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Characterization of the mating-type genes in *Leptographium procerum* and *Leptographium profanum*

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ABSTRACT

Leptographium procerum and the closely related species *Leptographium profanum*, are ascomycetes associated with root-infesting beetles on pines and hardwood trees, respectively. Both species occur in North America where they are apparently native. *L. procerum* has also been found in Europe, China New Zealand, and South Africa where it has most probably been introduced. As is true for many other *Leptographium* species, sexual states have never been observed in *L. procerum* or *L. profanum*. The objectives of this study were to clone and characterize the mating type loci of these fungi, and to develop markers to determine the mating types of individual isolates. To achieve this, a partial sequence of *MAT1-2-1* was amplified using degenerate primers targeting the high mobility group (HMG) sequence. A complete *MAT1-2* idiomorph of *L. profanum* was subsequently obtained by screening a genomic library using the HMG sequence as a probe. Long range PCR was used to amplify the complete *MAT1-1* idiomorph of *L. profanum* and both the *MAT1-1* and *MAT1-2* idiomorphs of *L. procerum*. Characterization of the *MAT* idiomorphs suggests that the *MAT* genes are fully functional and that individuals of both these species are self-sterile in nature with a heterothallic mating system. Mating type markers were developed and tested on a population of *L. procerum* isolates from the USA, the assumed center of origin for this species. The results suggest that cryptic sexual reproduction is occurring or has recently taken place within this population.

Introduction

Sexual reproduction in ascomycetes is governed by the mating type (*MAT*) genes residing at the mating type locus (*MAT-1*) (Yoder *et al.* 1986; Turgeon & Yoder 2000). Although they occupy the same position in the genome, different mating type alleles have highly dissimilar sequences and gene contents and they are consequently referred to as idiomorphs named *MAT1-1* and *MAT1-2* (Metzenberg & Glass 1990; Turgeon & Yoder 2000). In heterothallic filamentous ascomycetes, individual isolates contain either the *MAT1-1* or the *MAT1-2* idiomorph and sexual reproduction occurs only when isolates containing different *MAT* idiomorphs interact.

The *MAT1-1* idiomorph has one to three genes, including the mandatory *MAT1-1-1* encoding a protein that contains alpha box sequences, together with one or a combination of the *MAT1-1-2*, *MAT1-1-3*, *MAT1-1-4* and *MAT1-1-5* genes (Coppin *et al.* 1997; Kronstad & Staben 1997; Turgeon 1998; Turgeon & Yoder 2000; Amselem *et al.* 2011). *MAT1-2* usually contains

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a single *MAT1-2-1* gene encoding for a protein with a high mobility group (HMG) domain (Coppin *et al.* 1997; Turgeon & Yoder 2000), but in some cases a second gene (*mat a-2*, *MAT1-2-2*; *MAT1-2-3* or *MAT1-2-4*) can be found within the *MAT1-2* idiomorph (Pöggeler & Kück 2000; Kanamori *et al.* 2007; Amselem *et al.* 2011; Martin *et al.* 2011). In contrast to heterothallic fungi, isolates of homothallic species harbor both *MAT1-1* and *MAT1-2* regions in their genomes, and sexual reproduction can occur in the absence of a second isolate (Nelson 1996). However, there are rare exceptions to this scheme, such as in the case of the homothallic *Lodderomyces elongisporus* and *Neurospora africana*, where both *MAT* idiomorphs are missing in the former species and only *MAT1-1* (*mat A*) is present in the latter species (Glass & Smith 1994; Butler *et al.* 2009).

The rapidly growing number of studies on fungal mating systems emphasizes the important role that they play in fungal biology. Information regarding structure and organization of *MAT* genes can be used to establish whether a fungal species reproduces in a homothallic or heterothallic fashion. Furthermore, such knowledge can be used to develop molecular markers in order to determine the mating types of isolates, replacing the laborious and time consuming process of developing and crossing mating tester strains (Steenkamp *et al.* 2000; Scherrer *et al.* 2005; Ramirez-Prado *et al.* 2008; Brewer *et al.* 2011). The ability to identify mating types using molecular markers is also useful when it is necessary to select isolates of opposite mating type for genetic experiments, and this facilitates the discovery of sexual states of species known only by their asexual morphs (Kück & Pöggeler 2009).

The high level of variation in mating type genes offers considerable potential to resolve questions regarding phylogenetic relatedness in species complexes (Turgeon 1998; O'Donnell *et al.* 2004). Furthermore, information relating to the mating type genes has been used to develop hypotheses relating to the evolution of sexuality and the origins of homothallism and heterothallism in fungi (Yun *et al.* 1999; Fraser & Heitman 2004; Butler *et al.* 2009; Nygren *et al.* 2011). The frequency and distribution of different mating types has also been used to assess sexual reproduction and the preferred mode of reproduction in natural populations (Linde *et al.* 2003; Rau *et al.* 2005; Groenewald *et al.* 2006; Stergiopoulos *et al.* 2007).

Leptographium procerum and the closely related species, *Leptographium profanum*, are ascomycetes associated with root-infesting beetles on pine and hardwood trees respectively (Jacobs *et al.* 2006). While there is no evidence that *L. profanum* is a pathogen, *L. procerum* has been associated with root and root collar diseases of pine in the Eastern United States (Wingfield 1986; Wingfield *et al.* 1988). Both species are apparently native to North America, and *L. profanum* is a little-known species that has not been found outside the USA (Jacobs *et al.* 2006). In contrast, *L. procerum*, is a very well-known species that has apparently been introduced into various countries including those in Europe, New Zealand, and South Africa (Jacobs & Wingfield 2001; Linnakoski *et al.* 2012). The most recent introduction of *L. procerum* has been into China where the fungus has apparently contributed to the death of thousands of native *Pinus tabuliformis* trees (Lu *et al.* 2009a; Lu *et al.* 2009b).

Sexual states of *Leptographium* species have traditionally been treated in the genus *Grosmannia* (Zipfel *et al.* 2006). De Beer and Wingfield (2013) showed that the generic delineation of the two genera is problematic based on currently available data, and that comprehensive multigene phylogenies will be necessary to resolve this question. In view of the discontinuation of the dual nomenclature system (Hawksworth 2011), De Beer and Wingfield (2013) suggested that all 34 *Grosmannia* species be treated in *Leptographium sensu lato*, but to avoid unnecessary name changes (Wingfield *et al.* 2012), current species names in the two genera should be maintained until the generic boundaries have been resolved. At present, 59 of the 93 species in *Leptographium sensu lato*, including *L. procerum* or *L. profanum*, are known only by their asexual states (De Beer & Wingfield 2013). This is despite substantial effort to search for a sexual state in *L. procerum* (M.J. Wingfield, unpublished). Both *L. procerum* and *L. profanum* are thus considered mitosporic species.

Based on the consistent absence of observed sexual states in *L. procerum* and *L. profanum*, we have hypothesized that both fungi have heterothallic mating type systems. The aims of this study were to test this hypothesis by cloning and characterizing the mating type idiomorphs of *L. procerum* and *L. profanum* and to develop mating type markers for both species. A further aim was to investigate the mating type frequency in a population of *L. procerum* isolates that might provide evidence of sexual recombination in nature.

Materials and methods

Fungal isolates

Isolates of *LeptographiumL. procerum* and *LeptographiumL. profanum* (Table 1) used for cloning of mating type genes were obtained from the Culture Collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. Isolates of *L. procerum* used to assess the mating type ratio in a natural population of the fungus were collected in the eastern USA during Oct. of 2009 and have been preserved in CMW.

DNA isolation

Fungal mycelium was grown in liquid medium containing 2 % malt extract and 0.2 % yeast extract (YM broth) for 3–5 d. Mycelium was harvested by centrifugation and lyophilized. DNA was isolated from the lyophilized mycelium using the method described by Aljanabi & Martinez (1997) with minor modifications. After extraction, DNA was resuspended in 200 µl of Tris-EDTA buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) containing 0.02 mg ml^{-1} of RNase A. After 30 min incubation at 37 °C, samples were extracted twice with an equal volume of Phenol/Chloroform/Isoamyl Alcohol (25:24:1, v/v). DNA was then precipitated from the solution by adding 1/10 volume of 3 M sodium acetate and 2.5 volume of absolute ethanol. Samples were incubated at −20 °C for 1 h and then centrifuged at 10 000 *g* for 30 min at 4 °C. The pellets were washed twice with 70 % ethanol, air dried and resuspended in Tris–HCl 10 mM, pH 8.0. This DNA was used for Southern analyses

Table 1 – Fungal isolates used in this study.

Species	Isolate number		Host	Origin	Mating type
	CMW[a]	CBS[b]			
L. procerum	10		*Pinus nigra*	Unknown	*MAT1-1*
	12	118578	*P. strobus*	Farmington, USA	*MAT1-1*
	13	115211	Unknown	Unknown	*MAT1-1*
	45	118580	*P. sylvestris*	Minnesota, USA	*MAT1-2*
	216		*P. taeda*	Sabie, South Africa	*MAT1-1*
	10216		*P. strobus*	Burlington, USA	*MAT1-1*
	25627		*P. tabuliformis*	Shanxi, China	*MAT1-1*
	Isolates of *L. procerum* used for assessment of the mating type ratio in a natural population were collected in the eastern USA during 2009.				
L. profanum	10550		Dogwood	USA	*MAT1-2*
	10552	120307	Hickory	USA	*MAT1-2*
	10553		Hickory	USA	*MAT1-2*
	10554	120226	*Nyssa sylvatica* (Blackgum)	USA	*MAT1-1*
	10555		*N. sylvatica* (Blackgum)	USA	*MAT1-1*

a CMW = Culture Collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.
b CBS = Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

and long range PCR. DNA samples used for PCR detection of mating-type genes were prepared from fresh mycelium using PrepMan™ Ultra reagent (Applied Biosystems, California, USA) following the protocol suggested by the manufacturer.

Cloning the mating-type idiomorphs from Leptographium profanum

Cloning of MAT1-2 *in Leptographium profanum*

Degenerate primers NcHMG1 and NcHMG2 (Arie *et al.* 1997) were used to obtain partial sequence of HMG-box of *MAT1-2-1* in *L. profanum*. The PCR mixture, 25 μl total volume, consisted of 2.5 μl 10× PCR reaction buffer, 2.5 mM $MgCl_2$, 200 μM each dNTP, 1 μM of each primer, 1 U FastStart *Taq* DNA Polymerase (Roche Diagnostics GmbH, Mannheim, Germany) and 20–50 ng of genomic DNA. PCR reactions were performed in an Eppendorf MasterCycler® gradient (Eppendorf, Hamburg, Germany) using the following conditions: an initial denaturation at 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 55 °C annealing for 30 s, and 72 °C extension for 60 s, with a final extension at 72 °C for 8 min. Amplified products were separated in a 1.5 % (w/v) agarose gel, stained with GelRed (Biotium, Inc., California, USA) and visualized under UV light. Bands of expected size (approximately 300 bp), were excised and DNA fragments were recovered from the gel using a QIAquick Gel Extraction Kit (Qiagen, Inc., California, USA). The purified DNA fragments were ligated into pGEM®-T vector (Promega, Wisconsin, USA) and transformed into *Escherichia coli* JM109. Plasmids were extracted from the positive clones and sequenced using T6 an SP7 primers.

Specific primers (P-HMG-F: 5′-CTCAACCTGTCGCTTGATTTC-3′ and P-HMG-R: 5′-TATCGTAAGGACCACCACAAGG-3′) were designed from HMG-box sequences of *L. profanum*. These primers were then used to screen a number of *L. profanum* isolates for the presence of *MAT1-2-1*. The PCR reaction mixture and thermal profile used was the same as that for the degenerate primers NcHMG1 and NcHMG2, except that only 0.2 μM of each primer was used. PCR products were separated using agarose gel electrophoresis, stained with GelRed and examined under UV light. Isolates that gave a band around 224 bp were then assigned as representing the *MAT1-2* genotype; isolates that did not give an expected product were putatively assigned as representing the *MAT1-1* genotype.

Southern blotting was carried out with genomic DNA from two *L. profanum* *MAT1-2* isolates (CMW10550 and CMW15002). A DIG-labeled probe was synthesized by using a PCR DIG probe synthesis kit (Roche), following the manufacturer's protocol and using primers P-HMG-F and P-HMG-R, and pGEM®-T containing the HMG box sequence from *L. profanum* as template. The genomic DNA was extracted from lyophilized fungal mycelium as described above. Five micrograms of each DNA sample were digested to completion with *Eco*RI and *Hin*dIII (Fermentas, Vilnius, Lithuania). After digestion, DNA fragments were precipitated using ethanol and resuspended in 20 μl of TE buffer. The fragments were then separated in a 0.8 % agarose gel. The DIG-labeled DNA molecular weight marker VII (Roche) was used as a size standard for electrophoresis and Southern hybridization. The DNA fragments were transferred from agarose gel onto a Nylon membrane (Roche) using the capillary transfer method, and fixed to the membrane under UV irradiation. This membrane was used for Southern hybridization with the DIG-labeled probes. The hybridization and detection was carried out using reagent from DIG-High prime DNA labeling and detection kit (Roche) following the manufacturer's protocols.

Results obtained from Southern blot showed exactly the same pattern for the two *L. profanum* isolates tested. The *Eco*RI digested fragments gave a hybridized signal of about 4 kb, while the *Hin*dIII digested fragments gave a signal of over 8 kb. A selection was then made to use *Hin*dIII-digested DNA from CMW10552 to construct a partial genomic library for screening of the *MAT1-2* idiomorph. Ten micrograms of genomic DNA from CMW10552 was completely digested with *Hin*dIII and separated on a 0.8 % agarose gel. Fragments with sizes corresponding to the hybridized signal were excised from the gel and purified using QIAquick gel extraction kit (Qiagen). The fragments were cloned into the pBluescript II KS (+) phagemid vector (Stratagene, California, USA) and

transformed into *E. coli* JM109. This library was then screened for clones harboring *MAT1-2* by means of colony hybridization using the same DIG-labeled probe that was used for Southern hybridization. Plasmids from the positive clones were extracted and inserts were sequenced by primer walking (sequencing primers are available on request). Genes present in the inserts were predicted using FGENESH+ (Salamov & Solovyev 2000) (http://linux1.softberry.com). Mating type genes and other Open Reading Frames (ORFs) were identified by BLAST against the NCBI database.

Cloning of MAT1-1 *in Leptographium profanum*

The *MAT1-1* idiomorph in *L. profanum* was obtained using long range PCR with primers PSeq-7 (5′-AGGATGGGAAGGGATTCT -3′) and Pseq-8 (5′-CAGACCGGGAGATTGACTC-3′) designed from the regions flanking the *MAT1-2* idiomorphs. PCR was performed using these primers on a DNA sample of a *MAT1-1* isolate as determined based on the absence of PCR product when amplified with primers targeting the *MAT1-2* HMG box. The long range PCR mixture, 50 μl total volume, consisted of 5 μl 10× PCR reaction buffer with 27.5 mM $MgCl_2$, 500 μM each dNTP, 0.3 μM of each primer, 0.75 μl expand long template enzyme mix (Roche Applied Science) and 200 ng of genomic DNA. The PCR thermal cycle consisted of an initial denaturation at 95 °C for 5 min, followed by ten cycles of 94 °C for 10 s, 53 °C annealing for 30 s, and 68 °C extension for 8 min, followed by 25 cycles of 94 °C for 15 s, 55 °C annealing for 30 s, and 68 °C extension for 8 min (plus 20 s cycle elongation for each successive cycle), and a final extension at 72 °C for 8 min. Amplified products were separated by agarose gel electrophoresis. Fragments of the expected size were excised from the gel, purified using the QIAGEN gel extraction kit and sequenced by primer walking. Genes were predicted using FGENESH+ (Salamov & Solovyev 2000). *MAT* genes were identified using the predicted amino acid sequences and BLASTp against the NCBI database.

Cloning the mating-type idiomorphs of Leptographium procerum

The mating type idiomorphs (*MAT1-1* and *MAT1-2*) in *L. procerum* were obtained by long range PCR using primers PSeq-7 and PSeq-12 (5′-TAGCCGTGGGATGGAGGTTG-3′) designed from the flanking regions of the mating type idiomorphs of *L. profanum*. Various *L. procerum* isolates were first screened with P-HMG-F and P-HMG-R primers to identify their putative mating type. Genomic DNA from two *L. procerum* isolates of opposite mating type (CMW45 as *MAT1-2* and CMW216 as *MAT1-1*) were used in long range PCR's in order to obtain both *MAT1-1* and *MAT1-2* idiomorphs. The PCR protocol and thermal cycles used were the same as those used to obtain *MAT1-1* in *L. profanum* as described above. The PCR products were excised from the gel, purified and sequenced as described above. Genes were predicted using FGENESH+ and manually compared with those in *L. profanum*.

Phylogenetic analyses of the HMG and α domain amino acid sequences

Deduced amino acid sequences of HMG and α domain were compared to those of other ascomycetes from GenBank using BLASTp search. Representative sequences were downloaded, the dataset was compiled and aligned using an online version of MAFFT 6 (Katoh & Toh 2008). Neighbor joining analyses were performed using MEGA 5.01 (Tamura *et al.* 2011) with 1000 bootstrap replicates.

Development of multiplex PCR-based mating type markers for Leptographium profanum and Leptographium procerum

The PCR-based mating type markers for *L. profanum* and *L. procerum* were developed based on sequences of *MAT* genes of these species. For detection of the *MAT1-2* idiomorph, primers P-MAT2-F (5′-CGATGGTGAAGTATGTGATTGA -3′) and P-MAT2-R (5′-TTCAGCCTCATCGCCAGT-3′) were designed from the conserved and unique regions of *MAT1-2-1* from both *L. profanum* and *L. procerum*. Primers P-MAT1-F (5′-ATGGCCGA TGAAGACTGCT-3′) and P-MAT1-R (5′-CTTCCGAATTTCCTTG GATT-3′) were designed from the *MAT1-1-3* gene region for both species for the detection of the *MAT1-1* idiomorph. All primers were selected to have the same annealing temperature and to result in different amplicon sizes. These primers were combined in a multiplex PCR to detect mating types of isolates of both species. A number of *L. profanum* and *L. procerum* isolates (Table 1) were selected to test the amplification success and the specificity of the primers.

The multiplex PCR reaction consisted of 2.5 μl 10× PCR reaction buffer, 2.5 mM $MgCl_2$, 200 μM each dNTP, 0.2 μM of each primer (both *MAT1-1* and *MAT1-2* primers), 1 U FastStart *Taq* DNA Polymerase (Roche) and 20–50 ng of genomic DNA. PCR cycling conditions were an initial denaturation at 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 55 °C annealing for 30 s, and 72 °C extension for 60 s, with a final extension at 72 °C for 8 min. PCR products were separated on a 2 % agarose gel, stained with GelRed and examined under UV light. Isolates presenting a product of 620 bp were designated as *MAT1-2*, while isolates presenting a product of 273 bp were designated as *MAT1-1*.

Sexual compatibility tests

Leptographium profanum and *L. procerum* isolates of opposite mating type as determined by the multiplex PCR were paired in culture in an attempt to induce the formation of teleomorph structures. Four *L. profanum* isolates (two *MAT1-1* and two *MAT1-2*) and ten *L. procerum* isolates (five *MAT1-1* and five *MAT1-2*) were paired in all possible combinations (including both specific and interspecific pairings) on water agar plates containing pine twigs known to stimulate the formation of ascomata in related fungi (authors, unpublished). The plates were incubated at 15 °C and 25 °C in the dark and examined monthly for 1 y for the presence of ascomata.

Distribution of mating-type genes in a population of Leptographium procerum

The newly developed primers P-MAT1-F and P-MAT1-R (for *MAT1-1*) and P-MAT2-F and P-MAT2-R (for *MAT1-2*) were used to investigate the mating type ratio in a population of *L. procerum*. Twenty isolates of *L. procerum* collected during 2009 on native *Pinus resinosa* and *Pinus strobus* infested with

the bark beetle *Dendroctonus valens* (Coleoptera: Scolytinae) growing in Pennsylvania, Massachusetts and New Hampshire, USA, were used in this study. DNA extraction was carried out using PrepMan™ Ultra reagent (Applied Biosystems). The multiplexing PCR mixture and thermal profile used for these primers was as described in section 2.5 above. The mating type of each isolate was assigned based on the size of the corresponding PCR product.

Results

Cloning the mating-type idiomorphs in Leptographium profanum

Cloning of MAT1-2 in Leptographium profanum

PCR with the degenerate primers NcHMG1 and NcHMG2 yielded expected bands of around 300 bp for isolates of *L. profanum* and *L. procerum*. The bands were successfully recovered from the gel and cloned in to pGEMT-easy. Sequencing with T7 and SP6 primers confirmed that these are part of the HMG boxes of *MAT1-2-1*, having high levels of similarity with known *MAT1-2-1* sequences of *Ophiostoma novo-ulmi* and *Ophiostoma himal-ulmi* (Paoletti *et al.* 2005). New HMG box specific primers (P-HMG-F and P-HMG-R) were developed and successfully amplified HMG box sequences from *L. profanum* and *L. procerum*.

Southern blot analysis of genomic DNA from two *L. profanum* isolates using the HMG box as probes resulted in similar hybridization patterns and gave a signal at about 4 kb and over 8 kb for *Eco*RI and *Hin*dIII digested fragments, respectively. A partial genomic DNA library was constructed for *Hin*dIII-digested fragments and colony hybridization resulted in three positive clones in approximately two thousand colonies screened. Sequencing plasmids obtaining from these clones confirmed the presence of a 10.5 kb insert. Gene prediction using FGENESH+ showed that the insert harbored a complete *MAT1-2* idiomorph (GenBank accession number KC883457) consisting of a *MAT1-2-1* gene (996 bp in size, predicted ORF of 867 bp) and a truncated version of *MAT1-1-1* (1558 bp in size, predicted ORF of 1341 bp) (Fig 1). The partial sequence of the cytoskeleton assembly control *(SLA)* gene flanking the *MAT1-2* idiomorph was also obtained, however neither a DNA lyase gene sequence nor any other ORF was found in the other flanking region of the *MAT1-2* idiomorph. *MAT1-2-1* contained a typical HMG box similar to that known in the *MAT1-2-1* gene of other ascomycetes and had two introns of 61 and 68 bp. The second intron resides within the HMG box and split the codon encoding for serine as is found in many ascomycetes. Alignment of the HMG box DNA and deduced amino acid sequences showed high similarity with sequences from other ascomycetes (Fig 2). BLAST analysis of *MAT1-2-1* deduced amino acid sequence showed 75 % and 65 % similarity with the predicted protein sequence of *MAT1-2-1* from *Grosmannia clavigera* (GenBank accession number: ACXQ02000048) and *O. himal-ulmi* (Paoletti *et al.* 2005) respectively. A truncated version of *MAT1-1-1* with a 217 bp intron was also found on the *MAT1-2* idiomorph (Fig 1). BLAST analysis of its deduced amino acid sequence showed 36 % similarity to the predicted *MAT1-1-1* gene product from *O. novo-ulmi* subsp. *novo-ulmi*. However, sequence analysis showed that the α domain was absent (Fig 3), suggesting that this gene product would not be fully functional.

Cloning of MAT1-1 in Leptographium profanum

Long range PCR using primers (PSeq-7 and PSeq-8) flanking the *MAT1-2* idiomorph, yielded an expected PCR product of about 9.1 kb for a *L. profanum MAT1-1* isolate (CMW10555). Direct sequencing of this PCR product confirmed the presence of a complete *MAT1-1* idiomorph (GenBank accession number KC883458). Gene prediction using FGENESH+ and BLAST analysis showed that this fragment contained a partial sequence of the *SLA* gene and a complete *MAT1-1* idiomorph with each of the *MAT1-1-1*, *MAT1-1-2* and *MAT1-1-3* genes (Fig 1). No other ORF was found in the fragment. *MAT1-1-1* was 2251 bp in length, had one intron of 52 bp and contained a conserved α domain. BLASTp analysis of the MAT1-1-1 protein sequence showed 36 % and 33 % similarity with predicted *MAT1-1-1* gene products from *Ophiostoma novo-ulmi* subsp. *novo-ulmi* (Jacobi *et al.* 2010) and *Ophiostoma quercus* (Wilken *et al.* 2012) respectively. Alignment of the α domain nucleotide and amino acid sequences with those of other ascomycetes showed a high level of similarity (Fig 2). The *MAT1-1-2* gene was 1171 bp in length (predicted ORF of 849 bp) and contained five introns of 84, 76, 56, 50 and 56 bp respectively. BLASTp analysis of the *MAT1-1-2* deduced amino acid sequence showed 29 % similarity with the predicted *MAT1-1-2* gene product from *O. novo-ulmi* subsp. *novo-ulmi*. *MAT1-1-3* was 498 bp in length (predicted ORF of 405 bp) and contained one intron of 93 bp. BLASTp analysis of the *MAT1-1-3* deduced amino acid sequence showed 44 and 42 % similarity with *MAT1-1-3* gene products from *O. quercus* and *O. novo-ulmi* subsp. *novo-ulmi* respectively. The fact that the *MAT* locus contained either the *MAT1-1* or the *MAT1-2* idiomorph suggests that *L. profanum* is a heterothallic species.

Cloning the mating-type idiomorphs in Leptographium procerum

The long range PCR using primers PSeq-7 and PSeq-12 resulted in PCR products of about 7.7 kb in the *L. procerum MAT1-1* isolate (CMW216) and about 6.4 kb in the *L. procerum MAT1-2* isolate (CMW45). Sequences determined for these PCR products confirmed that the *MAT1-1* and *MAT1-2* idiomorphs from *L. procerum* had been successfully identified. The gene elements and organization of the *MAT1-1* and *MAT1-2* idiomorphs in *L. procerum* were similar to those in *L. profanum*. The *MAT1-1* idiomorphs (GenBank accession number KC883456) consisted of *MAT1-1-1*, *MAT1-1-2* and *MAT1-1-3* genes (Fig 1) with very high sequence similarity to those in *L. profanum*. The *MAT1-1-1* gene was 2260 bp in size (predicted ORF of 2208 bp) and contained one intron of 52 bp. A conserved α domain was detected in the sequence of the *MAT1-1-1* gene. The *MAT1-1-2* gene was 1040 bp in size (predicted ORF of 771 bp) and had three introns of 76, 56 and 137 bp. *MAT1-1-3* was 498 bp in size (predicted ORF of 384 bp) and contained one intron of 114 bp. The *MAT1-2* idiomorphs (GenBank accession number KC883455) consisted of a *MAT1-2-1* gene together with a truncated version of a *MAT1-1-1* gene (Fig 1). The sequences of these two genes were also very similar to those of *L. profanum*. The truncated *MAT1-1-1* gene lacked an α domain sequence (Fig 3), was 1950 bp in size

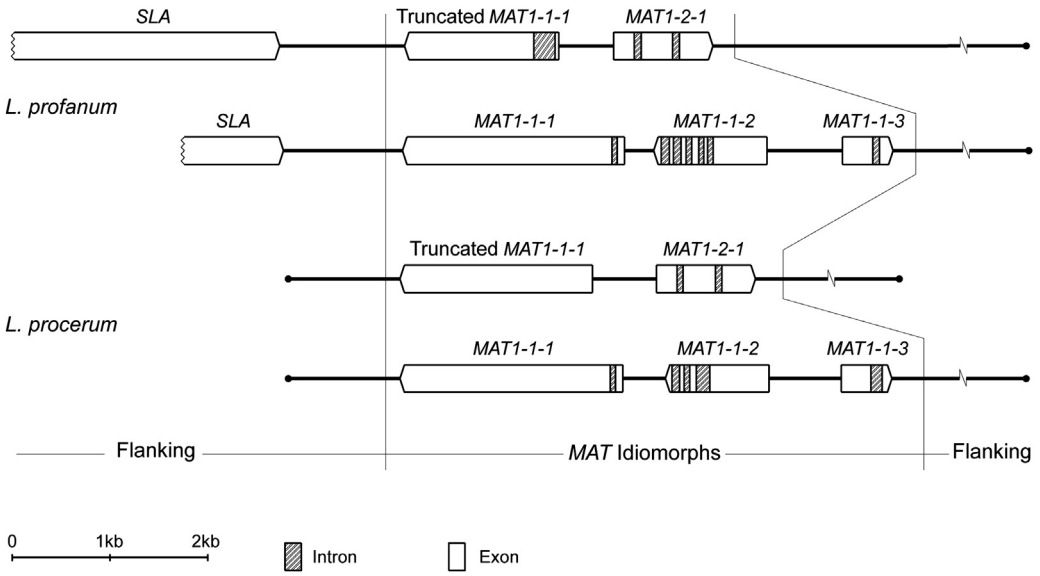

Fig 1 – Organization of the mating type loci of *L. procerum* and *L. profanum*. The introns were predicted using the program FGENESH+ (Salamov & Solovyev 2000) and thus the presence and size of the introns are putative.

and had no intron. *MAT1-2-1* was 993 bp in size (predicted of ORF 867), contained a conserved HMG-box sequence and had two introns of 60 and 66 bp. The gene elements and structures of *MAT* idiomorphs in *L. procerum* suggested that this species is heterothallic.

Phylogenetic analyses of the HMG and α domain amino acid sequences

Phylogenetic trees with largely similar topology and strongly supported monophyletic clades were obtained from phylogenetic analyses of the HMG and alpha domain amino acid sequence (Fig 4). Similar topologies were also observed when DNA sequences were used (data not shown). The HMG and alpha domain protein sequences of Ophiostomatoid fungi formed a monophyletic clade with strong bootstrap support. Three sub-clades emerged for *Leptographium sensu lato*, *Sporothrix* and *Ophiostoma* also with strong bootstrap support. Well supported clades were also observed for other ascomycetes included in the analyses i.e. the Sordariales, Hypocreales, Capnodiales and Pleosporales (Fig 4).

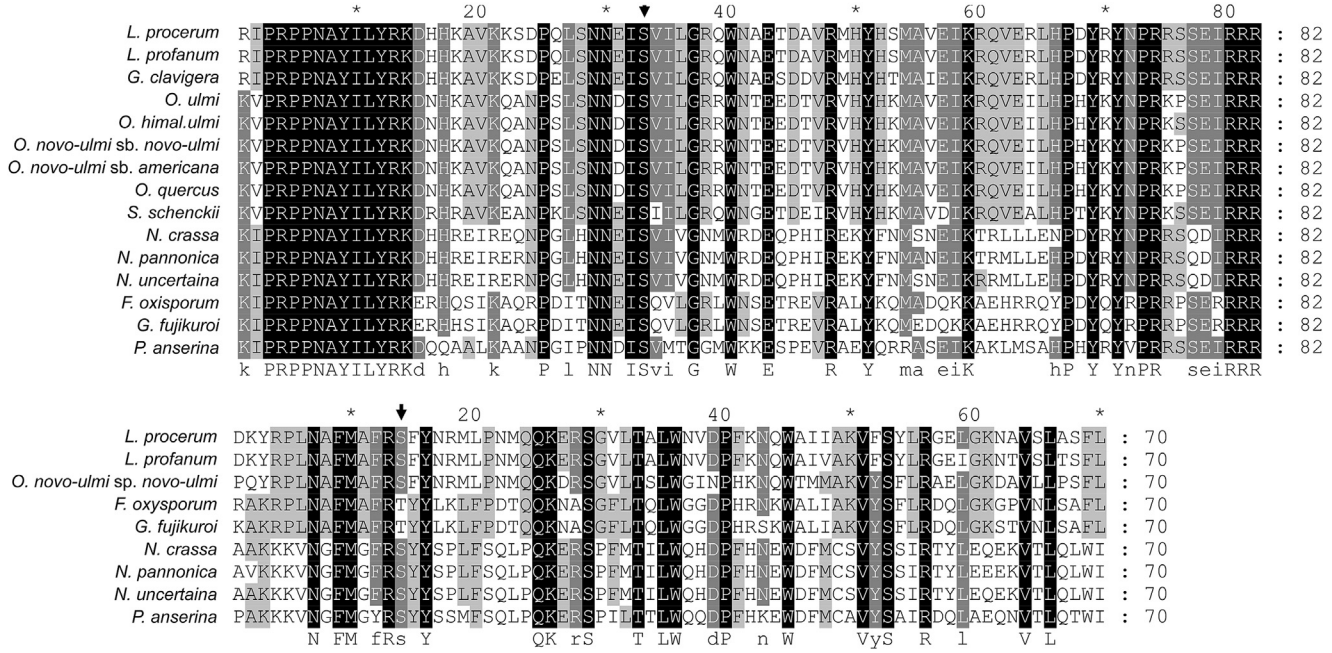

Fig 2 – Alignment of amino acid sequences of the α domain (top) and HMG-box domain (bottom) from *L. procerum*, *L. profanum* and other ascomycetes. Black arrows indicate intron positions.

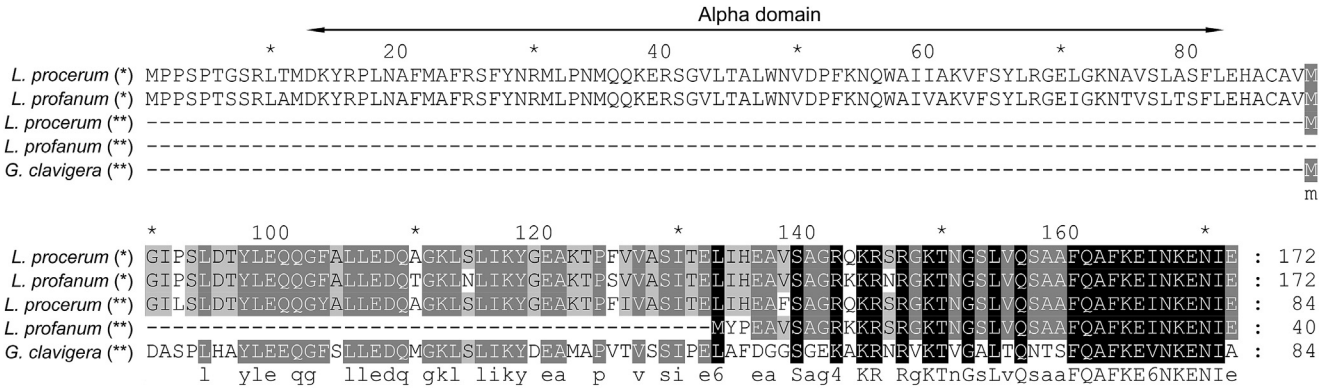

Fig 3 – Alignment of N-terminal amino acid sequences of MAT1-1-1 (*) and truncated MAT1-1-1 (**) from *L. procerum*, *L. profanum* and *G. clavigera* indicating that alpha domain sequences are missing in truncated MAT1-1-1 in all three species.

Development of multiplex PCR-based mating type markers for Leptographium profanum and Leptographium procerum

The primer pairs P-MAT1-F, P-MAT1-R, P-MAT2-F and P-MAT2-R consistently amplified the partial *MAT1-1* and *MAT1-2* idiomorphs from both *L. profanum* and *L. procerum* isolates in a multiplex PCR assay. *MAT1-1* isolates resulted in a unique band of 273 bp, whereas *MAT1-2* isolates gave a unique band of 620 bp. The different PCR products of the two mating types were differentiated easily using agarose gel electrophoresis (Fig 5).

Sexual compatibility tests

The year-long experiment to test for sexual compatibility between isolates of *L. procerum* and *L. profanum* having opposite mating type did not produce ascomata under any of the conditions tested.

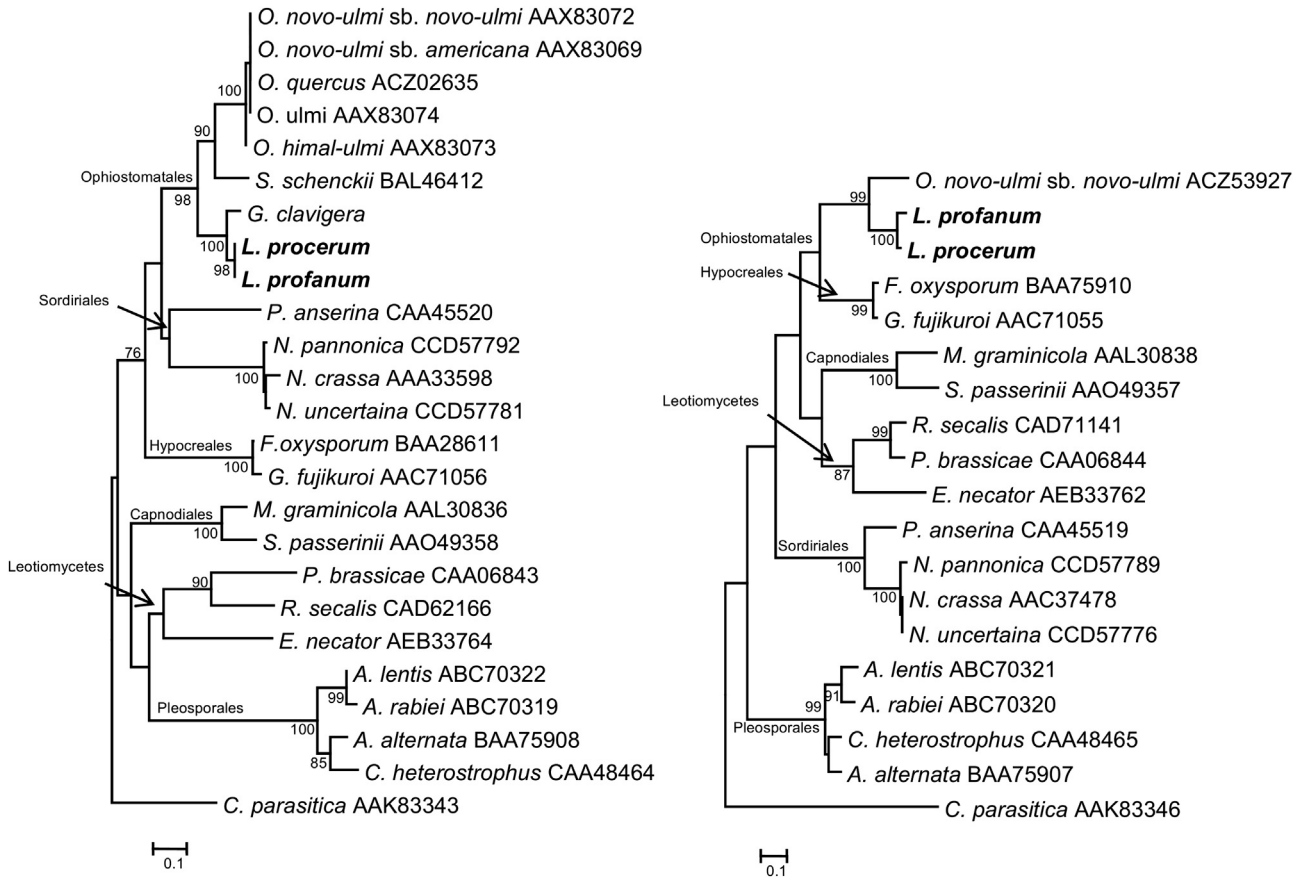

Fig 4 – Neighbor joining trees derived from analysis of the HMG-box domain (left) and α domain (right) amino acid sequences from *L. procerum*, *L. profanum* and other ascomycetes. Bootstrap values (1000 replicates) above 75 % are indicated at nodes. GenBank accession numbers are presented after the species name.

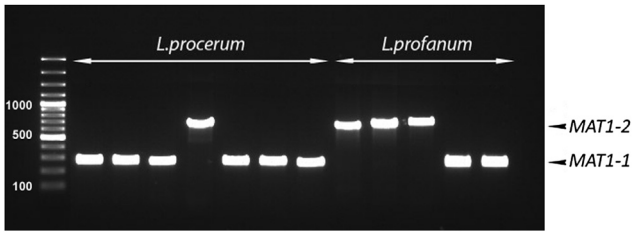

Fig 5 – Results obtained from a multiplex PCR assay to determine the mating genotype of isolates of *L. procerum* and *L. profanum*. From left to right, DNA samples from *L. procerum* CMW10; CMW12; CMW13; CMW45; CMW216; CMW10216; CMW25627; and *L. profanum* CMW10550; CMW10552; CMW10553; CMW10554; CMW10555 were tested. All fragments were separated in 2 % agarose gel, stained with GelRed and visualized under UV light. The molecular weight marker used was GeneRuler™ 100 bp Plus DNA Ladder (Fermentas).

Distribution of mating-type genes in a population of Leptographium procerum

Both mating types were detected in a population of *L. procerum* from the USA. The ratio of *MAT1-1* to *MAT1-2* was 1:1 for 20 isolates tested (Fig 6). Isolates of opposing mating types were found in close proximity to each other, and in some cases originated from a single beetle gallery.

Discussion

This is the first study to identify and completely characterize the mating type loci in any species of *Leptographium*. Using robust molecular genetic evidence, both *L. procerum* and *L. profanum* were shown to have heterothallic mating systems. This is also the first report describing the complete mating type locus in a heterothallic member of the *Ophiostomatales*. Together with the *MAT1-1* idiomorph from *G. clavigera* (DiGuistini *et al.* 2011), partial *MAT1-1* and *MAT1-2* idiomorphs from *O. novo-ulmi* (Paoletti *et al.* 2005; Jacobi *et al.* 2010), partial *MAT1-1* and *MAT1-2* idiomorphs from *O. quercus* (Wilken *et al.* 2012), the mating type loci characterized in this study provide insights into the structure and gene orientation of the mating type locus in the *Ophiostomatales*.

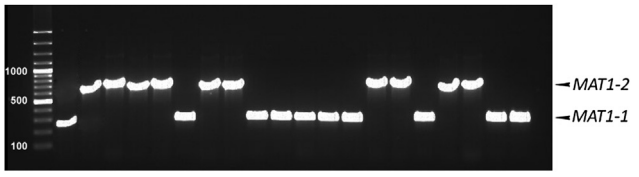

Fig 6 – PCR amplicons resulting from the multiplex PCR (using *MAT1-1* and *MAT2-1* primers) of a population of *L. procerum* isolates from the USA. The ratio of *MAT1-2* and *MAT1-2* isolates equivalent to 1:1 infers that sexual reproduction is occurring or has recently taken place within this population. All fragments were separated in 2 % agarose gel, stained with GelRed and visualized under UV light. The molecular weight marker used was GeneRuler™ 100 bp Plus DNA Ladder (Fermentas).

The *MAT1-1* idiomorphs in *L. procerum* and *L. profanum* were shown to have very similar sequences and they had a structure typical of those found in other Sordariomycetes, especially those in the *Ophiostomatales*. The *MAT1-1* idiomorphs identified in *L. procerum* and *L. profanum* were shown to have three mating type genes i.e. *MAT1-1-1*, *MAT1-1-2* and *MAT1-1-3* with similar order and orientation to those of *Gibberella fujikuroi*, *Fusarium oxysporum* (Yun *et al.* 2000; Martin *et al.* 2011), *Cryphonectria parasitica* (McGuire *et al.* 2001), *Podospora anserina* (Debuchy *et al.* 1993) and *Neurospora crassa* (Ferreira *et al.* 1996). The sequences of *MAT1-1* genes in these two fungi are very similar and correspond with those known in other Ophiostomatoid fungi (Table 2). Typical conserved α domains were found in *MAT1-1-1* genes of both *L. procerum* and *L. profanum*, each having one intron in a similar position to that of other Ascomycetes. The nucleotide and amino acid sequences of alpha domains were almost identical for the two species, with only a few differences in the nucleotide sequences and one difference in the amino acid sequence. The predicted amino acid sequences in the alpha domains of *L. procerum* and *L. profanum* were also very similar to those of *O. novo-ulmi* subs. *novo-ulmi*, and somewhat less so to those in *F. oxysporum* and *G. fujikuroi*.

In contrast to the *MAT1-1* idiomorphs, the *MAT1-2* idiomorphs in *L. procerum* and *L. profanum* had an atypical structure when compared to those of other ascomycetes. In addition to a mandatory *MAT1-2-1* gene, the *MAT1-2* idiomorphs in these two species harbor a second ORF that encodes a putative peptide that is highly homologous to the *MAT1-1-1* gene product from the *MAT1-1* idiomorphs. However, sequence analyses showed that the conserved alpha domain was missing, suggesting that these are truncated *MAT1-1-1* genes. We were also able to identify the truncated *MAT1-1-1* in the *MAT1-2* idiomorphs in *G. clavigera* (GenBank accession number: ACXQ02000048) (DiGuistini *et al.* 2011) by comparing its sequence with those of *L. procerum* and *L. profanum*. The presence of a truncated *MAT1-1-1* gene in *MAT1-2* idiomorphs in these three species suggests that this organization could be common in some, if not all, *Leptographium sensu lato* species. Most of the *MAT1-2* from other ascomycetes have only *MAT1-2-1* gene (Coppin *et al.* 1997; Turgeon & Yoder 2000). However, similar structures have also been observed in the *MAT1-2* idiomorphs of few other heterothallic Sordariomycetes, where pseudo-genes, partial sequences, or homologs of *MAT1-1* genes are present in the *MAT1-2* idiomorph (Yokoyama *et al.* 2003; Kanematsu *et al.* 2007; Wilken *et al.* 2012).

The presence of the truncated *MAT1-1-1* in the *MAT1-2* locus provides opportunities to study the evolution of the *MAT* locus in these fungi. Contrary hypotheses on the evolution of homothallism and heterothallism in fungi have been debated for a very long time. Most studies aim to provide arguments and evidence supporting the hypothesis that homothallic fungi evolved from heterothallic ancestors (Yun *et al.* 1999; Nygren *et al.* 2011; Gioti *et al.* 2012). Whereas, only a few others support an opposing hypothesis: that heterothallism arose from homothallism (Geiser *et al.* 1998; Amselem *et al.* 2011). The structure of the *MAT* loci in *L. procerum* and

Table 2 – Identity comparison of predicted transcript and deduced amino acid sequences of mating type genes of *L. procerum* and *L. profanum* to mating type genes from other species in the Ophiostomatales.

	L. procerum				*L. profanum*			
	MAT1-1-1	MAT1-1-2	MAT1-1-3	MAT1-2-1	MAT1-1-1	MAT1-1-2	MAT1-1-3	MAT1-2-1
L. procerum	–	–	–	–	93/91	88/89	90/89	96/96
L. profanum	93/91	88/89	90/89	96/96	–	–	–	–
G. clavigera	–	–	–	75/75	–	–	–	74/73
S. schenckii	–	–	–	38/34	–	–	–	38/35
O. ulmi	–	–	–	42/39	–	–	–	41/39
O. novo-ulmi sb. *novo-ulmi*	36/31	28/20	34/30	43/40	35/30	27/18	35/31	41/39
O. novo-ulmi sb. *americana*	–	–	–	42/39	–	–	–	41/39
O. himal-ulmi	–	–	–	43/40	–	–	–	42/39
O. quercus	54/45[a]	–	44/40[a]	42/39	54/43[a]	–	45/41[a]	41/39

The values are presented as percent of transcript identity/percent of protein identity.
– Not applicable (due to the unavailability of sequences).
a Partial sequences were compared (due to the unavailability of complete *O. quercus MAT* sequences).

L. profanum, with the presence of a portion of *MAT1-1-1* on the *MAT1-2* idiomorph, presents possible evidence to address the controversy. However, the data obtained in the present study alone are not adequate to resolve the question. The presence of a portion of the *MAT1-1-1* on the *MAT1-2* idiomorphs could have resulted from translocation or unequal crossover of *MAT1-1* idiomorphs. This concept is often used to explain the evolution of homothallism from heterothallic ancestors. However, it is also possible that the truncated *MAT1-1-1* gene resulted from incomplete deletions of *MAT1-1* genes from the *MAT1-2* idiomorph, supporting the notion that heterothallism evolved from homothallism. Thorough structure and sequence comparisons of the *MAT* loci in the heterothallic *L. procerum* and *L. profanum*, with other homo- and heterothallic *Leptographium sensu lato* species will be necessary to fully resolve this question.

Phylogenetic analyses of HMG and α domain amino acid and nucleotide sequences grouped *L. procerum* and *L. profanum* with other Ophiostomatoid fungi in a distinct clade, separated from other ascomycetes. This suggests that mating type genes could potentially be used to resolve phylogenetic questions in the Ophiostomatales. Mating type genes have been applied in combination with other nuclear genes to resolve phylogenetic relationships and to describe new species (Turgeon 1998; O'Donnell *et al.* 2004). However, it is widely accepted that reproductive genes are prone to inter-specific gene transfer and this could lead to conflicts with other gene trees. The application of *MAT* genes as phylogenetic markers should thus be undertaken with care and in conjunction with other gene regions (Inderbitzin *et al.* 2005; Paoletti *et al.* 2006; Strandberg *et al.* 2010; Martin *et al.* 2011).

Despite many previous efforts to find these structures, the sexual states of *L. procerum* and *L. profanum* remain to be discovered. This is also the case for many other *Leptographium* species. The heterothallic nature of *L. procerum* and *L. profanum*, and possibly many other *Leptographium* species, provides some explanation for the low success rate in mating tests with these fungi. Clearly, the mating process requires the presence of both mating types, which can now be experimentally determined. However, suitable mating conditions are also required and given the complex conditions of bark beetle galleries in which these fungi occur, determining these conditions will remain a challenge.

Both mating types were present in a natural population of *L. procerum* from the USA, the purported center of origin for this species. This suggests that the potential for genetic recombination exists and that sexual recombination can occur. This also suggests that a sexual state exists for this fungus. The mating type ratio of 1:1 confirms that recombination is occurring frequently. More intensive studies are required to discover the sexual state and to investigate sexual reproduction of these fungi. Knowledge of the mating type loci and the development of PCR based mating type makers in this study will be valuable in the quest for sexual states and to better understand the processes involved in the reproduction of *L. procerum* and *L. profanum*.

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