E-mail: verkley@cbs.knaw.nl

Received 21 January 2004; accepted 15 July 2004.

Mycosphaerella punctiformis, the type species of the genus *Mycosphaerella*, is epitypified by material collected on *Quercus robur* in The Netherlands. The teleomorph is described *in planta*, and the *Ramularia* anamorph, for which the new name *R. endophylla* is proposed, and the *Asteromella* spermatial state are characterized *in vitro*. Sequence data of the nuclear ribosomal DNA are presented and analyzed together with other *Mycosphaerella* spp. with *Ramularia* and several other anamorphs. Several strains originating from *Quercus*, *Acer* and *Tilia* showed diverging ITS sequences, indicating that the *M. punctiformis* complex may comprise more than a single phylogenetic species, but this could not be confirmed by the analysis of our dataset. An endophytic phase is established for the first time in the life-cycle of *M. punctiformis*, as the species was repeatedly isolated from surface sterilized green healthy leaves of *Quercus robur* in summer at the epitype locality.

INTRODUCTION

The genus *Mycosphaerella* is one of the largest genera of ascomycetes, comprising many plant pathogens of economically important crops, but also saprobic species. Teleomorph morphology is relatively simple and uniform in *Mycosphaerella*, but the genus is unequalled in the diversity of the associated anamorphs. Indeed, 27 anamorphic genera have been associated with *Mycosphaerella* (von Arx 1983, Sutton & Hennebert 1994), 23 of which were accepted by Crous *et al.* (2000). Klebahn (1918) and Laibach (1922) suggested segregating groups of species from *Mycosphaerella* based on their association with a particular anamorph, but genera they proposed did not become widely used. Recent molecular studies indicate that characters used to define the anamorph genera, such as conidiomatal structure, and conidial shape, size, and septation, are not always phylogenetically informative, and that some generic concepts for the anamorphs need to be revised (Crous *et al.* 2000, Crous, Kang & Braun 2001, Verkley *et al.* 2004). However, a group of species with *Cladosporium* anamorphs was recently segregated under the name *Davidiella* (Braun *et al.* 2003); it is a close sister group of other *Mycosphaerella*.

Mycosphaerella punctiformis, the type species of *Mycosphaerella*, was originally described as *Sphaeria punctiformis* from fallen leaves of *Quercus robur*. Microscopical examination of the lectotype material of *M. punctiformis* deposited in L, confirmed the identity. However, the over 200 yr old herbarium specimen does not provide an unambiguous application of the name, because recent molecular work has shown that *M. punctiformis* as currently circumscribed comprises cryptic species that are morphologically indistinguishable. Several strains in the CBS collection that had been morphologically identified as *M. punctiformis* from *Quercus*, *Acer* and *Tilia*, were found to be heterogeneous in their sequences of the internal transcribed spacer (ITS) region of the nuclear ribosomal RNA gene array. As no ex-type strain is available, we tried to obtain ribosomal DNA from the type material of *M. punctiformis*, but failed. In accordance with Art. 9.7 of the *Code*, we sought to settle the application of the name by selecting an epitype for *M. punctiformis*. The main purpose of this paper is to epitypify *M. punctiformis* with material recently collected from the type host *Quercus robur* in The Netherlands, and to give a full phenotypic characterization of the teleomorph, and (syn)anamorphs in culture. Because the anamorph will be the only sporulation observed in most ecological and endophyte studies, we consider it useful to also formally name this conidial state. Fresh ascomata of *M. punctiformis* were collected on dead fallen leaves of the type host *Quercus robur*, checked for agreement with the lectotype material, and ascospore isolates were made. We also obtained ecological data from a biodiversity study of foliar ascomycetous endophytes of *Quercus* in the epitype locality. We sequenced the ITS region of rDNA of the available strains of *M. punctiformis*, and also included a number of additional taxa in the sequence analyses to investigate the phylogenetic relationships of *M. punctiformis* with other *Mycosphaerella* species with *Ramularia* and several other anamorphs. Furthermore, partial small subunit (SSU) sequences of the ex-epitype strain of *M. punctiformis* were analysed with other data available in order to obtain further support for the phylogenetic position within the *Mycosphaerella* clade.

MATERIALS AND METHODS

Isolation from fruit bodies on decaying leaves and endophytic mycelia from green leaves

Strains used in this study are listed in Table 1. Dead fallen leaves with ascomata were collected in March to May of 2002 and 2003 in the forested area 'De Stompert' in The Netherlands, from three mature trees of *Quercus robur*. Leaves were incubated in a moist chamber for several hours in the laboratory at *ca* 20 °C. They were then cut into square pieces and glued to the inside of Petri dish lids to allow ascospores to be discharged on to 2% malt extract agar (MEA). Germinating ascospores were examined after 24 h, illustrated and transferred to MEA. Fresh green leaves from the same trees were collected monthly between May and November, put in plastic bags and transported to the lab. On the same day, leaves were sterilized in domestic bleach water (5% chlorine) for 5 min, followed by three rinses in sterile water. Small squares of about 0.5 cm^2 were placed onto MEA with 50 ppm streptomycin, aureomycin and penicillin to inhibit bacterial growth, placed on the laboratory bench in diffuse daylight, and regularly checked for fungal growth. Mycelia growing out of the margin were transferred to 2% MEA and oatmeal agar (OA; Centraalbureau voor Schimmelcultures 2001) and preliminarily identified morphologically.

Phenotypic characterization

For microscopic examination, fruiting structures were mounted in tap water. Line drawings were made with a drawing tube, and photographic images with a Nikon Coolpix 995 digital camera. For the description of colony features and sporulating structures, isolates were transferred onto OA and 3% MEA plates and placed in an incubator at 15 ° under n-UV (12 h rhythm). Colours are described according to Rayner (1970).

DNA extraction and sequencing

Strains were transferred from agar cultures to 2 ml liquid medium (2% malt extract) and incubated on a rotary shaker (300 rpm) for 3 wk at room temperature. Liquid cultures were transferred to 2-ml tubes, centrifuged and washed twice with sterile water. DNA was extracted using the FastDNAkit (Omnilabo 6050073, BIO 101, CA) according to the manufacturer's instructions. For ITS sequence analysis a part of the ribosomal RNA gene cluster was amplified by PCR using primer pairs V9G (de Hoog & Gerrits van den Ende 1998) and LR5 (Vilgalys & Hester 1990). Part of the 18S rRNA gene (SSU) was amplified using primers NS1 and NS4 (White *et al*. 1990). PCR was performed in 50 µl reaction volumes, each reaction containing 10–100 ng of genomic DNA, 25 pM of each primer, 40 µM dNTP, 1.0 unit Supertaq DNA polymerase and 5 µl 10× PCR buffer (SphaeroQ, Leiden). PCR was performed in an Applied Biosystems (Foster City, CA) thermocycler with the following program: 1 min at 95 °, 30 cycles (1 min 95 °, 1 min 55 °, 2 min 72 °) followed by a final extension of 5 min at 72 °. PCR products were cleaned with GFX columns (Amersham Pharmacia, NJ) and analyzed on a 2% agarose gel to estimate concentrations. ITS1 and ITS4 (White *et al*. 1990) were used as internal sequencing primers for the ITS region. The SSU region was sequenced using the PCR primers. Sequencing was performed with the BigDye terminator chemistry (Part no. 403049, Applied Biosystems) following the manufacturer's instructions. The sequencing products were cleaned with G50 Superfine Sephadex columns (Amersham Pharmacia 17-0041-01), and separated and analyzed in an ABI Prism 3700 DNA Analyzer (Applied Biosystems). Forward and reverse sequences were matched using SeqMan (DNAstar, Madison, WI).

Phylogenetic analyses

Pairwise and global alignment of consensus sequences were performed in Bionumerics 3.0 (Applied Maths, Kortrijk, Belgium), and manually adjusted where necessary. Parsimony analysis was done using PAUP v. 4.0b10 (Swofford 2003). The heuristic search was performed with the following parameters: characters unordered with equal weight, random taxon addition, branch swapping with tree bisection-reconnection (TBR) algorithm, branches collapsing if the maximum branch length was zero. Maxtrees was set at 10 000. Alignment gaps were treated as missing characters. Parsimony bootstrap analyses were performed using the full heuristic search option, random stepwise addition, and 1000 replicates, with maxtrees set at 100.

Neighbour-joining analyses was performed using PAUP, with GTR (Gamma = 0.5, and rates for variable sites equal), and 1000 neighbour-joining bootstrap replications to test the stability of clades. BLAST searches in GenBank revealed highest similarity to species of *Mycosphaerella*. GenBank accession numbers, taxon names and other information about the sequences from GenBank used in this study are given in Table 1. GenBank accession numbers (marked with *) of sequences generated in this study are also given in Table 1. A strain of *Davidiella tassiana* (sub *Mycosphaerella tassiana*) was defined as outgroup for the ITS dataset and sequences of *Botryosphaeria* species were used as outgroup for the SSU dataset. The alignments and trees were lodged in TreeBASE (study accession S1126).

RESULTS

Phylogenetic analyses

The alignment of the ITS sequences comprised 513 characters, of which 168 (36%) were parsimony-informative. 23 of these characters were excluded from the analysis because they were positioned in small insertions/deletions or regions with ambiguous position homology. Furthermore, 322 uninformative characters were also excluded, so that 145 characters were used in the parsimony analysis. In the neighbour joining analysis in total 213 characters were included, as constant characters were excluded, but autapomorphic characters were included to obtain accurate branch lengths in the phylogram. The heuristic search yielded 580 most parsimonious trees (MPT) of 535 steps (*C.I.* = 0.505, *R.I.* = 0.878, *R.C.I.* = 0.443, and homoplasy index = 0.495). The strict consensus tree is shown in Fig. 1. Several highly supported multi-taxon clades were the same in the parsimony and neighbour joining analyses (neighbour joining trees not shown). Among these was a clade comprising all included strains with *Ramularia* anamorphs (parsimony 99%/neighbour joining minimum 100%), which in the parsimony analysis formed a sister group to the clade with the cereal pathogens *Mycosphaerella graminicola* and *Septoria passerini* (100/92). The support for the two clades together was, however, lower (61/<50). Further highly supported clades were the one with *Cercospora* spp. (90/97), a clade with *M. crystallina*, *M. heimii*, *M. heimioides* and *M. colombiensis* (99/95), and a clade with *M. africana*, *M. keniensis*, *M. aurantia*, *M. hedericola*, *Mycosphaerella* sp. (from *Coprosma* sp.), *M. confusa*, and *Passalora fulva* (91/81). The *Ramularia* clade was rather unresolved in parsimony and neighbor joining analyses. In the parsimony analysis, only a clade comprising four strains identified as *M. punctiformis* from *Quercus*, *Acer* and *Tilia* was well-supported (100/95). With their closest sister *M. phacae-frigidae*, these strains also obtained good support in both analyses (91/77).

BLAST results of the SSU sequence of *M. punctiformis* (AY490775) supported the close association of *M. punctiformis* with other *Mycosphaerella* species. The alignment of the SSU sequences included 1067 characters, of which 1006 were constant, 21 were parsimony uninformative and 40 were parsimony informative. The heuristic search yielded eleven most parsimonious trees of 81 steps (C.I. 0.852, R.I. 0.919, R.C. index 0.783, and H.I. 0.148). The strict consensus tree is shown in Fig. 2. The topology of the eleven trees only differed in the internal ordering of groups in the *Mycosphaerella* clade. Two main clades are delimited in the SSU tree, the first clade contains isolates of *Mycosphaerella* (98% bootstrap support) and the other isolates of *Davidiella* (100% bootstrap support). The sequence of *M. punctiformis* groups closest to the sequences of a *Mycosphaerella* sp. isolated from *Acacia* (AY251116) and a sequence of *Septoria tritici* (AY251117). However, this association does not have significant bootstrap support.

Phenotypic characterization (Figs 3–10)

A description of the teleomorph *in planta*: *Leaf spots* not observed. *Ascomata* developing on fallen dead leaves, predominantly hypophyllous, black, subepidermal, erumpent to superficial, globose, 70–110 μm diam; apical ostiole 5–10 μm wide; wall consisting of 2–3 layers of medium brown textura angularis. *Asci* aparaphysate, fasciculate, bitunicate, subsessile, cylindrical, straight, 8-spored, 30–45 × 5–7(–9) μm. *Ascospores* multiseriate, overlapping, hyaline, guttulate, thin-walled, straight, fusoid-ellipsoidal with obtuse ends, widest just above the septum, medianly 1-septate, constricted at the septum, tapering towards both ends, but more prominently towards the lower end, (6–)8–9(–10) × (2–)3 μm (av. 9 × 3 μm). Germinating ascospores hyaline, distorting, forming germ tubes 4–6 μm diam apically, parallel to the long axis from both ascospore cells, and simultaneously also laterally, from one or both ascospore cells, at an angle of 90 ° or less to the long axis (Germination pattern D; Crous 1998).

Free conidia possibly belonging to *M. punctiformis* were occasionally observed in late summer on older leaf lesions caused by pathogens such as *Septoria quercicola* and *Discula* sp.

Colony description (diffuse daylight, 15 °): *Colonies* on OA reaching 28–31 mm diam in 27 d, spreading (low), sometimes in the centre with some elevated mycelium, margin even or slightly lobed, glabrous, pale Honey to Olivaceous Buff or Rosy Vinaceous to Rosy Buff, colony surface glabrous or with appressed pure white aerial hyphae or conidiophores; in the centre submerged and superficial mycelium Rosy Buff to Salmon and concolourous on reverse, or becoming Dark Violet to dark Purple due to the deposition of violet pigment on the outer surface of vegetative hyphae, surrounding medium then often becoming Coral to red by diffusing pigments, and Coral to Flesh on reverse. In a few isolates, the colony was dominated by olivaceous colours (underneath a white covering of

Table 1. Fungal isolates included for ITS and SSU sequence analyses (in alphabetical order of the teleomorph names).

GenBank accession no.				
ITS	SSU	Teleomorph	Anamorph	Origin
	U42476	*Botryosphaeria rhodina*	*Lasiodiplodia theobromae*	No data available
	U42477	*B. ribis*	*Fusicoccum* sp.	No data available
AY251078	AY251096	*Davidiella tassiana*	*Cladosporium herbarum*	ATCC 66670 (=‘STE-U 5101’); CCA-treated Douglas-fir pole, New York, USA
	AY251094	*Davidiella* state unknown	*Cl. cladosporioides*	ATCC 66669 (=‘STE-U 5100’); Creosote-treated southern pine pole, New York, USA
	AY251092	*Davidiella* state unknown	*Cl. colocasiae*	STE-U 4323; *Colocasia esculenta*, Fiji Islands
	AY251098	*Davidiella* state unknown	*Cl. sphaerospermum*	CBS 188.54 (=‘STE-U 3686’, ATCC 11290)
	AY251097	*Davidiella* state unknown	*Cl. uredinicola*	ATCC 46649 (=‘STE-U 5390’); Fungicolous on *Cronartium fusiforme* f. sp. *quercum* on *Quercina nigra* leaves, Alabama, USA
AF173314		*Mycosphaerella africana*	Unknown	STE-U 794 (ex-type); *Eucalyptus viminalis*, South Africa
AY490773		*M. africana*	Unknown	CBS 680.95 (=STE-U 796; ex type); *Eucalyptus viminalis*, South Africa
AY150331		*M. aurantia*	Unknown	CBS 110500; *Eucalyptus globulus*, Australia
AF222838		*M. colombiensis*	*Pseudocercospora colombiensis*	STE-U 1106; *Eucalyptus*, Colombia
AF362058		?*M. confusa*	*Ps. rubi*	CBS 256.35
AF222839		*M. crystallina*	*Ps. crystallina*	STE-U 801 (ex type); *Eucalyptus bicostata*, South Africa
AY490757		*M. crystallina*	*Ps. crystallina*	CBS 681.95, STE-U 802 (ex type); *Eucalyptus bicostata*, South Africa
AY266152		*M. fijiensis*	*Ps. fijiensis*	ATCC 22116, PF7; *Musa* sp., Philippines
AY266150		*M. fijiensis*	*Ps. fijiensis*	ATCC 36054, PFD9; *Musa* sp., Honduras
AF181706		*M. musicola*	*Ps. musae*	ATCC 22115; *Musa* sp., Philippines
AY288148		*M. musicola*	*Ps. musae*	PM11, ATCC 36143; *Musa*, Honduras
AY152590		*M. laricina*	*Pseudocercospora* sp.	CBS 326.52; *Larix decidua*, Switzerland
AY152595		*M. fragariae*	*Ramularia grevilleana*	CBS 259.36; *Fragaria* sp., The Netherlands
AY152597		*M. fragariae*	*R. grevilleana*	CBS 719.84; *Fragaria* sp., The Netherlands
AY152596		*M. fragariae*	*R. grevilleana*	CBS 298.34; *Fragaria* sp., The Netherlands
AF297235		*M. fragariae*	*R. grevilleana*	ATCC 24113; *Fragaria* sp., Illinois, USA
AF173312		*M. fragariae*	*R. grevilleana*	STE-U 656; *Fragaria* sp., South Africa
AY152601		*M. graminicola*	*Septoria tritici*	CBS 100330 (=IPO 6566.1); *Triticum aestivum*, The Netherlands
AY152602		*M. graminicola*	*S. tritici*	CBS 100335; *Triticum aestivum*, The Netherlands
AY152603		*M. graminicola*	*S. tritici*	CBS 392.59; *Triticum aestivum*
AF181692		*M. graminicola*	*S. tritici*	IPO 323; *Triticum aestivum*, The Netherlands
AF181693		*M. graminicola*	*S. tritici*	T1; *Triticum aestivum*, Minnesota, USA
AF362068	AY251117	*M. graminicola*	*S. tritici*	STE-U 658; *Triticum* sp., South Africa
AY152581		*M. grossulariae*	*S. ribis*	CBS 235.37; *Ribes nigrum*, The Netherlands
AY490772		*M. hedericola*	*Unknown*	CBS 441.86; *Hedera helix*, France
AF452508		*M. heimii*	*Pseudocercospora heimii*	CMW5705
AF452509		*M. heimii*	*Ps. heimii*	CMW5707
AF222841		*M. heimii*	*Ps. heimii*	No data available
AF452512		*M. heimii*	*Ps. heimii*	CMW5713
AF222842		*M. heimioides*	*Ps. heimioides*	STE-U 1312; *Eucalyptus*, Indonesia
AF173300		*M. keniensis*	Unknown	STE-U 1084; *Eucalyptus grandis*, Kenya
AY490768		*M. latebrosa*	*Septoria aceris*	CBS 183.97; *Acer pseudoplatanus*, The Netherlands
AY152553		*M. latebrosa*	*S. aceris*	CBS 687.94; *Acer pseudoplatanus*, The Netherlands
AY490769	AY251114	*M. latebrosa*	*S. aceris*	CBS 652.85; *Acer pseudoplatanus*, The Netherlands
AY152600		*M. marksii*	Unknown	CBS 682.95 (=‘STE-U 842’); *Eucalyptus grandis*, South Africa

AY152599		*M. parkii*	*Stenella parkii*	CBS 387.92 (=‘STE-U 353; ex type); *Eucalyptus grandis*, Brazil
AY490758		M. *phacae-frigidae*	*Ramularia* sp.?	CBS 234.55; *Phaca frigida*, Switzerland
AY152583		*M. populicola*	*Septoria populicola*	CBS 100045; *Populus trichocarpa*, Washington, USA
AY152584		*M. populicola*	*S. populicola*	CBS 100052; *Populus trichocarpa*, Washington, USA
AY152585		*M. populicola*	*S. populicola*	CBS 100044; *Populus trichocarpa*, Washington, USA
AY152586		*M. populicola*	*S. populicola*	CBS 100051; *Populus trichocarpa*, Washington, USA
AY152587		*M. populicola*	*S. populicola*	CBS 100047; *Populus trichocarpa*, Washington, USA
AY490759		‘*M. punctiformis*’	*Ramularia* sp.	CBS 515.69; *Acer pseudoplatanus*, The Netherlands
AY490760		‘*M. punctiformis*’	*Ramularia* sp.	CBS 742.79; *Tilia* sp., Germany
AY152593		‘*M. punctiformis*’	*Ramularia* sp.	CBS 943.97; *Quercus* sp., The Netherlands
AY152594		‘*M. punctiformis*’	*Ramularia* sp.	CBS 184.97; *Acer pseudoplatanus*, The Netherlands
AY490761		*M. punctiformis*	*R. endophylla*	CBS 942.97; *Quercus* sp., Belgium
AY490762		*M. punctiformis*	*R. endophylla*	CBS 113871 (SS); *Quercus robur*, The Netherlands
AY490763	AY490775*	*M. punctiformis*	*R. endophylla*	CBS 113265 (SS; ex-epitype); *Quercus robur*, The Netherlands
AY490764		*M. punctiformis*	*R. endophylla*	CBS 113868; leaf endophyte *Quercus robur*, The Netherlands
AY490765		*M. punctiformis*	*R. endophylla*	CBS 113869; leaf endophyte *Quercus robur*, The Netherlands
AY490766		*M. punctiformis*	*R. endophylla*	CBS 113870; leaf endophyte *Quercus robur*, The Netherlands
AF222848		*M. punctiformis*	*R. endophylla*	KC1
AY152591		*M. pyri*	*Septoria pyricola*	CBS 222.31; *Pyrus communis*
AY152592		*M. pyri*	*S. pyricola*	CBS 640.72; *Pyrus communis*, The Netherlands
AY490767		*M. rubella*	*Ramularia* sp.?	CBS 288.49; *Angelica sylvestris*
AY152575		*M. ulmi*	*Phloeospora ulmi*	CBS 344.97; *Ulmus glabra*, Austria
AY490774		*Mycosphaerella* sp.	*Septoria* sp. (in culture)	CBS 113113; *Coprosma* sp., New Zealand
AY490771		*Mycosphaerella* sp.	*S. quercicola*	CBS 663.94; *Quercus robur*, The Netherlands
	AY251115	*Mycosphaerella stromatosa*	*Pseudocercospora stromatosa*	STE-U 1731; *Protea* sp., South Africa
	AY251116	*Mycosphaerella* sp.		STE-U 3837; *Acacia* sp., Venezuela
AF173310		*Mycosphaerella* state unknown	*Ramularia collo-cygni*	STE-U 2045; *Hordeum* sp., Germany
AJ417496		*Mycosphaerella* state unknown	*Ramularia* sp.	‘ascomycete 2’; *Quercus robur*, Germany
AY259131	AY251110	*Mycosphaerella* state unknown	*Ramulispora sorghi*	STE-U 905; *Sorghum* sp., South Africa
AY259132	AY251111	*Mycosphaerella* state unknown	*R. sorghi*	STE-U 906; *Sorghum* sp., South Africa
AY166268		*Mycosphaerella* state unknown	*Cercospora apii*	CA1, ATCC 12246
AY152576		*Mycosphaerella* state unknown	*C. beticola*	CBS 539.71; *Beta vulgaris*, Romania
AY266165		*Mycosphaerella* state unknown	*C. beticola*	MPPD12120, CB4; *Beta vulgaris*, Minnesota, USA
AY152577		*Mycosphaerella* state unknown	*C. kikuchii*	CBS 128.27 (ex type); *Glycine max*, Japan
AY166260		*Mycosphaerella* state unknown	*C. kikuchii*	CK 39; *Glycine max*, Illinois, USA
AY266161		*Mycosphaerella* state unknown	*C. kikuchii*	CK 35; *Glycine max*, Illinois, USA
AY260078	AY251104	*Mycosphaerella* state unknown	*C. zebrina*	STE-U 3955; *Trifolium pratense*, Canada
AY152572		*Mycosphaerella* state unknown	*Septoria apiicola*	CBS 395.52 (=IMI 092627); *Apium* sp., The Netherlands
AY152573		*Mycosphaerella* state unknown	*S. apiicola*	CBS 389.59; *Apium graveolens*, Italy
AY152574		*Mycosphaerella* state unknown	*S. apiicola*	CBS 400.54 (=IMI 092628); *Apium graveolens*, The Netherlands
AY152588		*Mycosphaerella* state unknown	*S. castaneicola*	CBS 102377; *Castanea sativa*, The Netherlands
AF279582	AF279583	*Mycosphaerella* state unknown	*S. epambrosiae*	*Ambrosia artemisiifolia*
AY490770		*Mycosphaerella* state unknown	*S. hippocastani*	CBS 411.61; *Aesculus hippocastanum*, Germany
AY152563		*Mycosphaerella* state unknown	*S. lamiicola*	CBS 109113; *Lamium album*, Austria
AY152564		*Mycosphaerella* state unknown	*S. lamiicola*	CBS 102328; *Lamium album*, The Netherlands
AF181697		*Mycosphaerella* state unknown	*S. passerinii*	ATCC 26516; *Hordeum vulgare*, Minnesota, USA
AF181699		*Mycosphaerella* state unknown	*S. passerinii*	P78; *Hordeum vulgare*, Minnesota, USA
	AY251108	*Mycosphaerella* state unknown	*Passalora dodonaeae*	STE-U 1223; *Dodonaea* sp., South Africa

Table 1. (*Cont.*)

GenBank accession no.				
ITS	SSU	Teleomorph	Anamorph	Origin
AY251069	AY251109	*Mycosphaerella* state unknown	*Pas. fulva*	CBS 119.46 (=‘STE-U 3688’); *Lycopersicon esculentum*, The Netherlands
	AY251103	*Mycosphaerella* state unknown	*Pas. janseana*	CBS 145.37 (‘STE-U 4303’); *Oryza sativa*, Arkansas, USA
AY152559		*Mycosphaerella* state unknown	*Septoria scabiosicola*	CBS 102336; *Knautia arvensis*, The Netherlands
AY152561		*Mycosphaerella* state unknown	*S. scabiosicola*	CBS 182.93; *Succissa pratensis*, France
	AY251095	*Sphaerulina polyspora*	Unknown	CBS 354.29 (=‘STE-U 4301’)
	AY251113	? *Sphaerulina rhemiana*	*S. rosae*	CBS 355.58 (=‘STE-U 4302’); leaf of *Rosa* sp.
	AY251106	Unknown	*Pseudocercospora angolensis*	CBS 149.53 (=‘ATCC 11669); leaf of *Citrus sinensis*, Angola
	AY251107	Unknown	*Ps. protearum* var. *leucadendri*	STE-U 1869; *Leucadendron* sp., South Africa

* GenBank accession no. of LSU sequence = AY490776.

conidiophores) and greyish Sepia to Hazel or Olivaceous on reverse.

Colonies on MEA reaching 21–30 mm diam in 27 d, restricted and up to 5 mm high in the centre, margin weakly to distinctly lobed, glabrous or finely felty of pure white aerial hyphea, Buff, pale Olivaceous or Rosy Buff, colony surface Pale Vinaceous or Pale Violet, and then often the surrounding medium becoming Coral to red by diffusing pigments, or greyish, but largely covered by pure white aerial hyphae or conidiophores; reverse Dark Purple to Blood Colour, or Fawn to Vinaceous Buff with Dark Brick, Brick and Cinnamon areas.

TAXONOMY

Ramularia endophylla Verkley & U. Braun, **sp. nov.** (Figs 11–16)

Conidiophora unicellulares (=cellulae conidiogenae), simplicia, subcylindrica vel cylindrica, (6–)10–30 × 2.5–4(–5) µm, recta vel geniculata-sinuosa, hyalina, levia; cicatrices conidiales leniter incrassatae et fuscae, circa 1 µm latae; *conidia* hyalina, levia vel sublevia, hila incrassata, fusca, refractiva, 0.5–1(–1.5) µm lata; conidia primaria solitaria, ovoidea, ellipsoidea vel subcylindrica, continua, apice rotundato, basin versus leniter attenuata, 6–15 × 2–4 µm; conidia secundaria catenata, saepe ramicatenata, in OA praecipue ellipsoidea vel cylindrica, in MEA ovoidea vel ellipsoidea-fusiformia, recta vel curvata, 0–1-septata, in OA 7–29 × 3–4(–5) µm, in MEA (4–)7–10(–15) × (3–)4–5 µm.

Typus: **The Netherlands:** *Utrecht*: Soesterberg, ‘De Stompert’, on dead leaf of *Quercus robur* (‘B3’), April 2003, *G. Verkley s.n.* [ex-epitypus *Mycosphaerella punctiformis*] (CBS 113265–holotypus; culture kept metabolically inactive, in liquid nitrogen and lyophilized).

Conidiophores simple, subcylindrical or cylindrical, (6–)10–30 × 2.5–4(–5) µm, straight or geniculate-sinuous, hyaline, smooth-walled, arising from terminal or intermediary hyphal cells at the colony surface, often without a basal septum; conidial scars somewhat thickened and darkened, about 1 µm wide; *conidia* formed holoblastically, hyaline, walls smooth to minutely roughened, hila conspicuous, thickened, darkened, refractive, 0.5–1(–1.5) µm wide; *primary conidia* solitary, ovoid, or ellipsoid to subcylindrical, aseptate, rounded at the top and somewhat attenuated towards the base, 6–15 × 2–4 µm; *secondary conidia* catenate, often in branched, acropetal chains, on OA predominantly ellipsoid to cylindrical, on MEA ovoid to ellipsoid-fusiform, straight to curved, 0–1-septate, ends with a single hilum rounded to attenuated, branching ends often with hila on short projections, on OA 7–29 × 3–4(–5) µm, on MEA (4–)7–10(–15) × (3–)4–5 µm.

Asteromella ***spermatial state***

Description *in vitro*: *Spermogonia* submerged or on the agar surface, pycnidial, globose, mostly aggregated in larger complexes containing several merging cavities and one or several rather undifferentiated ostioles, black to dark brown; *conidiomatal walls* composed of

Fig. 1. Strict consensus tree of 580 most parsimonious trees of 535 steps obtained in a heuristic search of 168 parsimony-informative characters of the ITS1-5.8SrDNA-ITS2 region calculated in PAUP. Numbers at the branches are bootstrap values obtained from 1000 replications and rounded to the nearest integer, shown only for branches supported by more than 50%. Species are labelled by teleomorph name, if known (anamorph names are given in Table 1).

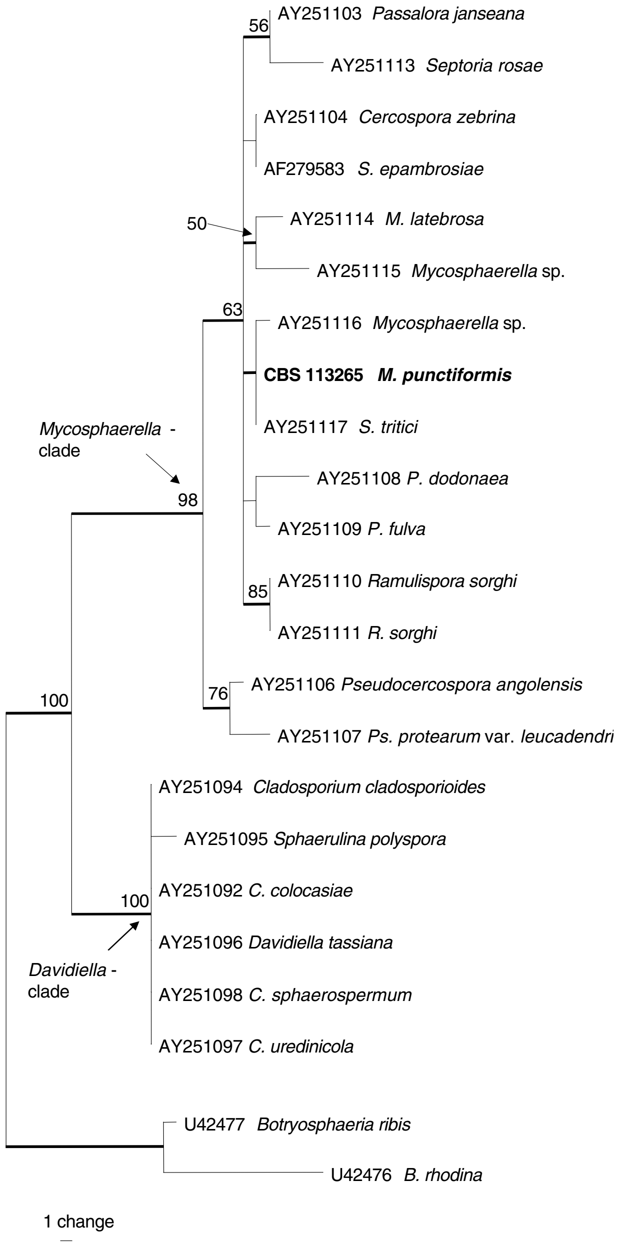
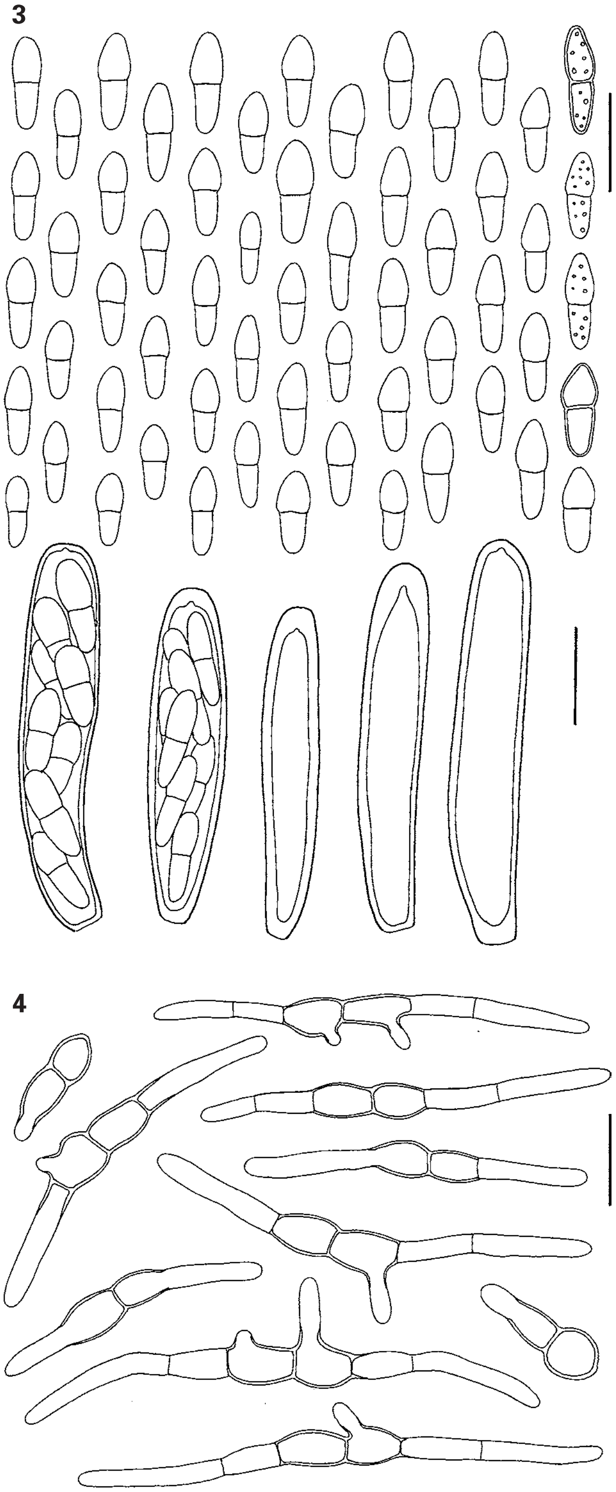


Fig. 2. One of eleven most parsimonious trees obtained from a heuristic search of the SSU sequence alignment. Bootstrap support values from 1000 replicates are shown at the nodes and the scale bar represents a single change. Branches that were maintained in the Strict consensus tree are thickened and the tree is rooted to *Botryosphaeria ribis* and *Botryosphaeria rhodina*.

an outer layer of thick-walled, brown textura angularis, and an inner layer of hyaline, irregular to isodiametric cells; *spermatogenous cells* phialidic, determinate, hyaline, discrete or integrated in simple, septate, more rarely branched, hyaline spermatiophores with acropleurogenous openings; s*permatia* ellipsoid to subcylindrical, with rounded ends, hyaline, smooth-walled, aseptate, 3–4(–5) × 1–1.5 µm, whitish in mass.

Mycosphaerella punctiformis (Pers.: Fr.) Starbäck, *Bih. Kongl. Svenska Vetensk.-Akad. Handl.* **15**(3, 2): 9 (1889).

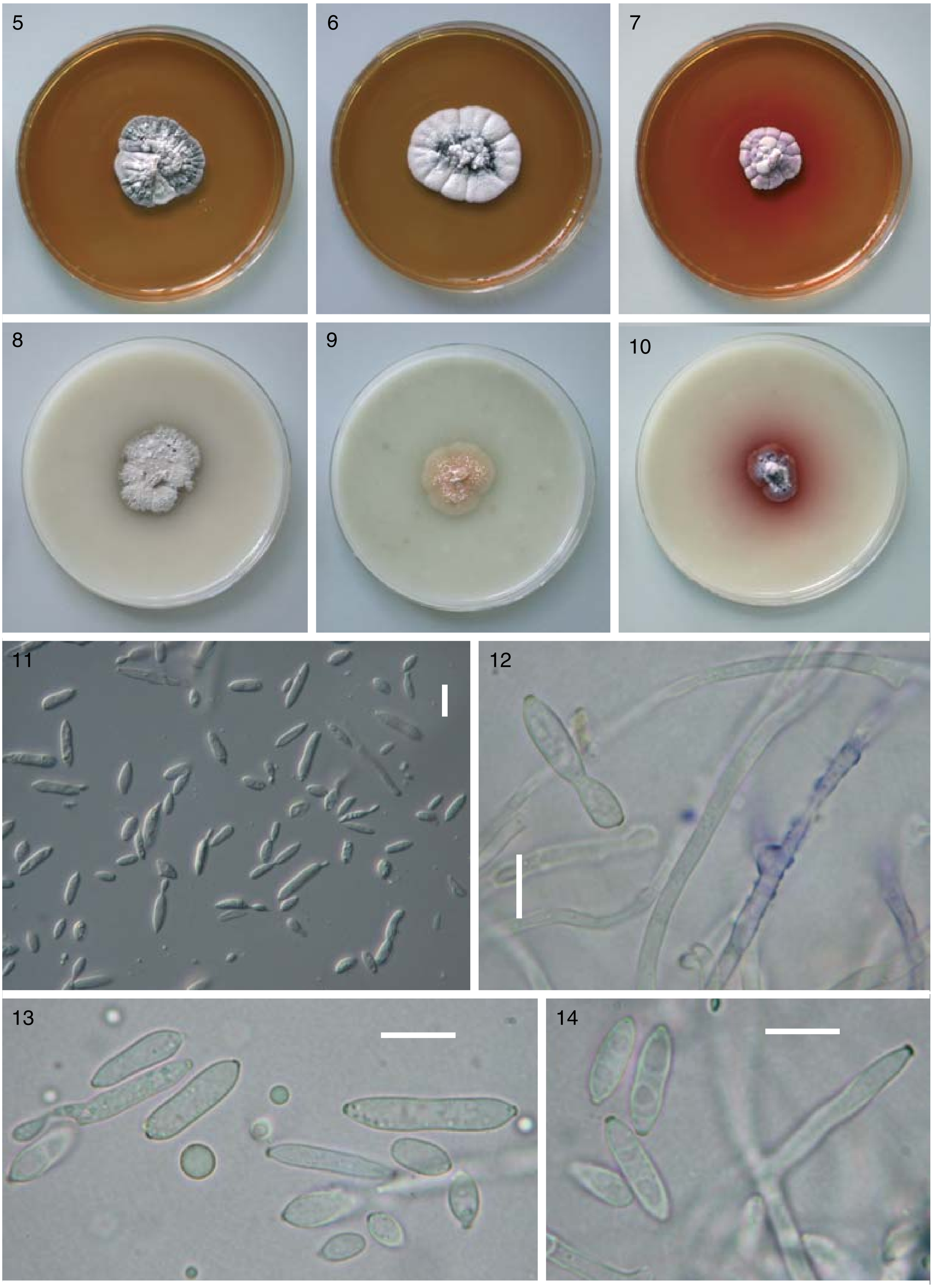


Figs 3–4. *Mycosphaerella punctiformis*, epitype (CBS herb. 7949). **Fig. 3.** Ascospores and asci *in planta*. **Fig. 4.** Germinating ascospores on MEA. Bars = 10 µm.

Sphaeria punctiformis Pers., *Ann. Bot.* (*Usteri*) **11**: 26. 1794 : Fr., *Syst. Mycol.* **2**: 525 (1823).

Sphaerella punctiformis (Pers.: Fr.) Rabenh., *Herb. Vivum Mycol.*, *ed. nov.*, *cent.* 3, no. 264 (1856).

Typus: **The Netherlands**: On lower surface of dead leaves of *Quercus* (*Fagaceae*), *Persoon s.n.* (L-Persoon – ***lectotypus hic designatus***); *Utrecht*: Soesterberg, 'De Stompert', *G. Verkley*



Figs 5–14. *Mycosphaerella punctiformis in vitro* (diffuse daylight, 18 °C). **Figs 5–7.** Isolates on MEA, after 27 d. **Fig. 5.** CBS 113870. **Fig. 6.** CBS 113868. **Fig. 7.** CBS 113869. **Figs 8–10.** Isolates on OA, after 27 d. **Fig. 8.** CBS 113870. **Fig. 9.** CBS 113869. **Fig. 10.** CBS 113868. **Figs 11–14.** Conidia and conidiogenous cells on OA. Bars = 10 µm.

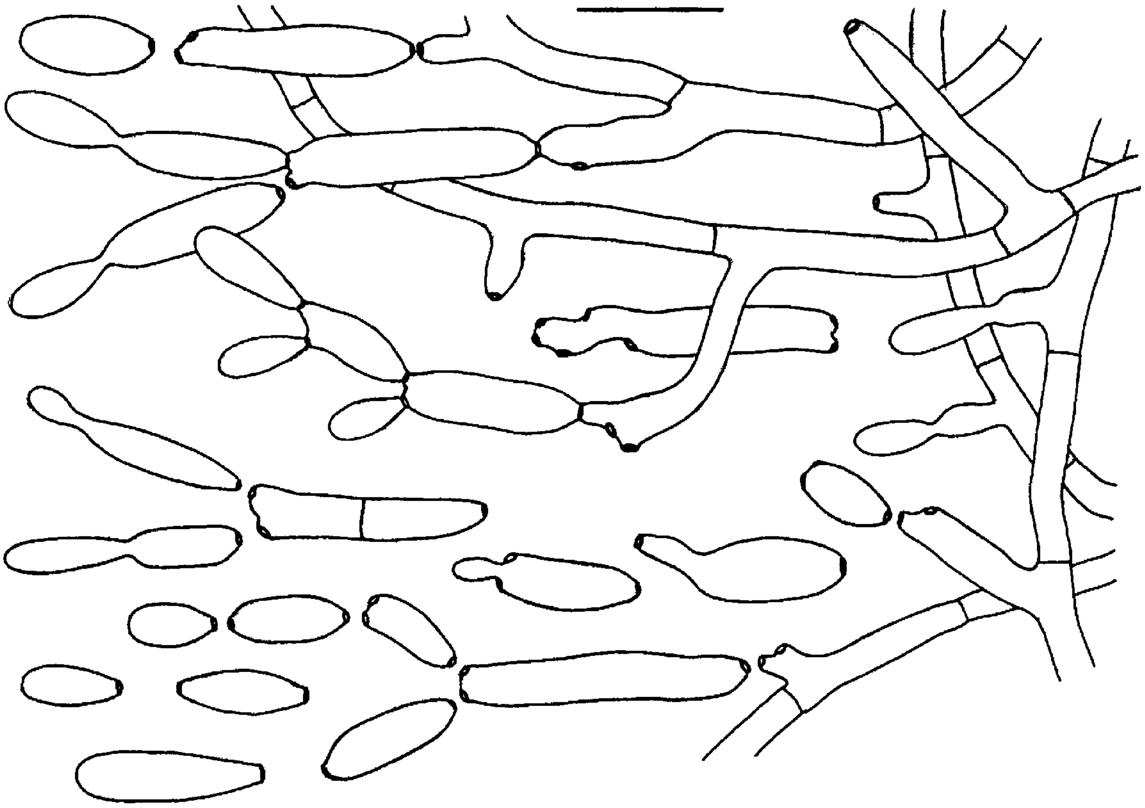


Fig. 15. *Mycosphaerella punctiformis* (CBS 113265 – ex-epitype). Conidiogenous cells and conidia on OA. Bar = 10 µm.

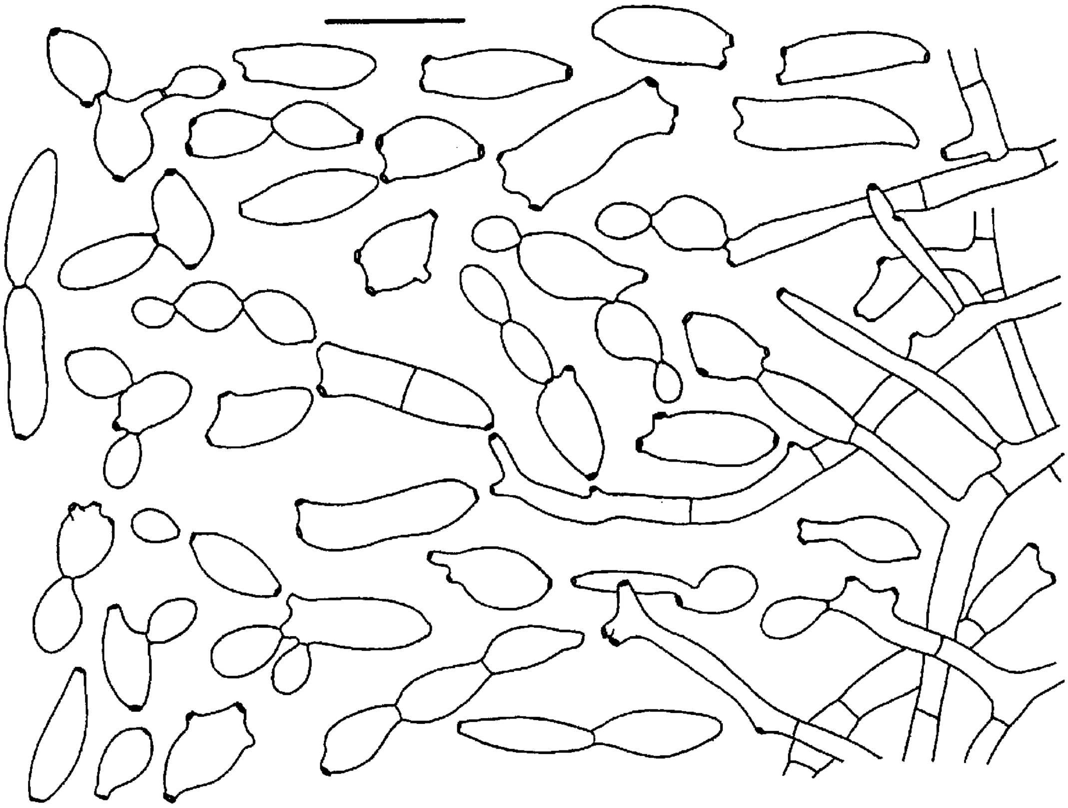


Fig. 16. *Mycosphaerella punctiformis* (CBS 113265 – ex-epitype). Conidiogenous cells and conidia on MEA. Bar = 10 µm.

s.n., on dead leaf of *Quercus robur* ('B3'), April 2003 (CBS herb. Nr 7949 – ***epitypus hic designatus***); living single ascospore (SS) culture CBS 113265 – (ex-epitype; also with the holotype of *Ramularia endophylla*).

The lectotype is the only material under this name in the Persoon herbarium that was not classified in another (often invalid) variety by himself. It is typical for the species, with cylindrical asci, and ascospores 8–10 × 2–3 µm.

Endophytic isolates examined: **The Netherlands**: *Utrecht*: Soesterberg, 'De Stompert', ex living leaf of *Quercus robur*, 'AugB3H8', Aug. 2002 (CBS 113868); *loc. cit.*, substr., 'AugB2L12', Aug. 2002 (CBS 113869), and 'AugB3H7' (CBS 113870).

DISCUSSION

Previous work showed that ITS sequences are fairly constant within most species of *Mycosphaerella*, and that some species may not even be discriminated by ITS sequences (Verkley *et al.* 2004). ITS sequence divergences among *Mycosphaerella* states which are identified as *M. punctiformis* found on dead leaves of *Quercus*, *Tilia*, and *Acer*, indicate that this morphospecies could in fact represent a species complex. *M. phacae-frigidae*, which grouped with four *M. punctiformis* strains, can be distinguished morphologically from *M. punctiformis* by the larger ascospores (11–13 × 3–3.5 µm in the holotype of *M. phacae-frigidae* in ZT; A. Aptroot, unpubl.). *M. punctiformis*, as we epitypify it here, has been fully characterized phenotypically on the basis of isolates from *Quercus*. Future work including morphological analysis of strains from other hosts, and also sequence analysis of additional genes, may provide evidence to delimit *M. punctiformis s. str.* from other cryptic species. The host range of *M. punctiformis* in this restricted sense is therefore still unknown. The characters of the teleomorph from which CBS 113265 was isolated comply with the original material of *M. punctiformis* in Persoon's herbarium in L. The main aim of the work presented here, is to link the name *M. punctiformis* to this material, and in accordance with Art. 9 of the *Code*, to epitypify *M. punctiformis* with herbarium specimen CBS 7949 (teleomorph on leaves), and an ex-epitype strain CBS 113265. Other *M. punctiformis* strains which originated from *Tilia*, *Acer*, and *Quercus* differ in ITS sequence by more than 20 positions from the epitype strain and other strains of *M. punctiformis s. str.* However, the ITS data proved insufficient to resolve possible cryptic species within the *M. punctiformis* complex. Therefore, all isolates studied here are for the moment considered as *M. punctiformis s. lat.*

We repeatedly isolated endophytic *Ramularia* strains from surface-sterilized, fresh, green leaves of *Quercus robur* trees collected between June and September. Because they were morphologically and genetically identical to the epitype strain, we were able to prove that *M. punctiformis* can asymptomatically colonize living *Quercus* leaves. Its presence becomes evident by the spermogonia, which develop in large numbers when oak leaves or parts hereof go into senescence naturally or due to activities of fungi or other invaders. Although *R. endophylla* conidia were occasionally seen near leaf lesions, we were unable to confirm that conidial sporulation of *M. punctiformis* does occur *in planta* or on dead leaves in nature. This is in accordance with Braun (1998), who listed the *Ramularia* anamorph of *M. punctiformis* as an insufficiently known taxon, formed in culture only. The life-cycle of *M. punctiformis* seems to be similar to that described in *M. buna*, a fungus with a *Pseudocercospora* anamorph which endophytically colonizes *Fagus crenata* foliage in Japan (Kaneko & Kakishima 2001, Kaneko, Kakishima & Tokumasu 2003).

On oaks in The Netherlands, *M. punctiformis* is commonly accompanied by the weakly pathogenic *Septoria quercicola*, which forms pycnidia within small leaf spots. We recently also discovered its teleomorph in small numbers on dead leaves, including those of the epitype specimen. The teleomorph of *S. quercicola* differs from *M. punctiformis* in the wider asci (35–50 × 9–12 µm) and longer ascospores (13–20 × 3.5–5 µm, av. 17 × 4.5 µm), which are not constricted at the septum and taper about equally towards both ends. Our ITS sequence analyses indicate that this *Mycosphaerella* species, which is probably different from all published species on oaks (Gilman & Wadley 1952) and for which an applicable name has not yet been found, is relatively distant from taxa of the *Ramularia* clade, as well as other taxa with *Septoria* anamorphs.

Host specificity in the *M. punctiformis* complex is still insufficiently known. Brefeld & Tavel (1891) regarded *M. punctiformis* as a plurivorous species. They noted that it was less abundant on oak than *M. maculiformis*, a species originally described from *Corylus*. According to Brefeld & Tavel, *M. maculiformis* can be distinguished from *M. punctiformis* by the more densely arranged ascomata, cylindrical asci and larger ascospores. However, they have been seen as synonymous for a long time, and the type specimens of both species were recently re-examined and found to contain (at least) morphologically indistinguishable fungi. Klebahn (1918) studied the ascomata of *M. punctiformis* on *Tilia*, *Corylus*, and *Quercus* and briefly described and illustrated the *Ramularia* anamorphs in culture. Klebahn noted that there were only minor differences between the teleomorphs from the various tree species, and that the isolates showed only some differences in pigmentation but were otherwise indistinguishable. He tentatively classified these fungi as host-specific forms of *M. punctiformis*. Von Arx (1949, Müller & von Arx 1962) considered *M. maculiformis* as a synonym of *M. punctiformis*, which he regarded as plurivorous. Later authors followed this concept (Barr 1972, Sivanesan 1984), but as is shown here, the situation is more complex and may involve more than one species.

All *Mycosphaerella* species with *Ramularia* anamorphs grouped in a single, monophyletic group which obtained high bootstrap support particularly in the parsimony analysis. This was also the case in earlier molecular studies, in which fewer taxa had been included (Crous *et al*. 2001, Goodwin, Dunkle & Zismann 2001, Verkley *et al*. 2004). As in those studies, *M. graminicola* and *Septoria passerinii* form the closest sister group, but support for the joined clades remains limited. The epitypification of the type species of *Mycosphaerella* will enable the unambiguous application of the name *M. punctiformis*, and facilitate the naming of possible future segregates from *Mycosphaerella*.

ACKNOWLEDGEMENTS

Irma van Kempen is kindly thanked for isolating and sequencing the oak endophytes, and Mieke Starink-Willemse for sequencing additional strains.

REFERENCES

Arx, J. A. von (1949) Beiträge zur Kenntnis der Gattung *Mycosphaerella*. *Sydowia* **3**: 28–100.

Arx, J. A. von (1983) *Mycosphaerella* and its anamorphs. *Proceedings of the Koninklijke Nederlandse Akademie van Wetenschappen, ser. C* **86**: 15–54.

Barr, M. E. (1972) Preliminary studies on the *Dothideales* in temperate North America. *Contributions of the University of Michigan Herbarium* **9**: 523–638.

Braun, U. (1998) *A monograph of Cercosporella, Ramularia and allied genera (phytopathogenic Hyphomycetes)*. Vol. 2. IHW-Verlag, Eching.

Braun, U., Crous, P. W., Dugan, F., Groenewald, J. Z. & de Hoog, G. S. (2003) Phylogeny and taxonomy of *Cladosporium*-like hyphomycetes, including *Davidiella* gen. nov., the teleomorph of *Cladosporium s. str. Mycological Progress* **2**: 3–18.

Brefeld, O. & Tavel, F. von (1891) *Untersuchungen aus dem Gesammtgebiete der Mykologie*. Vol. 10. *Ascomycetes II*. Commissions-Verlag von Heinrich Schöningh, Münster.

Centraalbureau voor Schimmelcultures (2001) *List of Cultures*. 35th edn. Centraalbureau voor Schimmelcultures, Utrecht.

Crous, P. W. (1998) *Mycosphaerella spp. and their Anamorphs associated with leaf spot diseases of Eucalyptus*. [Mycologia Memoir No. 21.] American Phytopathological Society Press, St Paul, MN.

Crous, P. W., Aptroot, A., Kang, J.-C., Braun, U. & Wingfield, M. J. (2000) The genus *Mycosphaerella* and its anamorphs. *Studies in Mycology* **45**: 107–121.

Crous, P. W., Kang, J.-C. & Braun, U. (2001) A phylogenetic redefinition of anamorph genera in *Mycosphaerella* based on ITS rDNA sequence and morphology. *Mycologia* **93**: 1081–1101.

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

Gilman, J. C. & Wadley, B. N. (1952) The ascigerous stage of *Septoria querceti* Theum. *Mycologia* **44**: 216–220.

Goodwin, S. B., Dunkle, L. D. & Zismann, V. L. (2001) Phylogenetic analysis of *Cercospora* and *Mycosphaerella* based on the internal transcribed spacer region of ribosomal DNA. *Phytopathology* **91**: 648–658.

Kaneko, R. & Kakishima, M. (2001) *Mycosphaerella buna* sp. nov. with a *Pseudocercospora* anamorph isolated from the leaves of Japanese beech. *Mycoscience* **42**: 59–66.

Kaneko, R., Kakishima, M. & Tokumasu, S. (2003) The seasonal occurrence of endophytic fungus, *Mycosphaerella buna*, in Japanese beech, *Fagus crenata*. *Mycoscience* **44**: 277–281.

Klebahn, H. (1918) *Haupt- und Nebenfruchtformen der Askomyzeten*. Borntraeger, Leipzig.

Laibach, F. (1922) Untersuchungen über einige *Ramularia*- und *Ovularia*-Arten und ihre Beziehungen zur Ascomycetengattung *Mycosphaerella* II. *Zentralblatt für Bakteriologie Abt. II*, **55**: 284–293.

Müller, E. & Arx, J. A. von (1962) Die Gattungen der didymosporen Pyrenomyceten. *Beiträge zur Kryptogamenflora der Schweiz* **11**(2): 1–922.

Rayner, R. W. (1970) *A Mycological Colour Chart*. Commonwealth Mycological Institute, Kew.

Sivanesan, A. (1984) *The Bitunicate Ascomycetes and their Anamorphs*. J. Cramer, Vaduz.

Sutton, B. C. & Hennebert, G. (1994) Interconnections amongst anamorphs and their possible contribution to ascomycete systematics. In *Ascomycete Systematics: problems and perspectives in the nineties* (D. L. Hawksworth, ed.): 77–100. [NATO ASI SERIES No. 269.] Plenum Press, New York.

Swofford, D. L. (2003) *PAUP**: phylogenetic analysis using parsimony (*and other methods)*. Version 4.0b 10. Sinauer Associates, Sunderland, MA.

Verkley, G. J. M., Starink-Willemse, M., Iperen, A. van & Abeln, E. C. A. (2004) Phylogenetic analyses of *Septoria* species based on the ITS and LSU-D2 regions of nuclear ribosomal DNA. *Mycologia* **96**: 558–571.

Vilgalys, R. & Hester, M. (1990) Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. *Journal of Bacteriology* **172**: 4238–4246.

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

Corresponding Editor: H. T. Lumbsch