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™ 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™ 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

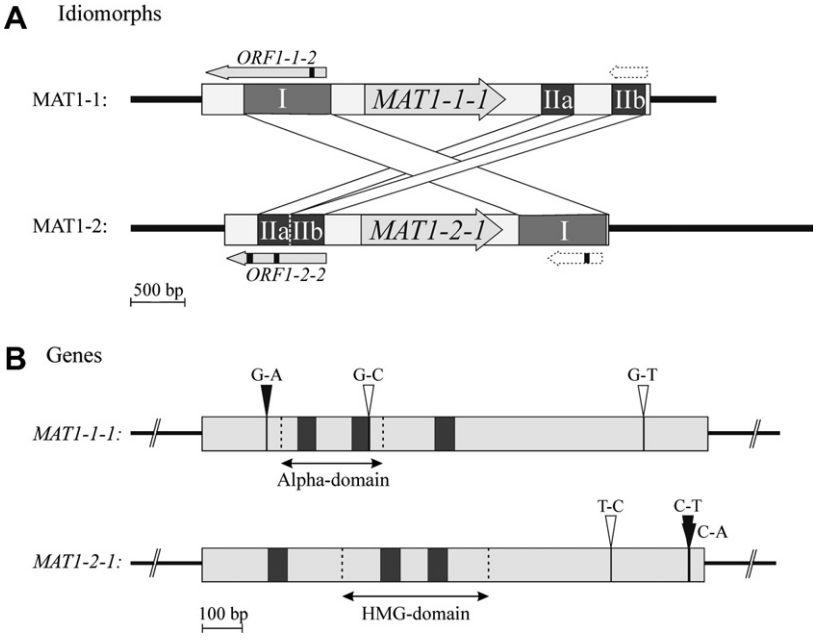

Fig. 1. In scale physical map of the mating-type idiomorphs (A) and the mating-type genes (B) of *C. fulvum*. (A) Idiomorphs are presented as boxes and their flanking regions as solid black lines. The positioning and transcriptional direction of the mating-type genes in each idiomorph is indicated by 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.

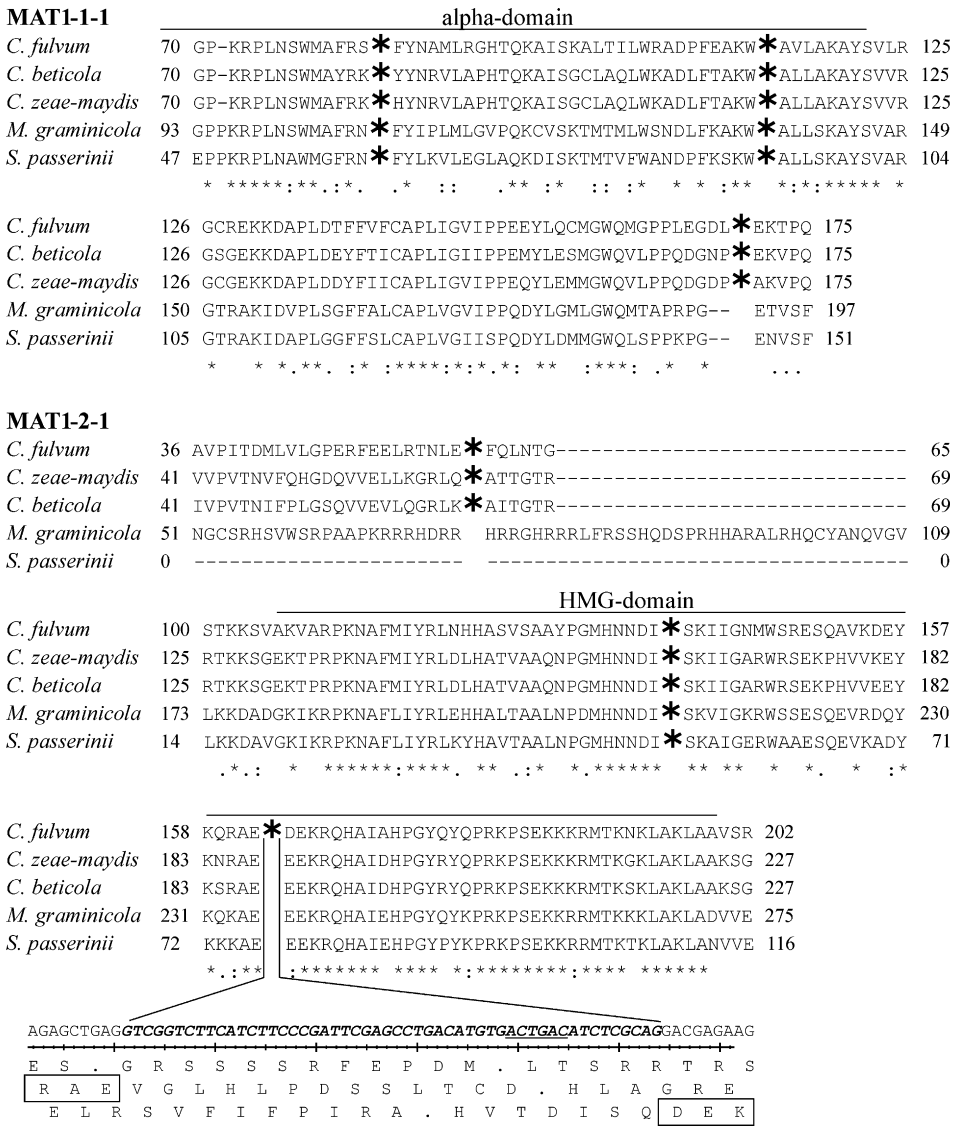

Fig. 2. Sequence alignments of MAT proteins from selected species of *Mycosphaerella* indicating the conserved positions of introns (∗) disrupting the corresponding DNA sequences. Alpha- and HMG domains are indicated as black lines above the alignment. The third intron predicted in *MAT1-2-1* of *C. 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	$N_{strains}$ ($N_{genot.}$)[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),

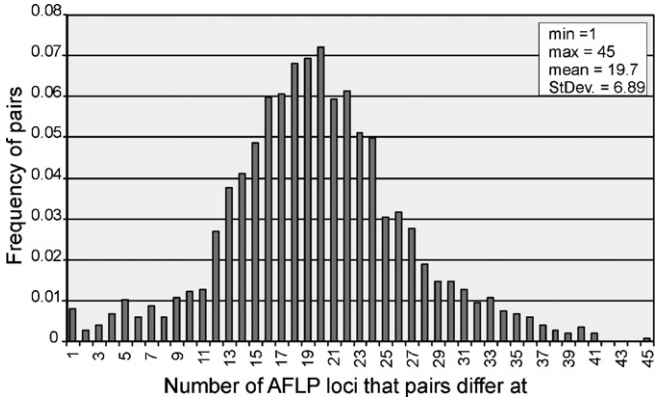

Fig. 3. Pair-wise comparison of 55 unique AFLP haplotypes showing the frequency of haplotype-pairs (*y*-axis) differing in one or more AFLP fragments (*x*-axis).

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

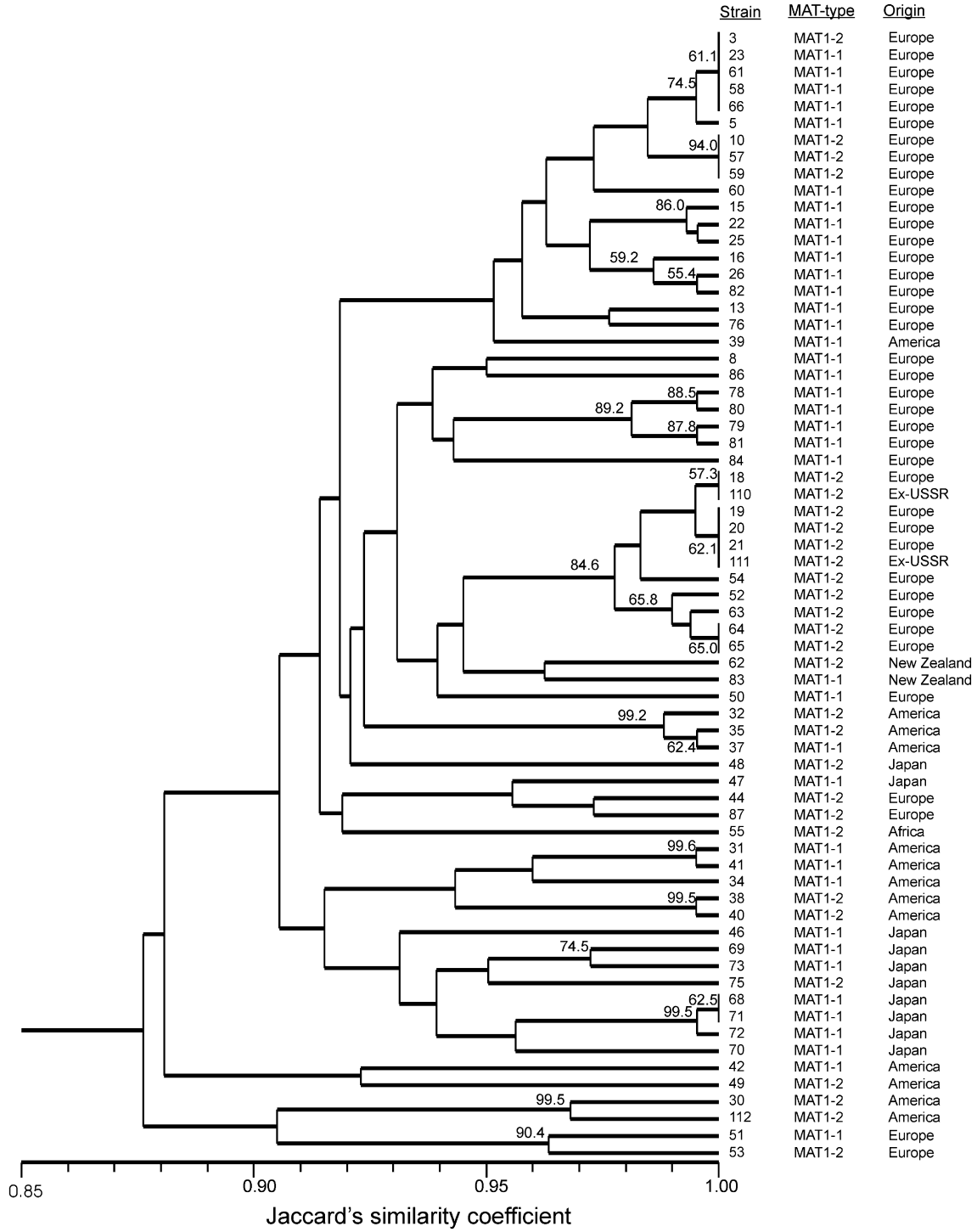

Fig. 4. UPGMA clustering of 67 strains of *C. fulvum* based on Jaccard's correlation coefficient, calculated from 255 AFLP fragments. Bootstrap values higher than 50% are shown above each branch. Mating-type as well as macro-geographic origin of the strains are also indicated.

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

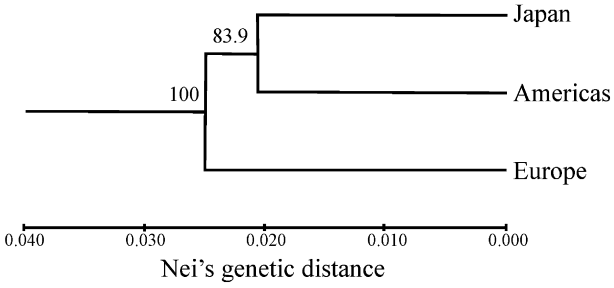

Fig. 5. UPGMA dendrogram generated based on Nei's (1972) genetic distances among the different *C. fulvum* geographic collections. Bootstrap support was obtained after 10,000 permutations over 255 AFLP fragments.

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.