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SHORT COMMUNICATION

Mónica García-Serrano · Emigdia Alfaro Laguna
Raúl Rodríguez-Guerra · June Simpson

Analysis of the *MAT1-2-1* gene of *Colletotrichum lindemuthianum*

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Abstract A single *MAT1-2-1* gene was identified from a mating pair of the filamentous ascomycete *Colletotrichum lindemuthianum*. The *MAT1-2-1* genes from both mating partners carried an open reading frame (ORF) of 870 bp encoding a putative protein of 290 amino acids that includes the highly conserved high mobility group (HMG) domain typical of the fungal *MAT1-2-1* genes. Three introns were confirmed within the *C. lindemuthianum* ORF, two of which were found to be conserved relative to a previously reported *MAT1-2-1* gene from *C. gloeosporioides*. The amino acid sequence of the HMG domain from *C. lindemuthianum MAT1-2-1* was also compared with those from other ascomycetes. These results suggest that although the *MAT1-2-1* genes are highly conserved among ascomycetes, the mechanism which defines mating partners in the genus *Colletotrichum* is distinct to the idiomorph system described for other members of this phylum.

Key words Ascomycetes · *Colletotrichum lindemuthianum* · *Glomerella MAT1-2-1* · TAIL-PCR

In most heterothallic, filamentous ascomycetes, a single mating-type locus (*MAT1*), first described for *Neurospora crassa* Shear and Dodge (Glass et al. 1988), controls sexual development. The *MAT1* locus has been defined in many different ascomycetes, and in the cases where sexual reproduction and mating have been studied in detail, the *MAT1* locus was shown to have two alleles or idiomorphs that are distinct in each member of a heterothallic mating pair (Coppin et al. 1997; Turgeon 1998; Poggeler 2001).

M. García-Serrano · E. Alfaro Laguna · J. Simpson (✉)
Department of Genetic Engineering, CINVESTAV, Campus Guanajuato, Apdo. Postal 629, C. P. 36821, Irapuato, Guanajuato, Mexico
Tel. +52-462-623-9667; Fax +52-462-624-5849
e-mail: jsimpson@ira.cinvestav.mx

R. Rodríguez-Guerra
Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP), Campo Experimental General Terán, Nuevo León, Mexico

These idiomorphs have been named *MAT1-1* and *MAT1-2* (Turgeon and Yoder 2000). *MAT1-1* contains an ORF that encodes a protein with an α-box domain, and in some genera additional ORFs are also found. The *MAT1-2* idiomorph normally contains a unique ORF that invariably encodes a protein with a highly conserved HMG (high mobility group) domain.

Heterothallic ascomycete strains carry either *MAT1-1* or *MAT1-2* and are sexually compatible when in contact with strains of the opposite mating type. In homothallic ascomycetous strains, a single individual normally contains both *MAT1-1* and *MAT1-2* idiomorphs (Coppin et al. 1997; Turgeon 1998; Poggeler 2001). However, a series of articles (Edgerton 1914; Lucas et al. 1944; Wheeler et al. 1948; Chilton and Wheeler 1949; Wheeler 1954) on *Glomerella cingulata* (Stoneman) Spauld & Schrenck (anamorph: *Colletotrichum gloeosporioides* (Penz.) Sacc.) and more recent reports on different species within the genus *Glomerella* (Cisar et al. 1996; Cisar and TeBeest 1999; Vaillancourt and Hanau 1999; Vaillancourt et al. 2000; Rodríguez-Guerra et al. 2005) suggest that control of sexual reproduction within this genus is different from that described for other filamentous ascomycetes.

Based on results obtained from classical genetic studies, Wheeler (1954) developed a model to describe how mating could be controlled genetically in *Glomerella*, proposing that most *Glomerella* strains are basically homothallic, but that pseudo- or unbalanced heterothallic strains may arise as a result of mutations in genes involved in the mating process. These strains are no longer capable of completing the sexual cycle by themselves, but on contact with another strain carrying a different mutation, complementation may occur and sexual reproduction achieved (Wheeler 1954). More recent molecular data on the *MAT1* locus in *Glomerella* have shown that in contrast to other filamentous ascomycetes, both members of a mating pair carry the *MAT1-2* idiomorph (Vaillancourt et al. 2000; Rodríguez-Guerra et al. 2005). This situation could arise under the model of Wheeler if different mutations either in the *MAT1-2* idiomorph or in other genes involved in the mating process occurred in each mating partner and were complemented

Table 1. Primers used to span the *MAT1-2-1* gene

Primer	Sequence	Primer	Sequence
F1	CATGCCGCAGTAAAGCAAATGGAC	AD1	NTCGASTWTSGWGTT
F2	AAACTTGGCAAAGCATGGAACGCA	AD2	NGTCGASWGANAWGAA
F3	CCTACTACCGCTACAACCC	AD3	NGTGNAGNANCANAGA
F4	TGGCAAAGGTTACTCCCATCGCCT	HMGDF	CCYCGYCCYCCYAAYGCNTAYAT
F5	TATTTTACATGCTGGTCAC	HMGDR	CGNGGRTTRTARCGRTARTNRGG
R1	CTGTAGCGGTAGTCGGGATG	HMGCLF	CATGCCGCAGTAAAGCAAAT
R2	TTTCGACAGTTTGAACCGA	HMGCLR	ATCATCAGACGTTCTTTGTG
R3	TTTGCTTTACTGCGGCATG	P5	GGGGTAGTCGAAAGAAACTG
R4	CGCCTACCCCCAGTTAGTATCATA	P3	CCAGACATCCTAGAATGATCTGTC
R5	CCGATGAAAACGTAGTCCC	C5	GATGCTGCGAGACTGTGCCAAGTT
R6	CAGTCTCGCAGCATCCAGA	C3	TTGGGGTATTTGCTCGCTAAACTG

by each other. Cisar and TeBeest (1999) reported that in *G. cingulata* multiple alleles occur at *MAT1-2*, which would also agree with the unbalanced heterothallism model if mutations between strains occurred mainly within this locus. The presence of the *MAT1-1* idiomorph has not been reported for any member of the genus *Glomerella* to date.

Colletotrichum lindemuthianum (Sacc. & Magn.) Scrib. is a haploid hemibiotroph, which is easily manipulated and transformed genetically under laboratory conditions (Perfect et al. 1999). Shear and Wood published the first report of sexual reproduction in *C. lindemuthianum* in 1913; however, this phenomenon was not reported again until 1970 by Kimati and Galli. who observed mating in laboratory cultures, followed by Batista and Chaves (1982), Bryson (1990), and Rodríguez-Guerra et al. (2005). The teleomorph (*Glomerella lindemuthiana* Shear) has never been observed under field conditions, and this species is generally still described as a filamentous deuteromycete for which no classical genetic analysis has been carried out. For other members of the genus, such as *C. gloeosporioides* (*G. cingulata*), sexual reproduction is common under field conditions.

The objective of this work was to characterize the *MAT1-2-1* gene from a mating pair of *C. lindemuthianum* strains.

Colletotrichum strains from different plant species were obtained from different locations. The avocado and papaya isolates were obtained from the fruits of those plants in the states of Michoacán and Campeche, Mexico, respectively. The isolate from pepper was obtained from the roots of pepper plants in Guanajuato State, and the isolates from pea, Mexican turnip, Hawthorne apple, and common bean were obtained from produce sold in the local market in Irapuato, Mexico. All strains were isolated and purified as described in González et al. (1998) and Rodriguez-Guerra et al. (2005). The sexually compatible *C. lindemuthianum* strains DGO 02 and MU 03 and the F_1 progeny of a cross between these strains have been described previously (Rodriguez-Guerra et al. 2005). All strains were maintained on acidified potato dextrose agar (PDA) (200 µl 85% lactic acid l^{-1}) and grown at 22°C for 10–15 days. DNA was obtained by the method of Raeder and Broda (1985) as described by González et al. (1998). The degenerate primers for amplification of the *MAT1-2* HMG domain reported by Arie et al. (1997) (HMGDF and HMGDR; Table 1) were used to amplify the *MAT1-2-1* HMG domain from the parental MU 03 and DGO 02 strains and from other *Colletotrichum* isolates obtained from different plant species, as described in Rodríguez-Guerra et al. (2005). Amplification products were separated on a 2% agarose gel and visualized by staining with ethidium bromide. Southern blotting was carried out using standard protocols (Sambrook et al. 1989). A 780-bp fragment obtained by polymerase chain reaction (PCR) using oligonucleotides C3 and C5 (see Table 1) was used as a probe. Amplification of the *MAT1-2-1* HMG domain from *C. lindemuthianum* using specific primers (HMGCLF/HMGCLR; Table 1) was carried out under the same conditions (Rodríguez-Guerra et al. 2005). The thermal asymmetrical interlaced (TAIL)-polymerase chain reaction (PCR) technique (Liu and Whittier 1995) was used to characterize the *MAT1-2-1* gene sequences flanking the conserved *MAT1-2-1* HMG domain from both MU 03 and DGO 02 strains. The degenerate primers AD1, -2, and -3 (Table 1) and PCR conditions were those described in Liu and Whittier (1995). Amplified fragments were visualized as described above.

Amplified HMG domain DNA fragments, the TAIL-PCR products, and a 2-kb sequence spanning the *MAT1-2* gene were cloned into the vector TOPO 4 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and sequenced on an ABI3700 sequencer. Nucleotide sequences of these DNA fragments were analyzed using a NCBI BLAST search and the Genescan program (Burge and Karlin 1997). Amino acid sequences were compared using Clustal W (Chenna et al. 2003), Jalview (Clamp et al. 2004), and the MEGA 4.0 program (Tamura et al. 2007). A consensus bootstrap dendrogram was produced using MEGA 4.0 (Tamura et al. 2007) and the neighbor-joining method.

Amplification of the conserved HMG region of the *MAT1-2-1* gene in *C. lindemuthianum* isolates MU 03 and DGO 02 revealed that the *MAT1-2-1* idiomorph was present in both partners of the mating pair (Rodríguez-Guerra et al. 2005). To extend this result, isolates of *Colletotrichum* from different plant species were analyzed using degenerate primers, and the *MAT1-2-1* gene was found to be present in all isolates tested (Fig. 1). One possible explanation is that *Colletotrichum* species have various copies of *MAT1-2-1* within their genome. However, Southern blot analysis of MU 03 and DGO 02 showed the presence of a single gene in both strains (Fig. 2). A *Hin*dIII-based restriction frag-

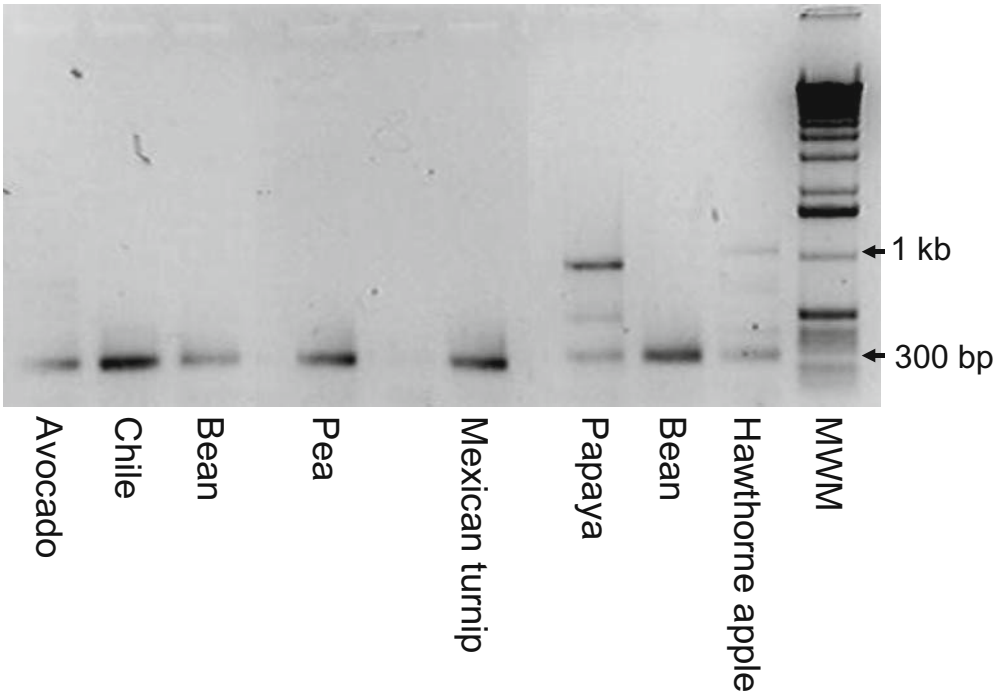

Fig. 1. *MAT-1-2* was found in all *Colletotrichum* isolates tested. Common names for each plant species from which samples were obtained are given: Hawthorne apples (*Crataegus mexicana*), Mexican turnip (*Pachyrizus erosus*). MWM; molecular weight markers

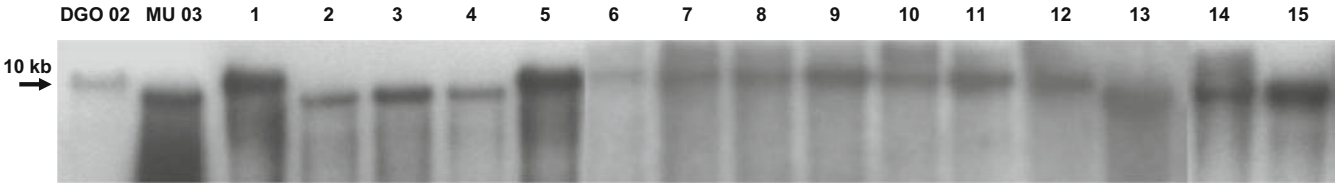

Fig. 2. Restriction fragment length polymorphism (RFLP) analysis of F_1 segregants of the DGO 02 × MU 03 cross. *DGO 02* and *MU 03* indicate the parental strains; *numbers* indicate F_1 segregants obtained from monoascospore cultures

ment length polymorphism (RFLP) analyzed in 150 F_1 individuals of the MU 03 × DGO 02 cross indicated that the *MAT1-2-1* gene segregates in a 1:1 ratio with 77 individuals having the DGO 02 genotype and 73 individuals with the MU 03 genotype. An example of part of the Southern blot data is shown for 15 F_1 segregants in Fig. 2.

To characterize the *MAT1-2-1* gene in more detail in both members of the *C. lindemuthianum* mating pair, TAIL-PCR reactions (Liu and Whittier 1995) were carried out using the primers described in Table 1. A 2-kb sequence spanning the *MAT1-2-1* gene was obtained for both MU 03 and DGO 02 (GenBank accession numbers EU23649, EU23650). Strains differed in only single base substitutions, and BLAST analysis of the sequences confirmed homology to the *MAT1-2-1* gene.

As already mentioned, mating pairs of most filamentous ascomycetes carry either the *MAT1-1* or the *MAT1-2* idiomorph at the *MAT1* locus; however, as shown here and in other reports in the genus *Glomerella* (Cisar et al. 1996; Cisar and TeBeest 1999; Vaillancourt et al. 2000; Rodríguez-Guerra et al. 2005), both mating partners carry the *MAT1-2* idiomorph. However, if one of the partners in the *Glomerella* mating pair carries a defective MAT1-2 idiomorph or nontranscribed *MAT1-2-1* gene, this would effectively reproduce the situation in other filamentous ascomycetes in which a functional *MAT1-2* idiomorph is present in only one member of the mating pair (Glass et al. 1988) and be in agreement with the model proposed by Wheeler (1954). However, the characterization of essentially identical sequences from both the DGO 02 and MU 03 *C. lindemuthianum* strains suggests that this gene is probably functional in both isolates.

Comparisons using the Genescan (Burge and Karlin 1997) program and the proposed protein sequence for a *G. cingulata MAT1-2-1* gene (GenBank AY357890) revealed an ORF comprising 870 nucleotides and including three potential introns (Fig. 3). Sequence analysis of cloned cDNAs produced by reverse transcription (RT)-PCR confirmed the presence of all three putative introns (data not shown). Translation of the putative messenger RNA gives a 290-amino-acid protein with a strong homology to other fungal *MAT1-2-1* proteins. Only 2 amino acid differences were identified between the strains MU 03 and DGO 02 (Fig. 4), and several in-frame ATGs were identified (data not shown).

Only one other complete sequence for a *Colletotrichum MAT1-2-1* gene is publicly available (*C. gloeosporioides*, GenBank AY357890). Based on Clustal W analysis, a com-

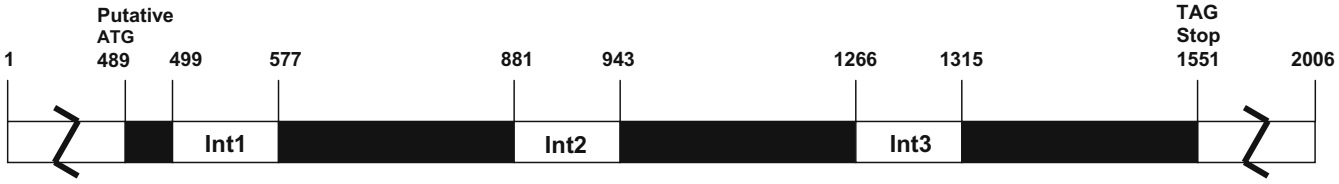

Fig. 3. Structure of *Colletotrichum lindemuthianum* MAT1-2-1 gene. *Black boxes* indicate exons; introns (*Int*) are indicated by *open boxes*. GenBank accession numbers are EU236949 and EU236950 for MU 03 and DGO 02, respectively

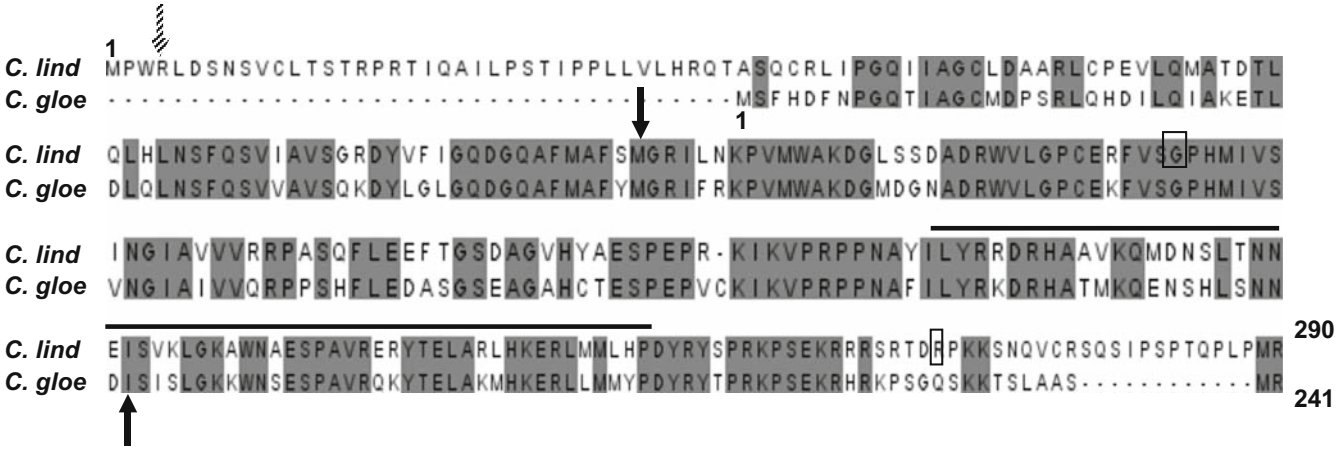

Fig. 4. Comparison of *Colletotrichum lindemuthianum* (*C. lind*) strain DGO 02 and *C. gloeosporioides MAT1-2-1* (*C. gloe*) protein sequences. *Shaded boxes* indicate conserved amino acids; *solid arrows* indicate conserved intron positions; *hatched arrow* indicates the position of a third intron in *C. lindemuthianum*; the *line above the sequence* indicates the HMG domain; *open boxes* indicate amino acid differences (R-K and G-S) between DGO 02 and MU 03. GenBank accession numbers are EU236949, EU236950, and AY357890 for MU 03, DGO 02, and the *C. gloeosporioides* sequence, respectively

parison between the putative protein sequences for the *C. lindemuthianum MAT1-2-1* and the *C. gloeosporioides MAT1-2-1* were carried out (Fig. 4). Of the 241 amino acids in the putative *C. gloeosporioides* protein, 159 (66%) were identical to those of the *C. lindemuthianum* protein and 222 (92%) were functionally conserved (Clustal conservation threshold >6). The positions of two introns, shown by solid arrows in Fig. 4, were found to correspond to the same amino acids within the two proteins. The putative *C. lindemuthianum* protein includes a stretch of 38 amino acids at the amino-terminal and 12 at the carboxy-terminal that are not found in the putative *C. gloeosporioides* protein reported in GenBank. The confirmed presence and conservation of the position of the introns in *MAT1-2-1* between *C. gloeosporioides* and *C. lindemuthianum* suggests that the deduced amino acid sequence for the *C. lindemuthianum MAT1-2-1* protein is the most probable.

No significant homology was observed in a search for regulatory sequences such as 5′-CTTTG-3′ (Philley and Staben 1994) or those associated with carbon metabolism or other regulatory factors (Glass et al. 1990; Debuchy and Coppin 1992; Leubner-Metzger et al. 1997) in either the 3′- or 5′-untranslated regions.

To compare the amino acid sequence of the highly conserved *MAT1-2-1* HMG box across other species and genera of ascomycetes, a comparison was first carried out using Protein BLAST. All the 100 sequences showing significant homology to the *C. lindemuthianum MAT1-2-1* sequence (e values from $2e^{-88}$ to $1e^{-11}$) corresponded to *MAT1-2-1* genes from other ascomycetes within the subphylum Pezizomycotina, with the exception of *Schizosaccharomyces pombe* Lindner and *Schizosaccharomyces kambucha* Singh & Klar (Taphrinomycotina). All samples from the genus *Glomerella* (anamorph: *Colletotrichum*) and one example from each genus (including both anamorph and teleomorph in some cases) of the Pezizomycotina were chosen to carry out cluster analysis using a conserved sequence of 58 amino acids spanning the highly conserved HMG domain (indicated as a solid line above the sequence in Fig. 4). The groups observed in the resulting dendrogram (Fig. 5) reflect the taxonomic classification within the Pezizomycotina, with few exceptions, down to the level of different orders. One group composed of Pleosporales, however, is found to be separated from the other group of Dothidiomycetes. The few examples of Lecanoromycetes and Leotiomycetes available were found to be dispersed throughout the cluster. *Colletotrichum lindemuthianum* groups within the genus *Glomerella* but was not included in the *G. cingulata* group as are *C. musae* (Berk & Curt.) von Arx and *C. fragariae* Brooks and is also separated from *G. graminicola* (Ces.) Wilson and *G. acutata* Guerber & J. C. Correll. One sample of *G. musae* was found to group with samples of *Fusarium* and may indicate a mistaken classification. The *Glomerella* group is closely associated with examples from the *Isaria* and *Cordyceps* genera, although these are insect pathogens rather than plant pathogens.

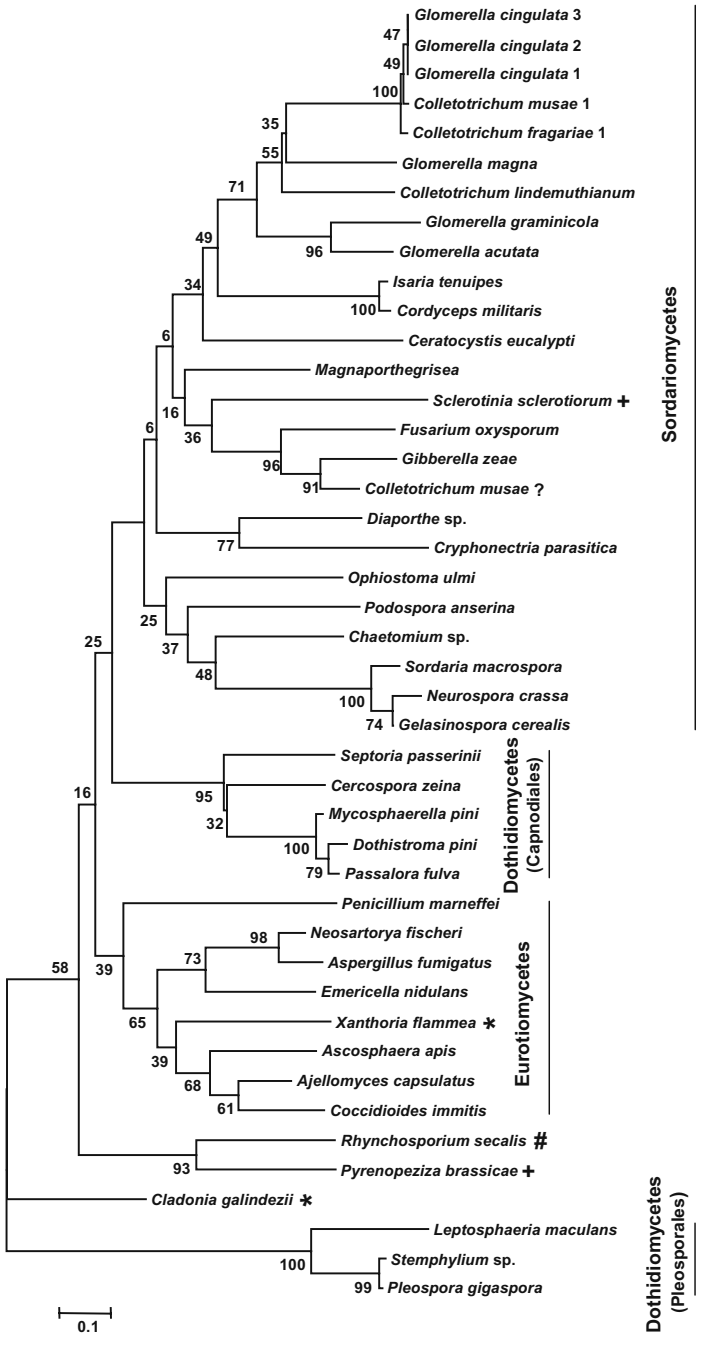

Fig. 5. Comparison of the *MAT1-2-1* high mobility group (HMG) domain in the Pezizomycotina. Groups associated with different classes are shown and *underlined*. *Numbers* indicate percentage conservation of nodes in 1000 bootstrap samples. *, Lecanoromycetes; +, Leotiomycetes; #, no definitive rank available; ?, possible misidentification

The cluster analysis confirmed the usefulness of the HMG domain as a taxonomic tool, even at the levels of order and family, as reported by Du et al. (2005). Previously *C. lindemuthianum*, *C. fragariae*, and *C. musae* were considered to be forms of *C. gloeosporioides* (Sutton 1992). The present results support this classification in the case of *C. fragariae* and *C. musae* but not in the case of *C. lindemuthianum*, which is separated from the *C. gloeosporioides* group.

In conclusion, the *MAT1-2-1* genes from *C. lindemuthianum* and *C. gloeosporioides* are strongly conserved in both gene structure and in the putative amino acid sequence of the encoded proteins. The presence of almost identical sequences in both parental *C. lindemuthianum* strains lends support to the hypothesis of pseudo-heterothallism proposed by Wheeler (1954). Further research should address the differences at the molecular and evolutionary levels that have led to the apparently unique mating system found in the genus *Colletotrichum*.

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