Mating type markers reveal high levels of heterothallism in *Leptographium sensu lato*

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Abstract

Species of *Leptographium sensu lato* (Ophiostomatales, Ascomycetes) are sap-stain fungi vectored by bark beetles (Coleoptera, Scolytinae) and some species cause or are associated with tree diseases. Sexual states have been reported for more than 30 species in this group and these have been treated in the sexual genus *Grosmannia*. No sexual state is known for at least 59 additional species and these reside in the genus *Leptographium*. The discovery of sexual states for species of *Leptographium* relies mainly on the presence of fruiting bodies on host tissue at the time of isolation and/or intensive laboratory mating studies, which commonly have a low levels of success. In this study, markers were developed to diagnose mating-type and to study sexual compatibility of species in *Leptographium sensu lato* using these markers. To achieve this objective, available mating type sequences for species of *Leptographium sensu lato* and *Ophiostoma* were obtained, aligned and used to design primers to amplify *MAT* genes in *Grosmannia* and *Leptographium* species. Using these primers, it was possible to amplify portions of the mating type genes for 42 species and to determine thallism, in many species for the first time. Surprisingly, the results showed that heterothallic and putatively heterothallic species are abundant (39 out of 42 species) in *Leptographium sensu lato*, and only three species were confirmed to be homothallic. The mating-type markers developed in this study will be useful for future studies concerning mating type and sexual compatibility of species in this genus.

Keywords: Heterothallism, homothallism, *Leptographium*, *Grosmannia*, mating type markers.

1. Introduction

Leptographium sensu lato is an ascomycete genus that includes both sexual and asexual species (De Beer and Wingfield, 2013). Species with known sexual states have been treated in the genus *Grosmannia* while those for which sexual states are unknown have been assigned names in *Leptographium* (Zipfel et al., 2006). There are currently 34 *Leptographium sensu lato* species with known sexual states. Of these, some have the ability to produce ascomata in cultures derived from single conidia or ascospores and are thus homothallic (Jacobs et al., 1998). Others are heterothallic and require crossing between isolates of opposite mating type to produce sexual structures (Duong et al., 2012; Jacobs et al., 1998; Masuya et al., 2005; Yamaoka et al., 2008). The remaining members of *Leptographium sensu lato* are known only by their asexual morphs.

Sexual compatibility in ascomycetes is determined by genes residing in the mating type (*MAT*) locus. The *MAT* locus has different alleles (idiomorphs) (Turgeon and Yoder, 2000), containing mating type genes that encode transcription factors controlling mate recognition and sexual processes (Metzenberg and Glass, 1990). In heterothallic ascomycetes, the mating types of isolates are determined by the presence of corresponding *MAT* idiomorphs in the haploid genome. Individuals of heterothallic ascomycetes have either the *MAT1-1* or *MAT1-2* idiomorph in their haploid genome and sexual reproduction occurs only when isolates of opposite mating type interact. In contrast, individual strains of homothallic ascomycetes contain both the *MAT1-1* and *MAT1-2* idiomorphs in their genomes and they are, therefore, self-fertile. While this is generally true, there are some exceptions, such as in the case of *Ophiostoma quercus* (Wilken et al., 2012).

The structure and gene content of the *MAT* loci have been used to gain insights into the sexual compatibility of many species originally believed to be asexual. Typically, most of these purported asexual species have been found to have fully-functional heterothallic mating systems (Kück and Pöggeler, 2009). Thus, mating-type markers have been developed for numerous important fungi and these have been used to determine whether sexual recombination might occur in natural populations of, for example, plant pathogens (Groenewald et al., 2006; Linde et al., 2003; Paoletti et al., 2005b; Wada et al., 2012). Mating type markers have also been useful to determine the mating type of individual isolates, thus replacing the traditionally tedious approach of crossing isolates in culture with tester strains of known mating type (Santos et al., 2010). Importantly, application of the growing knowledge regarding the *MAT* locus in fungi has facilitated the discovery of sexual cycles in many fungi

of clinical or industrial relevance that were thought to be asexual (Horn et al., 2009; Seidl et al., 2009).

Mating type gene sequences and the structure of the *MAT* locus are known for ten species of *Leptographium sensu lato* (including *Grosmannia*). These include *Grosmannia clavigera and* its closely related species (Tsui et al., 2013), *Leptographium procerum* and *L. profanum* (Duong et al., 2013). The *MAT* loci of these species have structures typical of those of heterothallic ascomycetes with both of the *MAT* idiomorphs present in an individual haploid genome. The *MAT1-1* idiomorphs have three mating type genes namely *MAT1-1-1*, *MAT1-1-2* and *MAT1-1-3*. But notably, besides the *MAT1-2-1* gene, the *MAT1-2* idiomorphs, all of these species have a truncated version of *MAT1-1-1*, lacking the functional alpha domain (Duong et al., 2013; Tsui et al., 2013). The presence of the truncated *MAT1-1-1* on the *MAT1-2* idiomorph has also been noted in species of *Ophiostoma* (Comeau et al., 2015; Tsui et al., 2013), a sister genus of *Leptographium sensu lato*, suggesting that the truncation event might share an evolutionary history among these two genera and perhaps also with other genera in the *Ophiostomatales*.

Most species in *Leptographium sensu lato* are known as only mitosporic fungi. Based on the results of Duong et al. (2013) and Tsui et al. (2013), we hypothesized that many of these species might actually have heterothallic mating systems. This would explain the low level of incidence of sexual states encountered for these fungi in nature or in culture. The aims of this study were thus to develop mating type markers in order to diagnose mating type and to consider the possible role that sexual reproduction might play in a relatively large collection of *Leptographium sensu lato* species.

2. Material and methods

2.1 Cultures, growth conditions and DNA extraction

Fungal isolates used in this study (Table 1) were obtained from the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. Single hyphal tip or single conidium cultures were grown in YM broth (2 % malt extract and 0.2% yeast extract) for 3 to 5 days. Mycelium was harvested by centrifugation and DNA was extracted using PrepMan™ Ultra reagent (Applied Biosystems, California, USA) following the methods described in Duong et al. (2012).

Table 1. Fungal isolates used in this study, including GenBank accession numbers of partial sequences of the *MAT1-2-1* and *MAT1-1-3* genes. Mating types and thallism of isolates are also indicated. Abbreviations and symbols used in the table are explained in the footnote. Accession numbers of MAT sequences used in the phylogenetic analyses are printed in italic. Sequences generated in this study are printed in bold type.

Species	**Isolate number**	**Insect/Host**	**Origin**	**Mating type**	***MAT1-2-1***	***MAT1-1-3***	**Thallism**
G. aenigmatica	CMW2199	*Ips typographus japonicus*	Japan	NA	***KT779243***	***KT779220***	HO
G. alacris	CMW621	*Pinus pinaster*	Portugal	MAT1-2	*KP171183*		HE
	CMW623	*Pinus pinaster*	Portugal	MAT1-1		*KP171181*	HE
	CMW1136	*Pinus taeda*	USA	MAT1-2	KJ528492		HE
	CMW2844	*Pinus pinaster*	South Africa	MAT1-2	KP171184		HE
G. americana	CMW2980	*Larix* sp.	USA	MAT1-2	***KT779263***		P-HE
G. aurea	CBS438.69	*Pinus contorta var. latifolia*	Canada	MAT1-1		*JX402951*	HE
	CMW29869	*Pinus contorta*	Canada	MAT1-2	***KT779253***		HE
G. clavigera	ATCC18086	*Pinus ponderosa*	Canada	MAT1-1		*JX402948*	HE
	KW1407	*Pinus contorta*	Canada	MAT1-2	*ACXQ02000048*		HE
G. huntii	CMW622	*P. pinaster*	Portugal	MAT1-1		***KT779227***	HE
	CMW654	*Pinus* sp.	USA	MAT1-2	***KT779250***		HE
	CMW1006	*Hylurgus ligniperda*	New Zealand	MAT1-2	**=KT779250**		HE
	CMW1015	Unknown	New Zealand	MAT1-1		**=KT779227**	HE
	CMW2824	*Pinus* sp.	USA	MAT1-2	**=KT779250**		HE
G. koreana	CMW14200	*Pinus densiflora*	Korea	MAT1-2	***KT779247***		HE
	CMW14201	*Pinus densiflora*	Korea	MAT1-1		***KT779222***	HE
G. piceiperda B[*]	CMW452	*Pseudotsuga menziesii*	USA	NA	***KT779246***	***KT779224***	HO
	CMW2811	*Picea rubens*	USA	NA	***KT779245***	***KT779223***	HO
G. piceiperda C[*]	CMW446	*Picea abies*	Norway	NA	***KT779244***	***KT779221***	HO
G. robusta	CMW710	Unknown	Unknown	MAT1-1		***KT779225***	P-HE
	CMW34175	*Pinus ponderosa*	USA	MAT1-1		**=KT779225**	P-HE
G. serpens	CMW191	*Pinus pinea*	Italy	MAT1-1		= **KT779226**	P-HE
	CMW192	*Pinus pinea*	Italy	MAT1-1		= **KT779226**	P-HE

Species	Isolate number	Insect/Host	Origin	Mating type	*MAT1-2-1*	*MAT1-1-3*	Thallism
-------	CMW289	*Pinus pinea*	Italy	MAT1-1		= **KT779226**	P-HE
	CMW290	*Pinus pinea*	Italy	MAT1-1		= **KT779226**	P-HE
	CMW304	*Pinus sylvestris*	Italy	MAT1-1		***KT779226***	P-HE
G. yunnanensis	CMW5152	*Pinus yunnanensis*	China	MAT1-2	***KT779249***		P-HE
L. abieticolens	CMW2866	*Abies balsamea*	USA	MAT1-1		***KT779228***	P-HE
L. abietinum	CMW2817	*Picea engelmannii*	USA	MAT1-2	***KT779264***		P-HE
L. albopini	CMW2065	*Pinus strobus*	USA	MAT1-2	***KT779251***		P-HE
L. alethinum	CMW3767	*Hylobius abietis*	UK	MAT1-1		***KT779229***	P-HE
L. bhutanense	CMW18650	*Pinus wallichiana*	Bhutan	MAT1-1		*KM491450*	HE
	CMW18652	*Pinus wallichiana*	Bhutan	MAT1-2	*KM491428*		HE
L. castellanum	CMW1988	*Hylurgus mickliki*	Spain	MAT1-1		**=KT779231**	P-HE
	CMW1989	*Hylurgus mickliki*	Spain	MAT1-1		**=KT779231**	P-HE
	CMW2320	*Pinus occidentalis*	Dominican Rep.	MAT1-1		**=KT779231**	P-HE
	CMW2321	*Pinus occidentalis*	Dominican Rep.	MAT1-1		***KT779231***	P-HE
L. celere	CMW12421	*Pinus semaonensis*	China	MAT1-2	***KT779261***		P-HE
L. douglasii	CMW2076	*Pseudotsuga menziesii*	USA	MAT1-1		***KT779230***	P-HE
L. gibbsii	CMW853	*Hylastes ater*	UK	MAT1-2	**=KT779258**		P-HE
	CMW1376	*Hylastes ater*	UK	MAT1-2	***KT779258***		P-HE
L. gracile	CMW12316	*Pinus armandii*	China	MAT1-2	KM491429		P-HE
	CMW12319	*Pinus armandii*	China	MAT1-2	KM491436		P-HE
L. longiclavatum	CMW20606	*Picea glauca*	Canada	MAT1-2	***KT779255***		HE
	CMW20607	*Pinus contorta*	Canada	MAT1-1		***KT779232***	HE
L. longiconidiophorum	CMW2004	*Pinus densiflora*	Japan	MAT1-1		*KM491452*	P-HE
L. lundbergii	CMW217	*Pinus sylvestris*	Sweden	MAT1-1		***KT779233***	HE
	CMW2190	*Pinus sylvestris*	Norway	MAT1-2	***KT779252***		HE
L. manifestum	CMW12436	*Larix olgensis*	China	MAT1-1		***KT779234***	P-HE
L. pineti	CMW3837	*Pinus* sp.	Indonesia	MAT1-1		***KT779235***	P-HE

Species	**Isolate number**	**Insect/Host**	**Origin**	**Mating type**	***MAT1-2-1***	***MAT1-1-3***	**Thallism**
L. pini-densiflorae	CMW5157	*Pinus densiflora*	Japan	MAT1-1		*KM491453*	HE
	CMW5162	*Pinus densiflora*	Japan	MAT1-2	*KM491438*		HE
L. procerum	CMW45	*Pinus sylvestris*	USA	MAT1-2	*KC883455*		HE
	CMW216	*Pinus taeda*	South Africa	MAT1-1		*KC883456*	HE
L. profanum	CMW10552	*Carya* sp.	USA	MAT1-2	*KC883457*		HE
	CMW10555	*Nyssa sylvatica*	USA	MAT1-1		*KC883458*	HE
L. pyrinum	CMW169	*Pinus resinosa*	USA	MAT1-2	***KT779262***		HE
	CMW3889	*Pinus jeffreyi*	USA	MAT1-1		***KT779236***	HE
L. sibiricum	CMW4481	*Abies sibirica*	Russia	MAT1-2	*KM491443*		P-HE
L. sinense	CMW38172	*Pinus elliottii*	China	MAT1-2	*KM491433*		P-HE
L. sinoprocerum	CMW26230	*Pinus tabuliformis*	China	MAT1-1		*KM491460*	HE
	CMW29990	*Pinus tabuliformis*	China	MAT1-2	*KM491447*		HE
L. terebrantis	SS394	*Pinus contorta* · *banksiana* hybrid	Canada	MAT1-2	*JX402935*		HE
	SS403	*Pinus contorta*	Canada	MAT1-1		*JX402956*	HE
L. truncatum	CMW644	*Hylastes* sp.	UK	MAT1-2	***KT779248***		HE
	CMW2402	*Pinus resinosa*	Canada	MAT1-1		***KT779237***	HE
L. wageneri v. ponderosae	CMW279	*Pinus ponderosae*	USA	MAT1-2	***KT779259***		HE
	CMW307	*Pinus contorta*	USA	MAT1-1		***=KT779239***	HE
L. wageneri v. *pseudotsugae*	CMW1533	*Pseudotsuga menziesii*	USA	MAT1-1		**=KT779239**	HE
	CMW154	*Pseudotsuga menziesii*	USA	MAT1-1		***KT779239***	HE
	CMW1541	*Pseudotsuga menziesii*	USA	MAT1-1		**=KT779239**	HE
	CMW2087	*Pseudotsuga menziesii*	USA	MAT1-2	***KT779260***		HE
L. wageneri v. *wageneri*	CMW53	*Pinus ponderosa*	USA	MAT1-2	= ***KT779259***		HE
	CMW493	*Pinyon Pine*	USA	MAT1-1		***KT779240***	HE
	CMW1828	*Pinus edulis*	USA	MAT1-1		**=KT779240**	HE
L. wingfieldii	CMW2096	*Pinus sylvestris*	France	MAT1-1		*JX402949*	HE

Species	**Isolate number**	**Insect/Host**	**Origin**	**Mating type**	***MAT1-2-1***	***MAT1-1-3***	**Thallism**
	CMW10221	*Pinus strobus*	USA	MAT1-2	***KT779256***		HE
L. yamaokae	CMW1935	*Pinus* sp.	Japan	MAT1-1		**=KT779241**	P-HE
	CMW1944	*Pinus* sp.	Japan	MAT1-1		**=KT779241**	P-HE
	CMW4726	*Pinus densiflora*	Japan	MAT1-1		***KT779241***	P-HE
	CMW4727	*Pinus densiflora*	Japan	MAT1-1		**=KT779241**	P-HE
	CMW4728	*Pinus densiflora*	Japan	MAT1-1		**=KT779241**	P-HE
	CMW4729	*Pinus densiflora*	Japan	MAT1-1		**=KT779241**	P-HE
Leptographium sp. X	CMW15470	*Pinus contorta*	Canada	MAT1-2	***KT779257***		HE
	CMW15493	*Pinus contorta*	Canada	MAT1-1		***KT779242***	HE
O. novo-ulmi sub. *novo-ulmi*	H327	*Ulmus* sp.	Slovakia	MAT1-1		*FJ858801*	HE
	V19	*Ulmus* sp.	Russia	MAT1-2	*AY887029*		HE
O. quercus	CMW27845	*Quercus* sp.	Canada	MAT1-1		*JQ319596*	HE
	CMW27847	*Quercus* sp.	UK	MAT1-2	*FJ865429*		HE

CMW = Culture Collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. NA = Not applicable; HE = Heterothallic; HO = Homothallic; P-HE = Putative heterothallic. * = Isolates referred to as '*G. piceiperda* B' and '*G. piceiperda* C' have been shown to represent distinct species in the *G. piceiperda* species complex (Linnakoski et al., 2012).

2.2 MAT primer design

The sequences of *MAT1-2-1* locus in *G. clavigera* and its relatives (Tsui et al., 2013), *L. procerum, L. profanum* (Duong et al., 2013), *O. ulmi* and *O. novo-ulmi* subsp. *novo-ulmi* (Paoletti et al., 2005a) were aligned and used to design primers to detect the *MAT1-2* idiomorph. The primers, Oph-HMG1 (5'- CGYAAGGAYMAYCACAAGGC -3') and Oph-HMG2 (5'- GGRTGAAGMMKCTCAACCTG -3'), were designed to amplify part of the HMG domain from the *MAT1-2-1* gene.

Because all the known *MAT* loci in *Leptographium sensu lato* have a truncated version of *MAT1-1-1* in the *MAT1-2* idiomorphs, we refrained from designing *MAT1-1* primers from the *MAT1-1-1* gene sequence. Primers Oph-MAT1F1 (5'- ATGKCCRATGARGAYTGCT -3') and Oph-MAT1R2 (5'- GGCGKTKGCRTTGTAYTTGTA -3') (Duong et al., 2015) were previously designed from the *MAT1-1-3* gene, which appears to be commonly present in *Leptographium sensu lato* (Duong et al., 2013; Tsui et al., 2013) and other *Ophiostoma* spp. for which the full *MAT* locus has been characterized (Comeau et al., 2015; Tsui et al., 2013), for detection of the *MAT1-1* idiomorph. In some species, where amplification with this primer combination failed, the primer Oph-MAT1F1 was used in combination with Oph-MAT1R1 (5'- GGCYYTRTGAAGYTTCTGTGC -3'), although this combination resulted in slightly shorter fragments.

2.3 PCR amplification, sequencing and mating type assignment

A PCR reaction mixture of 25 µl consisted of 2.5 µl 10 × PCR reaction buffer, 2.5 mM $MgCl_2$, 200 µM each dNTP, 0.8 µM of each primer (forward and reverse), 1 U FastStart *Taq* DNA Polymerase (Roche) and 20 to 50 ng of genomic DNA. The cycling conditions were an initial denaturation at 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 sec, 55 °C annealing for 30 sec, and 72 °C extension for 60 sec, with a final extension at 72 °C for 8 min. In most cases, the annealing temperature was at 55 °C for both *MAT1-1* and *MAT1-2* primers. However, when the PCR failed or when non-specific amplification was observed, the annealing temperature was adjusted in the range of 52 °C to 60 °C. Resulting PCR products were separated using 2% agarose gel electrophoresis, and gels were stained with GelRed (Biotium, Inc., California, USA) and examined under UV light.

The success of *MAT* marker amplification was confirmed by sequencing the obtained *PCR* amplicons. In cases where a single product was obtained, the PCR products were treated with exonuclease I and shrimp alkaline phosphatase (Exo-SAP) (Fermentas Inc., Hanover, MD,

USA) following the manufacturer's instructions, to remove excess primers and dNTPs. Where multiple bands were present, fragments of expected size were excised from the gel and sequenced using the same protocol. The treated or excised PCR products were directly sequenced using the same primers that were used for PCR amplification and the Big Dye®Terminator v. 3.1 cycle sequencing premix kit (Applied Biosystems, Foster City, California, USA).

Species that showed amplification products for both *MAT1-1* and *MAT1-2* primer combinations in a single isolate were designated as being homothallic. Species displaying amplification for both *MAT1-1* and *MAT1-2* primer combinations, but where only *MAT1-1* or *MAT1-2* could be detected in an individual isolate, were likewise designated as being heterothallic. Species where only the *MAT1-2* or *MAT1-1* idiomorph was detected were considered to be putatively heterothallic.

2.4 Phylogenetic analyses

In order to investigate the phylogenetic relationship between homothallic and heterothallic species investigated, phylogenetic analyses were conducted on a combined dataset of ITS2-LSU, partial *MAT1-2-1* and *MAT1-1-3* genes. The ITS2-LSU sequences for all species were obtained from GenBank, representing type isolates of each species, sequence accession numbers for these are presented elsewhere (De Beer and Wingfield, 2013; Yin et al., 2015). Sequences for regions of *MAT1-2-1* and *MAT1-1-3* genes were generated as described above (Table 1). All there gene regions were combined and aligned using an online version of MAFFT v. 7 (Katoh and Standley, 2013) .

Maximum likelihood (ML) and Bayesian inference (BI) analyses were carried out on the aligned dataset. Maximum likelihood analysis was conducted using RaxML v8.1.15 (Stamatakis et al., 2005) applying GTR+G model. A ML search for best-scoring ML tree followed by one thousand rapid bootstrap analysis, was conducted. Bayesian inference analyses were performed using MrBayes v. 3.1.2 (Ronquist and Huelsenbeck, 2003) applying the same models as used in the ML analysis. Four MCMC chains were run simultaneously for 5 million generations and tree sampling was conducted after every 100th generation. Twenty five percent of the trees sampled at the burn-in phase were discarded and posterior probabilities were calculated from the remaining trees.

3. Results

3.1 MAT primer design, PCR amplification and mating type assignment

MAT1-2 and MAT1-1 primers were successfully used in PCRs to amplify portions of *MAT1-2-1* and *MAT1-1-3* genes respectively. Primers Oph-HMG1 and Oph-HMG2 amplified part of HMG box of *MAT1-2-1* gene, resulting in PCR products of about 230 bp. Primers Oph-MAT1F1 and Oph-MAT1R2 amplified part of the *MAT1-1-3* gene, resulting in PCR products of about 450 bp. In cases where this MAT1-1 primer combination failed to amplify, for example in *G. aenigmaticum*, '*G. piceiperda* B' and '*G. piceiperda* C', primers Oph-MAT1R1 were successfully used in place of Oph-MAT1R2, resulting in slightly shorter PCR products of the *MAT1-1-3* gene. The relative primer binding positions on each of the MAT loci are presented in Fig. 1.

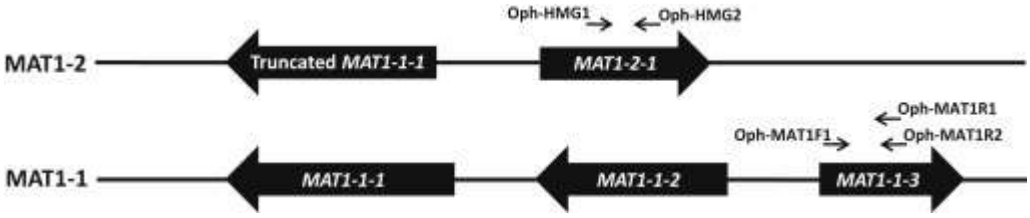

Figure 1. Relative binding sites of primers used to amplify MAT1-1 and MAT1-2 *in this study*. The organization of *MAT* locus presented in the figure was adapted from *MAT* loci in *L. procerum* and *L. profanum*.

In most cases, a single PCR product was obtained from each positive reaction. The identities of PCR products were confirmed as part of *MAT1-2-1* or *MAT1-1-3* genes by sequencing all positive amplifications. All obtained sequences were deposited in GenBank and accession numbers are presented in Table 1. Portions of the *MAT* genes for a total of 42 species residing in *Leptographium sensu lato* species were successfully amplified (Table 1). Based on the amplification profile, 20 species were identified as heterothallic, three species were homothallic and 19 species were tentatively assigned as heterothallic. Examples of *MAT* PCR amplification for some of the tested isolates are presented in Fig. 2.

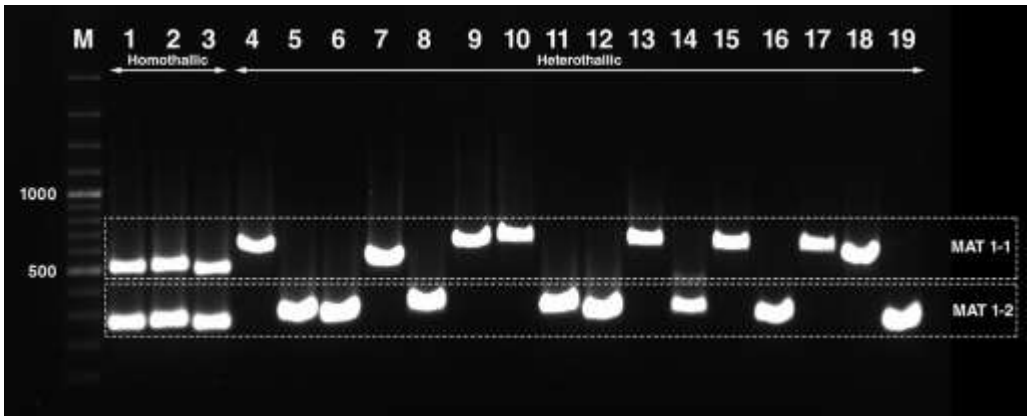

Figure 2. Agarose gel electrophoresis (2 % w/v) of *MAT1-1-3* (larger size bands- around 500 bp) and *MAT1-2-1* (smaller size bands, about 230 bp) PCR fragments from representative *Leptographium sensu lato* species. '*G. piceiperda* C' CMW446 (1); *G. aenigmatica* CMW2199 (2); '*G. piceiperda* B' CMW2811 (3); *G. alacris*: CMW623 (4), CMW621 (5); *G. koreana*: CMW14200 (6), CMW14201 (7); *G. huntii*: CMW1006 (8), CMW1015 (9); *L. bhutanense*: CMW18650 (10), CMW18652 (11); *L. longiclavatum*: CMW20606 (12), CMW20607 (13); *L. wageneri* var. *wageneri* CMW53 (14), CMW493 (15); *L. wageneri* var. *ponderosae* CMW279 (16), CMW307 (17); *L. wageneri* var. *pseudotsugae* CMW154 (18), CMW2087 (19). The molecular weight marker (M) used was GeneRuler™ 100 bp Plus DNA Ladder (Fermentas), the 1000 and 500 bp size fragments are indicated on the figure.

3.2 Phylogenetic analyses

The alignment of the ITS2-LSU region contained 604 characters, of which 450 characters were constant and 114 characters were parsimony informative. The alignment of *MAT1-1-3* gene region contained 493 characters, of which 191 characters were constant and 251 characters were parsimony informative. The alignment of *MAT1-2-1* gene region contained 215 characters, of which 90 were constant and 121 were parsimony informative.

ML and BI phylogenetic analyses of the combined dataset of ITS2-LSU, partial *MAT1-2-1* and *MAT1-1-3* genes resulted in trees with similar topology. In most cases, the species investigated grouped together to form species complexes as defined by De Beer & Wingfield (2013). Collectively, species from seven species complexes in *Leptographium sensu lato* were considered. Notably, three homothallic species as identified using MAT markers, *G. aenigmatica*, '*G. piceiperda* B' and '*G. piceiperda* C', resided in a single clade with high BI posterior probability and ML bootstrap support (Fig. 3). All the remaining species that were heterothallic or putatively heterothallic resided in six different species complexes (Fig. 3). These were the *G. clavigera* complex, *L. lundbergii* complex, *G. wageneri* complex, *G.*

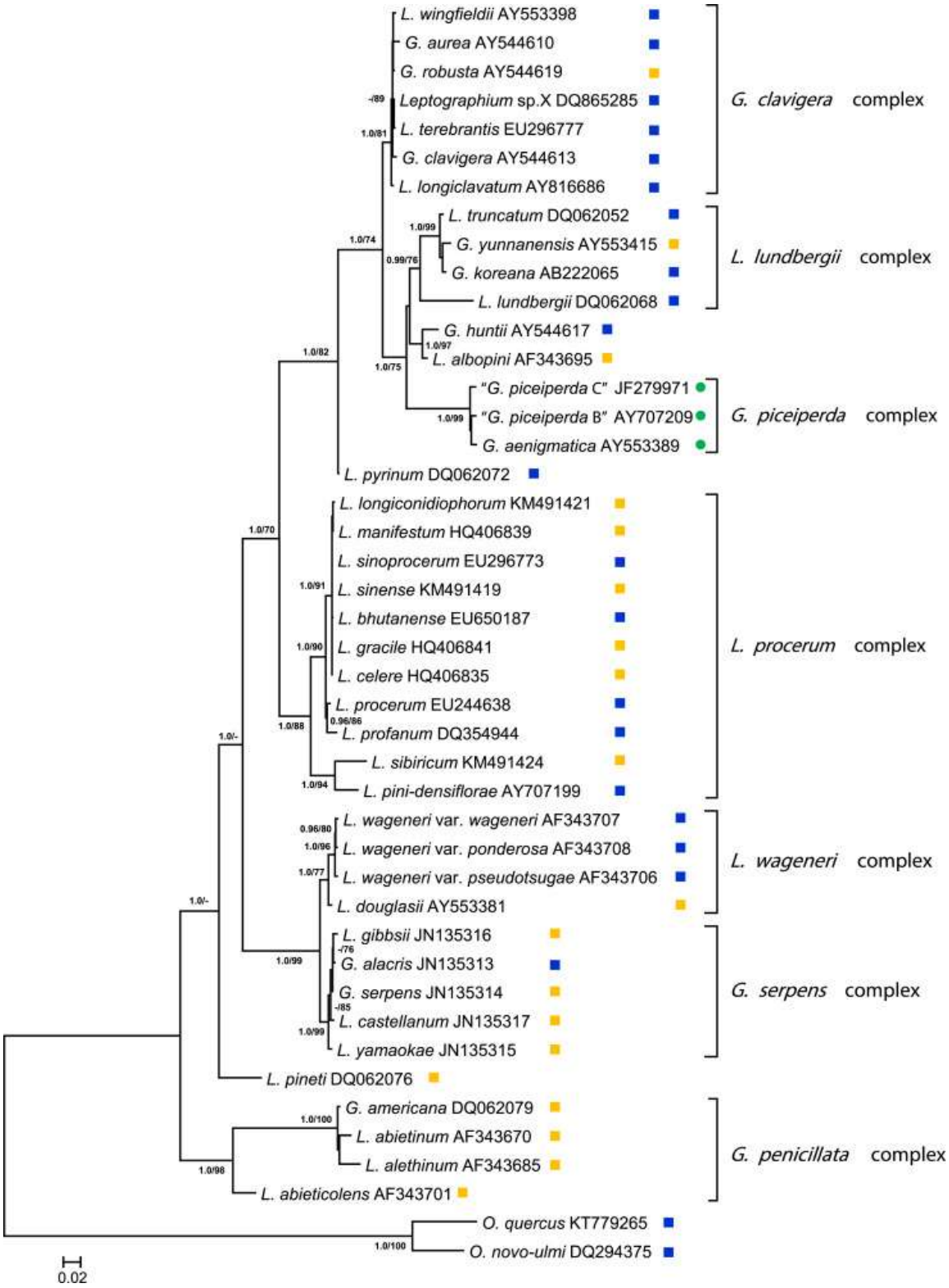

Figure 3. Phylogram derived from RaxML analysis of combined dataset of ITS2-LSU, *MAT1-1-3* and *MAT1-2-1* gene regions. Phylogenetic support is presented at nodes as Bayesian posterior probabilities (≥ 0.95) / ML bootstrap (≥ 70). Homothallic species are marked with green circles, heterothallic species are marked with blue squares and putatively heterothallic species are marked with yellow squares. ITS-LSU GenBank accession numbers are presented next to the species name. GenBank accession numbers for *MAT1-1-3* and *MAT1-2-1* used in phylogenetic analyses are presented in italic in Table 1.

serpens complex, *L. procerum* complex and *G. penicillata* complex. Of these, only the *G. wageneri* and *G. penicillata* complexes were well supported by BI and ML analyses.

4. Discussion

Molecular markers developed in this study made it possible to amplify and identify the mating strategy of 42 species residing in *Leptographium sensu lato*. Many species previously considered to be asexual were shown to be either heterothallic or putatively heterothallic, with individual isolates having only a single idiomorph. It will now be possible to attempt to induce sexual structures for these fungi in culture, by pairing isolates known to represent opposite mating types. Where this can be achieved, various genetic studies could also then be undertaken on these species that would otherwise not have been possible.

The lack of opposite mating type isolates in our possession negated the possibility for us to recover both MAT idiomorphs in a number of species included in this study. Thus, 19 species for which only *MAT1-1* or *MAT1-2* idiomorph could be recovered were designated as putatively heterothallic. With the MAT makers now available, it will be possible to confirm the heterothallic nature of these species when additional isolates become available for them. It is important to also recognize that primers described in this study could fail to amplify both MAT idiomorphs in some of these species and thus they could be homothallic. Although this is unlikely, the thallism of these species will need to be treated as putative until the opposite MAT idiomorphs to those detected in this study can be found.

Prior to this study, the mating types were known for only a small number of species residing in *Leptographium sensu lato*. Thus a particularly interesting outcome of this study was that the majority of species tested were either heterothallic or putatively heterothallic and this was in contrast to a relatively small number (three) of homothallic species detected. This finding is consistent with the fact that the greater number of species in *Leptographium sensu lato* have long been considered as asexual species (De Beer and Wingfield, 2013). Based on the results of this study, we believe that many species found only in the asexual form in nature are probably capable of reproducing sexually. It is plausible that their sexual states have not been seen due to their heterothallic nature and the fact that they have been collected in the absence of an opposite mating strain. This is similar to the situation for various other fungi, thought to be asexual but later shown to be heterothallic and where sexual states have recently been

discovered for some of the species (Horn et al., 2009; O'Gorman et al., 2008; Seidl et al., 2009).

Duong et al. (2012) were able to show that *G. alacris* is heterothallic by randomly crossing different isolates in all possible combinations. Thus, of the five species in the *G. serpens* complex (Duong et al., 2012), sexual sates have been found only in the case of *G. alacris*. Efforts to induce sexual states in the other four species did not result in ascomata. The present study has provided molecular evidence confirming that *G. alacris* is heterothallic, as are the other species in the *G. serpens* complex (Duong et al., 2012). In the present study, only a single mating type was found for isolates of *G. serpens* (*MAT1-1*), *L. castellanum* (*MAT1-1*), *L. yamaokae* (*MAT1-1*) and *L. gibbsii* (*MAT1-2*) and it will not be possible to attempt to produce sexual structures until strains of opposite mating type have been found. The results of this study explain why these fungi failed to produce sexual states in the study by Duong et al. (2012).

Goheen and Cobb (1978) reported the discovery of a sexual state in the important conifer root pathogen *G. wageneri*, which was found in the galleries of *Hylastes macer*. This form of the fungus has never again been seen and there has been doubt as to whether these authors had possibly collected a sexual state of some other ophiostomatoid fungus (Harrington and Cobb, 1988). The results of our study show clearly that *G. wageneri* is a heterothallic fungus and thus has the capacity to undergo sexual outcrossing. This provides strong evidence to suggest that Goheen and Cobb (1978) correctly identified the ascomata of this fungus in nature. Thus, it serves as an interesting example of a *Leptographium* sp. for which a sexual state has been found in nature only once and could never be produced in the laboratory (Wingfield, unpublished).

Species in *Leptographium sensu lato* have been assigned to different complexes based on their relatedness in phylogeny, morphological characters, as well as their ecology (De Beer and Wingfield, 2013; Linnakoski et al., 2012). Results of the present study showed that those species belonging to the same complex consistently share the same mode of sexual reproduction. Likewise, the only three homothallic species ('*G. piceiderda* B', '*G. piceiperda* C' and *G. aenigmatica*) considered in this study grouped in a single, well supported clade, consistent with the *G. piceiperda* complex previously defined (De Beer and Wingfield, 2013; Linnakoski et al., 2012). This suggests that these species might share a common homothallic ancestor. The remaining 39 heterothallic (or putatively heterothallic species) reside in six different species complexes. A number of other species residing in these six species

complexes could not be included in this study but based on the patterns observed, it is likely that they will also have a heterothallic mating system.

Patterns of distribution of sexual compatibility have previously been used to better understand the evolution of fungal mating systems in other fungi (Inderbitzin et al., 2005; Nygren et al., 2011; Yun et al., 1999). Likewise, the distribution of homothallic and heterothallic species provides an opportunity to gain insights into the origin and evolution of homothallism and heterothallism in *Leptographium sensu lato*. From the results of this study, it is reasonable to hypothesize that homothallism in *G. piceiperda* complex has evolved once from a heterothallic ancestor. A common heterothallic ancestor would thus best explain the current patterns of sexual compatibility in *Leptographium sensu lato*. However, the detailed structure of the *MAT* loci of species in the *G. piceiperda* complex, together with that in closely related heterothallic species such as those in the *G. clavigera* and *L. lundbergii* complexes, will be required to confirm this hypothesis.

5. Acknowledgements

This work was funded by members of the Tree Protection Co-operative Programme (TPCP) and the DST-NRF Centre of Excellence in Tree Health Biotechnology (CTHB) at the Forestry and Agricultural Biotechnology Institute (FABI), the National Research Foundation (NRF) and the University of Pretoria, Pretoria, South Africa.

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