Detection and phylogenetic analysis of mating type genes of *Ophiosphaerella korrae*

Tom Hsiang, Fajun Chen, and Paul H. Goodwin

Abstract: Portions of the mating type genes from *Ophiosphaerella korrae* (J. Walker & A.M. Smith) R.A. Shoemaker (= *Leptosphaeria korrae* J. Walker & A.M. Smith), a pathogenic fungus of grasses, were examined by PCR (polymerase chain reaction). For nine isolates of *O. korrae* from North America, both mating type genes were amplified, demonstrating that both MAT idiomorphs are detectable in this homothallic ascomycete. Amplified fragments from three isolates were sequenced, and parsimony analyses of *MAT1* nucleotide and protein sequences placed *O. korrae* in the basal position of a clade of Phaeosphaeriaceae and Pleosporaceae, whereas the *MAT2* nucleotide and protein data placed *O. korrae* in a clade with Pleosporaceae. The internal transcribed spacer (ITS) and 18S ribosomal DNA of *O. korrae* were also sequenced. The 18S sequences had insufficient variability to resolve the placement of *O. korrae*, whereas the ITS data placed it in Phaeosphaeriaceae. A total evidence analysis of Dothideomycetes with 18S, ITS, and MAT data placed *O. korrae* alongside *Phaeosphaeria* species, with moderate bootstrap support. However, the Kishino–Hasegawa test did not demonstrate this topology to be significantly different from one where *O. korrae* was placed with Pleosporales. Although *O. korrae* does not belong in *Leptosphaeria* based on ITS data, MAT data do not strongly support its placement in Phaeosphaeriaceae.

Key words: ascomycetes, mating type genes, ribosomal genes, taxonomy.

Résumé : Des gènes de compatibilité régissent la reproduction sexuelle chez plusieurs champignons. Les auteurs ont examiné, en PCR, des portions de gènes de compatibilité de l'*Ophiosphaerella korrae* (J. Walker & A.M. Smith) R.A. Shoemaker (= *Leptosphaeria korrae* J. Walker & A.M. Smith), un champignon pathogène des graminées. Chez neuf isolats de l'*O. korrae* de l'Amérique du nord, ils ont amplifié les gènes de compatibilité des deux types, et ils ont démontré qu'on peut détecter les deux idiomorphes MAT chez cet ascomycète homothallique. Ils ont séquencé les fragments amplifiés de trois isolats et des analyses en parcimonie du nucléotide *Mat1* et des séquences protéiniques situent l'*O. korrae* en position basale du clade des Phaeosphaeriaceae et Pleosporaceae, alors que le nucléotide *MAT2* et les données sur les protéines situent l'*O. korrae* dans un clade parmi les Pleosporaceae. Ils ont également séquencé l'espaceur interne transcrit (ITS) et l'ADN ribosomique 18S de l'*O. korrae*. Les séquences 18S n'ont pas assez de variabilité pour définir la localisation de l'*O. korrae,* alors que les données ITS le situent dans les Phaeosphaeriaceae. Une analyse de preuve totale chez les Dothideomycetes avec les données 18S, ITS et MAT situe l'*O. korrae* tout près d'espèces de *Phaeosphaeria*, avec un supporte en lacet modéré. Cependant, le test de Kishino-Hasegawa ne démontre pas que cette topologie est significativement différente de celle où l'*O. korrae* a été placé avec les Pleosporales. Bien que l'*O. korrae* n'appartienne pas au genre *Leptosphaeria* sur la base des données ITS, les données MAT ne supportent pas fortement sa localisation dans les Phaeosphaeriaceae.

Mots clés : ascomycètes, gènes de compatibilité sexuelle, gènes ribosomiques, taxonomie.

[Traduit par la Rédaction]

Introduction

Mating type (MAT) genes are known to regulate sexual reproduction in many fungi (Kronstad and Staben 1997; Turgeon 1998). MAT genes of ascomycetous yeasts and basidiomycetes share common structural and functional features but are different from those of filamentous ascomycetes (Pöggeler and Kück 2000). All tested filamentous heterothallic ascomycetes have a single mating locus with one of two alternate forms, *MAT1-1* and *MAT1-2*, which are termed idiomorphs instead of alleles because they lack significant sequence similarity (Coppin et al. 1997; Glass et al. 1988; Turgeon and Yoder 2000). In these heterothallic ascomycetes, isolates of opposite mating type, which have either a *MAT1-1* or a *MAT1-2* idiomorph, are required for sexual reproduction (Yun et al. 1999). In homothallic filamentous ascomycetes, two groups have been distinguished; in one group, both mating type genes have been detected, whereas in the other group, only one MAT gene can be detected (Glass et al. 1990; Pöggeler 1999). For brevity, the *MAT1-1-1* and the *MAT1-2-1* genes are referred to here as *MAT1* and *MAT2*, respectively.

Molecular analysis of MAT genes is a powerful tool to study evolutionary and phylogenetic patterns in fungi

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(Turgeon 1998). Turgeon (1998) claimed that MAT genes of *Cochliobolus* species were evolving at a faster rate than some other DNA sequences and that there was low MAT sequence variation within *Cochliobolus* species but high variation between species. As MAT genes are evolving rapidly but not recombining, they should give superior phylogenetic resolution than more commonly used sequences, such as the ribosomal internal transcribed spacer (ITS) regions, with only a fraction of the sequencing effort (Turgeon 1998). Turgeon (1998) was able to use MAT nucleotide sequences to distinguish between two groups of *Cochliobolus* species, which had not been possible with ITS sequences. Witthuhn et al. (2000) also compared ITS and MAT sequences in the *Ceratocystis coerulescens* complex and found that, although the phylogenetic trees were similar with both types of sequences, analyses of the MAT nucleotide sequences could separate certain *Ceratocystis* species and isolates, which could not be done using ITS sequences.

Traditionally, the isolation of MAT genes from fungi has been difficult. Strategies such as complementation of a mating type linked gene (Glass et al. 1988), heterologous hybridization (Picard et al. 1991), transformation (Turgeon et al. 1993), and genomic subtraction (Kang et al. 1994) have been used, but they can be very time consuming. A relatively simple polymerase chain reaction (PCR) approach to clone fungal mating type genes was developed by Arie et al. (1997) based on conserved elements of the mating type genes in several ascomycete species. The *MAT1-1* idiomorph has a conserved region called the alpha-box (Coppin et al. 1997), and the *MAT1-2* idiomorph has a conserved region called the high-mobility group (HMG) (Arie et al. 1997). Degenerate primers have been designed based on these conserved sequences in fungi to amplify these regions of the *MAT1* (Bennett et al. 1999) and *MAT2* genes (Arie et al. 1997; Bennett et al. 1999).

Ophiosphaerella korrae (J. Walker & A.M. Smith) R.A. Shoemaker (= *Leptosphaeria korrae* J. Walker & A.M. Smith) is a homothallic ascomycete that causes necrotic ring spot of *Poa pratensis* and spring dead spot of *Cynodon dactylis*. Walker and Smith (1972) placed this fungus in *Leptosphaeria*, but Shoemaker and Babcock (1989) proposed the transfer of *L. korrae* to *Ophiosphaerella* based on morphological features. Tisserat et al. (1994) supported the transfer of *L. korrae* to *Ophiosphaerella* based on the greater similarity in the ITS sequences of *O. korrae* to *Ophiosphaerella herpotricha* than to *Leptosphaeria maculans*. Although MAT sequences were not available in GenBank for any species of *Ophiosphaerella* (Phaeosphaeriaceae) or *Leptosphaeria* (Leptosphaeriaceae, Pleosporales), they were available for species of *Cochliobolus* and *Alternaria* (Pleosporaceae, Pleosporales) and *Phaeosphaeria* (Phaeosphaeriaceae), and therefore, comparisons of MAT sequences could be used to test the relationship of *O. korrae* to Phaeosphaeriaceae.

The objectives of this study were to (*i*) test PCR approaches for identifying MAT genes of *O. korrae*, (*ii*) determine the occurrence of one or both mating type genes in diverse isolates of *O. korrae*, and (*iii*) assess the congruence of phylogenetic trees produced from MAT nucleotide and protein sequences of *O. korrae* with those derived from ribosomal DNA sequences.

Material and methods

Fungal isolates and DNA preparation

Nine isolates of *O. korrae* (Table 1) were selected from a collection of 71 isolates from a previous study (Raffle and Hsiang 1998). These were chosen to represent five of the six different cross-continental genotypes identified by Raffle and Hsiang (1998). These isolates have been stored in the Department of Environmental Biology, University of Guelph, fungal culture collection. *Leptosphaeria maculans* isolate Leroy (ATCC 201609) was also examined. The method for DNA extraction was as previously described (Raffle and Hsiang 1998).

Mating type gene amplification

The primers Sn-ab$_1$ (5′-AARGCNYTNAAYGCNTTY-GTNGG) and Sn-ab$_2$ (5′-TCYTTNCCDAYYTGRTCN-CGDAT), modified from Bennett et al. (1999), were used to amplify part of the alpha-box region of *MAT1* in nine isolates of *O. korrae*. For the same *O. korrae* isolates, the primers ChHMG$_1$ (5′-AAGGCNCCNCGYCCNATGAAC) and ChHMG$_2$ (5′-CTNGGNGTGTAYTTGTAATTNGG), designed by Arie et al. (1997), were used to amplify part of the HMG box region of *MAT2*. Attempts were also made to amplify the mating type genes in *L. maculans* using these primers. The PCR volume was 15 μL and contained 1× PCR buffer (50 mM KCl, 10 mM Tris–HCl (pH 9.0), 0.1% Triton X-100), 2.5 mM $MgCl_2$, 200 μM of each dNTP, 2 μM of each primer, 0.5 U of *Tsg* polymerase (BioBasic Inc., Scarborough, Ont.) and 1–5 ng of genomic DNA. Amplifications of *MAT1* were performed with an initial denaturation step of 95°C for 2 min followed by 35 cycles of 94°C for 1 min, 60°C for 30 s, and 72°C for 1 min and then 72°C for 10 min. Amplifications of *MAT2* were performed with an initial denaturation step of 95°C for 2 min followed by 35 cycles of 94°C for 1 min, 50°C for 30 s, and 72°C for 1 min and then 72°C for 10 min.

Ribosomal DNA amplification

For *O. korrae* isolate LK20, the ITS region of genomic ribosomal DNA was amplified with the primer pair ITS1 and ITS4, and the 18S ribosomal gene was amplified using three primer pairs: NS1 and NS2, NS3 and NS4, and NS5 and NS8 as described by White et al. (1991). The 30-μL reaction mixture for each PCR amplification contained 10 ng of DNA, 1× PCR buffer (described above), 1.5 mM $MgCl_2$, 200 μM of each dNTP, 0.5 μM of each primer, and 1 U of *Tsg* DNA polymerase. Amplifications were performed with an initial denaturation step of 94°C for 2 min followed by 35 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 1 min and then 72°C for 10 min.

Sequencing and phylogenetic analyses

The PCR products of the putative *MAT1* and *MAT2* genes from three *O. korrae* isolates (LK10, LK20, and LK21) and PCR products of the 18S ribosomal gene and ITS of isolate LK20 were separated by electrophoresis on 1.2% agarose gels in modified TAE buffer (0.8 M Tris HCl (pH 8.0), 0.4 M sodium acetate, and 0.04 M Na_2EDTA). When bands of the expected size were found, they were extracted and purified with the Ultrafree-DA kit (Millipore, Bedford, Mass.).

Table 1. Isolates of *Ophiosphaerella korrae* from *Poa pratensis* used in this study.

Isolate No.[a]	Origin	Date	Genotype[b]
LK2	Kitchener, Ontario	1991	2
LK4	Orangeville, Ontario	1991	2
LK10	Kelowna, British Columbia	1991	1
LK13	Ottawa, Ontario	1991	2
LK20	Kitchener, Ontario	1991	n.e.
LK21	Thornhill, Ontario	1991	3
LK30	Markham, Ontario	1992	n.e.
LK56	Richland, Washington	1985	4
LK69	Coulee Dam, Washington	1985	5

[a]Isolates are stored in the Department of Environrmental Biology fungal culture collection at the University of Guelph.
[b]Based on randomly amplified DNA analysis (Raffle and Hsiang 1998); n.e., not examined.

The purified PCR products were sequenced on an Applied Biosystems 377A automated DNA sequencer (Perkin Elmer, Mississauga, Ont.) using both forward and reverse primers separately. These sequences have been deposited in GenBank (18S and ITS: AF486626, MAT1: AF4866624, and MAT2: AF4866625).

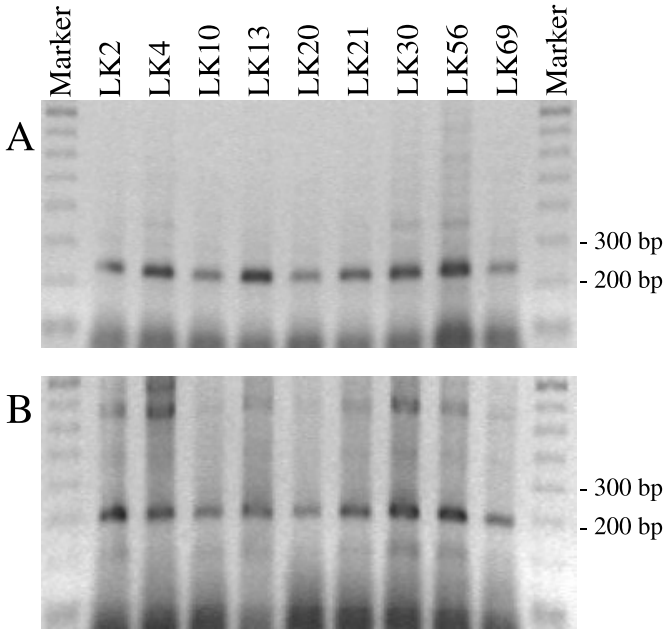
The sequences were submitted to BLAST searches in the GenBank database, and sequences of high similarity were downloaded. In addition to MAT sequences of Dothideomycetes identified by BLAST searches, MAT sequences of Sordariomycetes were downloaded to test the resolution at the class level by MAT data. Nucleotide sequences or predicted protein sequences were aligned using the program ClustalX version 1.81 (Thompson et al. 1997) with default parameters. The alignments were checked manually, and phylogenetic analyses were done on the following seven data sets. The nucleotide alignments for *MAT1* (14 sequences), *MAT2* (18 sequences), ITS (27 sequences), and 18S (26 sequences) were separately analysed as well as the protein sequences for *MAT1* (14 sequences) and *MAT2* (18 sequences). Finally, a total evidence analysis of nucleotide data was done involving six dothiodeomycete taxa plus an outgroup. Sequence alignments in Clustal format are available at http://www.uoguelph.ca/~thsiang/pubs/lkmat or upon request (first author at thsiang@uoguelph.ca).

For each data set, a strict consensus tree was derived from parsimony analysis with PAUP* version 4b9 using heuristic searches with 10 resamplings of random sequence additions and with tree bisection–reconnection in branch swapping (Swofford 2001). Gaps in the alignment were treated as missing characters. To compare different tree topologies, the Kishino–Hasegawa test, as implemented in PAUP*, was used. To examine the effect of altered sequence alignments on tree topologies, reduced gap penalties were used in ClustalX version 1.81 (pairwise and multiple gap opening and extension penalties reduced two- to threefold from default), and the resulting alignments were subjected to parsimony analysis for comparison with those derived using default gap penalties.

Results

All nine isolates of *O. korrae* (Table 1) produced bands of predicted size of 240 bp for *MAT1* and 290 bp for *MAT2* (Fig. 1), but a few additional bands for *MAT2* were observed using the ChHMG primers with some isolates (Fig. 1).

Fig. 1. Polymerase chain reaction amplification of a portion of (A) *MAT1* and (B) *MAT2* of *Ophiosphaerella korrae* isolates (inside lanes) using Sn-ab primers from Bennett et al. (1999) for *MAT1* and ChHMG primers from Arie et al. (1997) for *MAT2*. The two outside lanes for both gels are 100-bp markers.



Attempts to obtain the MAT genes of *L. maculans* with the Sn-ab and ChHMG primers were unsuccessful. Arie et al. (1997) tested four isolates of *L. maculans*, including known mating pairs, but were unable to amplify *MAT2* using the ChHMG primers. The only report of a MAT-like gene from *L. maculans* was by Cozijnsen et al. (2000), who found an amplified fragment length polymorphism sequence that shows very limited similarity with HMG sequences of mating type genes from ascomycetes.

For *O. korrae*, the *MAT1* fragment of 235 bp had a sequence that was most similar to *MAT1* of *Cochliobolus* and *Alternaria* with intron splice sites located at identical positions (Fig. 2). The *MAT1* nucleotide sequences of LK10 and LK20 were identical, and although LK21 had one nucleotide base difference, the predicted *MAT1* amino acid sequences of the three isolates were identical (Fig. 2). The 256-bp *MAT2* fragment of *O. korrae* had the highest sequence similarity with the *MAT2* genes of *Cochliobolus* and *Alternaria*,

Fig. 2. Comparison of *MAT1* sequences in *Ophiosphaerella korrae* (AF486624), *Alternaria alternata* (AB009451), *Cochliobolus homomorphus* (AF129741), and *Phaeosphaeria* avenaria (AF358822). Intron splice signals are shown in italics. Above each nucleotide sequence are the predicted amino acid sequences. The single site variation among three *O. korrae* isolates is double underlined (T for LK10 and LK20 but C for LK21). A star below a nucleotide position indicates a fully conserved site.

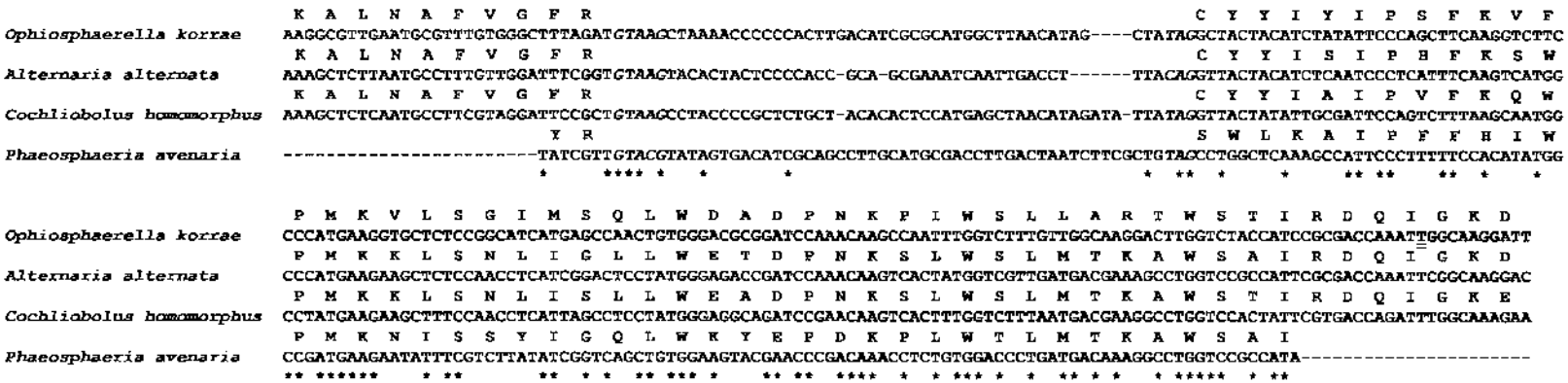


Fig. 3. Comparison of *MAT2* sequences in *Ophiosphaerella korrae* (AF486625), *Alternaria alternata* (AB009452), *Cochliobolus homomorphus* (AF129741), and *Phaeosphaeria* avenaria (AF358825). Intron splice signals are shown in italics. Above each nucleotide sequence are the predicted amino acid sequences. The single site variation among three *O. korrae* isolates is double underlined (C for LK20 and LK21 but T for LK10). A star below a nucleotide position indicates a fully conserved site.

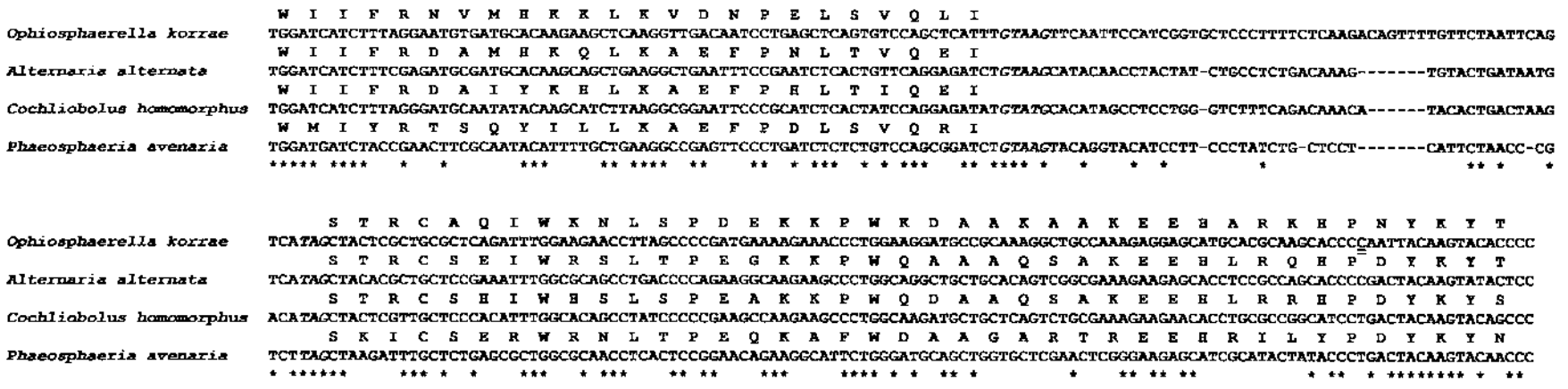
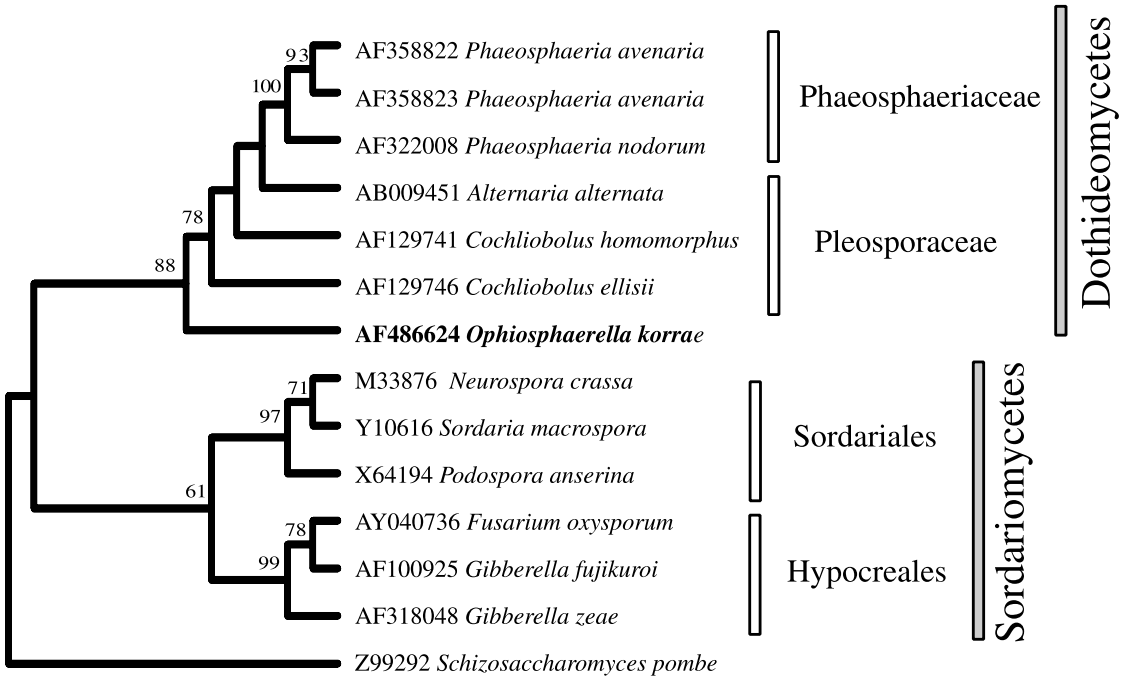


Fig. 4. A strict consensus of five equally parsimonious trees (149 steps, consistency index = 0.906, retention index = 0.907, homoplasy index = 0.094) based on partial *MAT1* protein sequences of various ascomycetes with a *Schizosaccharomyces pombe* sequence as the outgroup. GenBank accession numbers and species are given at the tips. The tree topology was derived from parsimony analysis of a protein sequence alignment from ClustalX using PAUP*. The aligned length of 51 amino acids included 47 polymorphic sites, 39 of which were phylogenetically informative. Numbers near each branch represent percentages out of 1000 bootstrap replications. In all five most parsimonious trees, *Ophiosphaerella korrae* was basal to Pleosporaceae and Phaeosphaeriaceae.



and an intron was found at identical positions in all three species (Fig. 3). The isolates LK20 and LK21 had identical *MAT2* nucleotide sequences, whereas LK10 had one nucleotide base difference from these. However, the three isolates had the same predicted *MAT2* amino acid sequences.

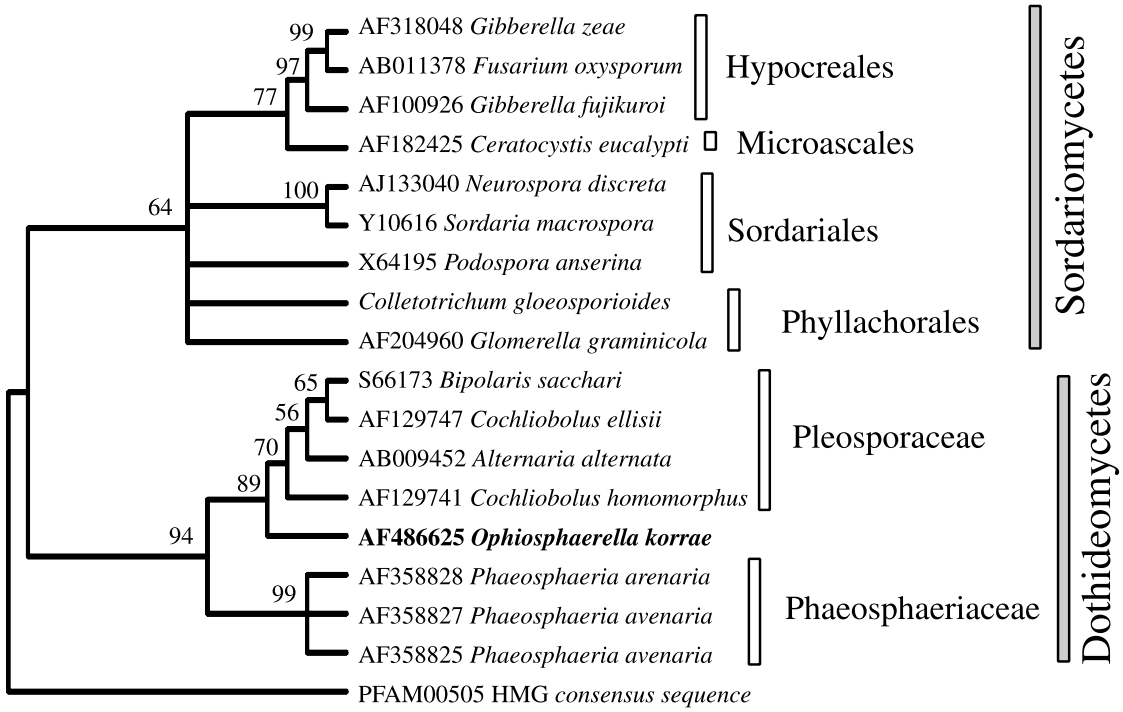
In parsimony analysis, *MAT* protein sequences belonging to Sordariomycetes or Dothideomycetes were observed in separate clades (Figs. 4 and 5). For *MAT1* sequences (Fig. 4), there was 61% bootstrap support for Sordariomycetes and 88% bootstrap support for Dothideomycetes. The five most parsimonious trees were each composed of 149 steps (consistency index = 0.906); in all five trees, *O. korrae* was in the basal position of a clade of Dothideomycetes (Fig. 4). For *MAT2* protein sequences (Fig. 5), there was 64% bootstrap support for Sordariomycetes and 94% bootstrap support for Dothideomycetes. The five most parsimonious trees were each composed of 289 steps (consistency index = 0.792); in all five trees, *O. korrae* was in the basal position of a clade of Pleosporaceae (Fig. 5). The high bootstrap support for Dothideomycetes in both *MAT* protein trees was probably due to the close relationship of the species tested, whereas low bootstrap support for Sordariomycetes may have been due to the more diverse representatives from this class.

Parsimony analysis of nucleotide sequences of *MAT2* (data not shown) improperly placed Hypocreales (Sordariomycetes) in a clade with Dothideomycetes rather than with Sordariales. In both most parsimonious trees (723 steps, consistency index = 0.657), *O. korrae* was in a clade with Pleosporaceae with 82% bootstrap support, and *Phaeosphaeria* was found in a sister clade (data not shown). Nucleotide sequence comparisons of *MAT1* were able to separate Dothideomycetes and Sordariomycetes (90 and 83% bootstrap support, respectively), but the single most parsimonious tree (868 steps, consistency index = 0.559) placed *O. korrae* in a position basal to clades of Pleosporaceae or Phaeosphaeriaceae (data not shown).

The dendrogram based on the ITS ribosomal DNA sequences also showed strong separation of Sordariomycetes from Dothideomycetes (Fig. 6). The single most parsimonious tree composed of 1530 steps (consistency index = 0.512) showed *O. korrae* within Phaeosphaeriaceae. *Ophiosphaerella korrae* was placed in a clade with other members of *Ophiosphaerella* (96% bootstrap support), and the sister clade contained members of *Phaeosphaeria* and *Paraphaeosphaeria* as well as *Leptosphaeria microscopica* (85% bootstrap support). *Leptosphaeria doliolum*, which is the type species of *Leptosphaeria*, and *L. maculans* were in a clade basal to Phaeosphaeriaceae and Pleosporaceae. *Leptosphaeria obiones* and *Leptosphaeria bicolor* formed the basal group for the Dothideomycetes in this tree, showing that *L. bicolor* does not belong within *Leptosphaeria*, as noted previously (Dong et al. 1998; Morales et al. 1995), nor does *L. obiones*. These results agree with previous ITS analyses showing that *O. korrae* does not belong within *Leptosphaeria* (Câmara et al. 2000; Tisserat et al. 1994; Wetzel et al. 1999*a*, 1999*b*).

The dendrogram based on 18S ribosomal DNA sequences (18 Dothideomycetes, seven Sordariomycetes, *Saccharomyces cerevisiae* as outgroup) showed strong separation of

Fig. 5. A strict consensus of five equally parsimonious trees (289 steps, consistency index = 0.792, retention index = 0.807, homoplasy index = 0.208) based on partial *MAT2* protein sequences of various ascomycetes with an HMG box consensus sequence as the outgroup (PFAM00505 in the GenBank conserved domain database). GenBank accession numbers and species are given at the tips, except for the sequence from *Colletotrichum gloeosporioides*, which was courtesy of L. Vaillancourt (University of Kentucky, Lexington, Ky., personal communication). The tree topology was derived from parsimony analysis of a protein sequence alignment from ClustalX using PAUP*. The aligned length of 65 amino acids included 61 polymorphic sites, 58 of which were phylogenetically informative. Numbers near each branch represent percentages out of 1000 bootstrap replications. All five most parsimonious trees placed *Ophiosphaerella korrae* with Pleosporaceae.



Sordariomycetes from Dothideomycetes with 100% bootstrap support for these two major branches (data not shown). The 15 most parsimonious trees were each composed of 637 steps (consistency index = 0.757). A large clade involving most members of Phaeosphaeriaceae with 55% bootstrap support showed little internal differentiation (bootstrap values <50%), reflecting the highly conserved nature of 18S sequences. Although some members of Pleosporales were outside this large clade (e.g., *Alternaria*), members of other pleosporaceous genera, such as *Cucurbitaria* and *Leptosphaeria*, were inside this large clade alongside *O. korrae*. There appears to be insufficient variation in the 18S ribosomal DNA sequences to resolve the relationship of *O. korrae* to Phaeosphaeriaceae or Pleosporaceae.

From the sequence of the 18S ribosomal DNA of *O. korrae*, a group I intron (Cech 1988) of 425 bp was identified at the 3′ end of the 18S, which is 30 bp upstream of the 5′ end of the ITS1. This sequence (AF486626) had 100% identity with the intron found in the same location in the *O. korrae* 18S sequences at GenBank (AF102187, AF102188, AF102189). Wetzel et al. (1999*a*) found the occurrence of this intron in 51 of 159 isolates of *O. korrae*, and similar introns have been found in other fungi (e.g., Shinohara et al. 1996; Suh et al. 1999). These introns are less well conserved between species than the 18S DNA. There is a 20% difference between the 18S intron of *O. korrae* and that of *O. narmari* (Wetzel et al. 1999*a*), while the 18S of *O. korrae* shares over 99% identity with other *Leptosphaeria* species. The 100% identity between the intron of *O. korrae* isolate LK10 from this study and *O. korrae* isolates from Wetzel et al. (1999*a*) helps to confirm that these isolates were indeed the same species.

To further test the placement of *O. korrae*, a total evidence analysis was conducted by combining 18S, ITS, *MAT1*, and *MAT2* data for *O. korrae*, *Phaeosphaeria nodorum*, *Phaeosphaeria avenaria*, *Cochliobolus ellisii*, *Cochliobolus homomorphus*, *Alternaria alternata*, and an outgroup, *Neurospora crassa* (Table 2). The aligned length of 2683 nucleotides included 773 polymorphic sites, of which 380 were phylogenetically informative. The single most parsimonious tree of 1234 steps (consistency index = 0.865) placed *O. korrae* alongside *Phaeosphaeria* with 70% bootstrap support (Fig. 7). However, the Kishino–Hasegawa test found no significant differences ($p > 0.05$) in the placement of *O. korrae* with either Pleosporaceae or Phaeosphaeriaceae (Fig. 7). Realignment of all seven data sets with reduced penalties for gaps resulted in most parsimonious trees that were congruent with trees from the original alignments with respect to the position of *O. korrae*.

Discussion

Conserved primers developed for ascomycete mating type genes were able to amplify portions of *MAT1* and *MAT2* from every isolate of *O. korrae* tested. Since *MAT1* and *MAT2* were detected in all nine isolates, *O. korrae* appears

Fig. 6. The single most parsimonious tree (1530 steps, consistency index = 0.512, retention index = 0.642, homoplasy index = 0.488) based on ITS nucleotide sequences of various ascomycetes with *Saccharomyces cereviseae* as the outgroup. GenBank accession numbers and species are given at the tips. The tree topology was derived from parsimony analysis of a protein sequence alignment from ClustalX using PAUP*. The aligned length of 538 nucleotides included 396 polymorphic sites, of which 310 were phylogenetically informative. Numbers near each branch represent percentages out of 1000 bootstrap replications.

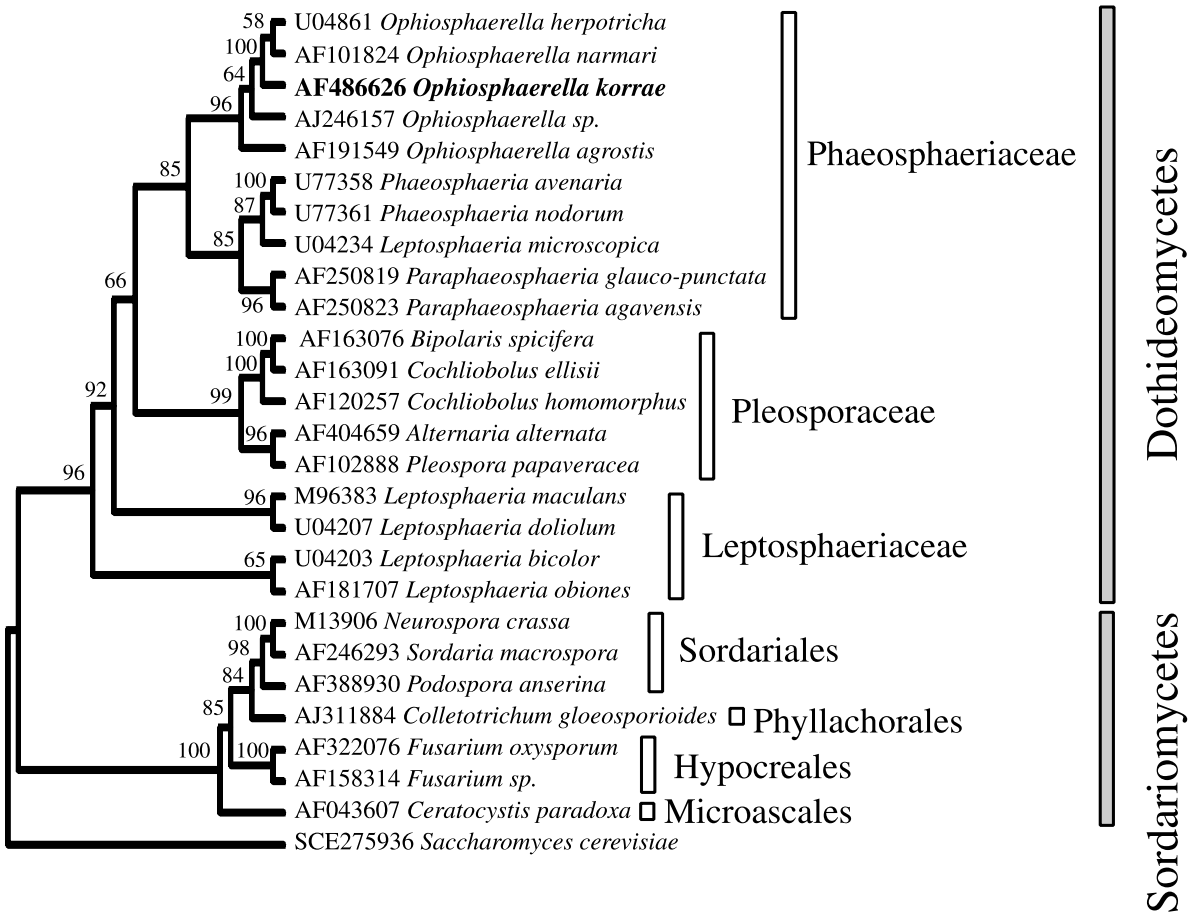


Table 2. GenBank nucleotide sequences used to construct total evidence analysis for *Ophiosphaerella korrae* and species of *Phaeosphaeria*, *Cochliobolus*, and *Alternaria* with *Neurospora crassa* as the outgroup.

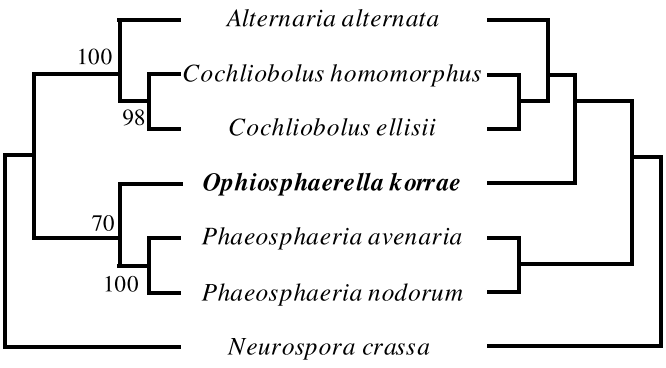
	18S	ITS	*MAT1*	*MAT2*
P. nodorum	U04236	U77361	AF322009	AF358828[a]
P. avenaria	U04236[b]	U77358	AF358823	AF358827
O. korrae	AF486626[c]	AF486626[c]	AF486624[c]	AF486625[c]
C. ellisii	U42479[d]	AF163091	AF129746	AF129747
C. homomorphus	U42479[d]	AF120257	AF129741	AF129741
A. alternata	U05194	AF404659	AB009451	AB009452
N. crassa	X04971	M13906	M33876	AJ133040[e]

[a] *P. arenaria* used here.
[b] *P. nodorum* used here.
[c] Sequenced in this study.
[d] *C. sativus* used here.
[e] *N. discreta* used here.

to belong to the group of homothallic ascomycetes where both MAT idiomorphs are present. In some homothallic filamentous ascomycetes, such as *Sordaria macrospora* and several *Cochliobolus* species, the two idiomorphs are fused together forming one chimeric gene at the mating type locus (Pöggeler and Kück 2000; Yun et al. 2000). The chromosomal relationships of *MAT1* and *MAT2* are not known for *O. korrae*, and attempts to determine whether *MAT1* and *MAT2* are linked or fused in *O. korrae* by nested PCR after mixing the two pairs of primers were unsuccessful.

Parsimony analyses with protein sequences of *MAT2* and *MAT1* and nucleotide sequences of *MAT1* were able to prop-

Fig. 7. Cladograms of combined nucleotide sequences (18S, ITS, *MAT1*, and *MAT2*) for *Ophiosphaerella korrae* and species of *Phaeosphaeria*, *Cochliobolus*, and *Alternaria*. GenBank accession numbers are given in Table 2. The tree on the left, where *Ophiosphaerella korrae* was placed with phaeosphaeriaceous species, was the single most parsimonious trees requiring 1234 steps (consistency index = 0.865, retention index = 0.707, homoplasy index = 0.135). Numbers near each branch represent percentages out of 1000 bootstrap replications. The tree on the right, where *O. korrae* was placed with pleosporaceous species, required 1238 steps (consistency index = 0.862, retention index = 0.700, homoplasy index = 0.138) but did not differ significantly from the tree on the left according to a Kishino–Hasegawa test in PAUP*.



erly place members of Dothideomycetes and Sordariomycetes in separate clades. *MAT2* nucleotide and protein data placed *O. korrae* with Pleosporaceae, while *MAT1* nucleotide and protein data placed *O. korrae* in a basal position for all Dothideomycetes tested.

Hawksworth et al. (1995) listed *Ophiosphaerella* alongside *Phaeosphaeria* in Phaeosphaeriaceae, whereas *Leptosphaeria* was placed in the Leptosphaeriaceae, with both families belonging to the Dothideales. Eriksson et al. (2001) revised the taxonomy of the Ascomycota using both morphological and molecular data, which was heavily based on ribosomal DNA sequences, and listed Leptosphaeriaceae (*Leptosphaeria*), Pleosporaceae (*Cochliobolus* and *Alternaria*), and Phaeosphaeriaceae (*Ophiosphaerella* and *Phaeosphaeria*) in Pleosporales (class Dothideomycetes). However, neither of the MAT protein sequence trees supported a closer relationship of *O. korrae* to *Phaeosphaeria* species than to *Cochliobolus* species, and therefore, the MAT data do not strongly support the placement of *O. korrae* in Phaeosphaeriaceae. The total evidence analysis, involving 18S, ITS, and MAT data, placed *O. korrae* in a clade with the two *Phaeosphaeria* species, but this clade had moderate bootstrap support, and the Kishino–Hasegawa test did not find this topology to be significantly better than one where *O. korrae* was in a clade with Pleosporales.

For fungi, there are probably a greater number of species with the full ITS sequence reported in GenBank than any other region of DNA. The ITS region has been used to resolve relationships among closely related genera and within genera (Cannon and Bridge 2000). Many phylogenetic studies or taxonomic revisions of *Ophiosphaerella* and related fungi have been based on ITS sequences, including Phaeosphaeriaceae (Khashnobish and Shearer 1996), *Leptosphaeria* (Morales et al. 1995), and *Ophiosphaerella* (Câmara et al. 2000; Wetzel et al. 1999*a*, 1999*b*). However, ITS nucleotide positions that vary between even closely related species are commonly unalignable because ITS regions are prone to insertions and deletions (Hershkovitz et al. 1999). For *O. korrae*, only two gaps (total of four gap positions) needed to be introduced when aligning the MAT nucleotide sequences from *Cochliobolus*, *Alternaria*, and *Phaeosphaeria* compared with 14 gaps (35 gap positions) needed to align the ITS sequences. In this alignment, the MAT sequences also had a higher proportion of phylogenetically informative sites (42% for both *MAT1* and *MAT2*) compared with the 25% for ITS sequences. Turgeon (1998) proposed that MAT sequences could provide equal or greater resolution than ITS sequences in phylogenetic analyses, and our analyses of MAT sequences clearly supported this assertion. Depending on the taxonomic level, reliance on a single region of DNA for taxonomic reclassification is risky without confirmation and support from other regions of DNA or from other characteristics. Our study found that the tree topologies derived from ITS, *MAT1*, and *MAT2* were not congruent for the position of *O. korrae*. Although *O. korrae* shows high relatedness to members of Phaeosphaeriaceae based on ITS sequences, the MAT sequence data do not strongly support the placement of *O. korrae* in Phaeosphaeriaceae. Analysis of additional sequences from *Leptosphaeria*, *Ophiosphaerella*, and *Phaeosphaeria*, particularly the type species *L. doliolum*, *Ophiosphaerella graminicola*, and *Phaeosphaeria oryzae*, as well as additional related species will help to clarify the taxonomic position of *O. korrae*.

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