

**Taxonomy, mating type distribution
and population biology
in species of *Leptographium* and *Grosmannia***

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**Taxonomy, mating type distribution
and population biology
in species of *Leptographium* and *Grosmannia***

by

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 Dr. Z. Wilhelm de Beer**

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Declaration

I declare that the dissertation/thesis, which I hereby submit for the degree *Philosophiae Doctor* at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

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Preface

Preface

Species in the ascomycete genus *Leptographium sensu lato* (Ophiostomatales) are specifically adapted to be vectored by primarily conifer-infesting bark beetles (Coleoptera, Scolytinae). Most species in the genus are saprophytes that cause blue stain in freshly exposed timber. However, under favorable conditions, some of these species can cause disease in their host trees and can thus result in severe economic losses. At present, *Leptographium sensu lato* accommodates more than 90 species, of which only 34 have known sexual states. Under the dual nomenclature system, these have been treated in the genus *Grosmannia*. The remaining species are known only by their asexual states and currently reside in *Leptographium*.

Various taxonomic problems remain unresolved in *Leptographium sensu lato*. Currently, nine species complexes are recognized in the genus. These complexes were defined based on the molecular phylogeny of ribosomal DNA sequences (De Beer and Wingfield 2013). However, many species in these complexes were described based solely on morphology or in some cases using sequence data for a single gene region. This raises concerns regarding species boundaries and the taxonomy of species in these complexes. Although *Leptographium sensu lato* includes both sexual and asexual species, limited information is available regarding the mating type genes and their ability to undergo sexual outcrossing. Furthermore, several species of *Leptographium sensu lato* have a global distribution, but little is known regarding their dispersal patterns or centers of origin.

The research presented in this thesis deals with a range of topics including the taxonomy, the genetic basis of mating systems, and population biology of selected species of *Leptographium* and *Grosmannia*. The taxonomy and biology of the ophiostomatoid fungi, including *Leptographium sensu lato*, have been thoroughly reviewed in recent publications (Jacobs and Wingfield 2001; Seifert et al. 2013). Furthermore, several extensive reviews have been published recently on many different aspects of fungal mating systems (Lee et al. 2010; Ni et al. 2011). For this reason and to avoid unnecessary repetition, I have dedicated **Chapter 1** to a review of the evolution of fungal mating systems and specifically to the transitions between homothallism and heterothallism. The literature relevant to this topic is summarized, discussed and used to formulate hypotheses in order to stimulate debate on this controversial topic.

Chapter 2 presents a phylogenetic revision of one of the species complexes in *Leptographium sensu lato*. New primers were developed for the calmodulin gene and these were applied, together with available primers for four other gene regions, in a phylogenetic study on a collection of isolates previously identified as *Grosmannia serpens*. Based on the

resulting multigene phylogeny, five different species could be delineated and described in the newly defined the *G. serpens* complex. This study presents the first comprehensive multigene phylogeny for any of the species complexes in *Leptographium sensu lato*.

Of the five species defined in the *G. serpens* complex in chapter 2, *Grosmannia alacris* is the most widely distributed. Isolates of this species have been identified from different continents including Europe, North America and Africa. In **Chapter 3**, I developed microsatellite markers to be used in a population study on this species. The possibility of applying these markers to other species in the *G. serpens* complex was also investigated.

Many species in the *Leptographium sensu lato* are treated as asexual species and it is unknown whether sexual reproduction occurs in them. The studies presented in **Chapter 4** aim to better understand the sexual systems in species of *Leptographium sensu lato* by characterizing the mating type (*MAT*) locus. Two species residing in the *L. procerum* complex, were used this study. These included *Leptographium procerum*, that has been introduced into China recently and is contributing to the death of thousands of native *Pinus tabuliformis* trees in that country, and its close relative, *Leptographium profanum*. The complete *MAT* loci of both *L. procerum* and *L. profanum* were obtained by screening a genomic library and long range PCR. The possibility of a sexual state existing in *L. procerum* was also investigated making laboratory crosses and by seeking evidence of sexual recombination in a naturally occurring population of the fungus.

The results obtained in chapter 4, together with *MAT* gene sequences for several other species in the Ophiostomatales that have recently become available, provided the opportunity to study the sexual systems in a large collection of species in *Leptographium sensu lato*. In **Chapter 5**, I developed primers (mating type markers) making it possible to predict whether a species is heterothallic or homothallic. The possibility of using mating type genes as phylogenetic characters was also investigated. The work in this chapter presents the first available information on the topic and it provides tools to study evolution of sexual reproduction in *Leptographium* and *Grosmannia*.

In **Chapter 6**, a population genetic study was undertaken on collections of *G. alacris* isolates from South Africa and the USA. This was achieved using the microsatellite markers developed in chapter 3. Additional mating type markers were also developed based on the sequences obtained in chapters 4 and 5 of this thesis. The genetic diversity, population structure, mating type frequency and distribution of populations of *G. alacris* from these two countries were considered. The data were analysed to better understand patterns of introduction and the mode of reproduction in these populations.

The studies presented in the five research chapters of this thesis are closely connected and aimed to resolve problems with regard to the taxonomy, sexual compatibility and population biology of selected *Leptographium* and *Grosmannia* species. Other than deepening our

understanding of the fungi studied, the results have also provided tools to enable similar studies on other species and species complexes in *Leptographium sensu lato*, other genera in the Ophiostomatales, and other Ascomycetes.

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Chapter 1

Evolution of fungal mating systems: heterothallism or homothallism as ancestral state?

Abstract

Sexual reproduction is ubiquitous and found in all kingdoms of life. The fungal kingdom exhibits an astonishing variety of sexual strategies with homothallism and heterothallism as the two dominant modes of reproduction. The evolution of fungal mating systems mostly involves switching between these two modes of sexual exchange. However, whether homothallism or heterothallism serves as the ancestral form is the subject of an ongoing debate. In this review, findings and arguments regarding these transitions are discussed. Clearly, transitions between heterothallism and homothallism have occurred in both directions and they vary between fungal groups. We propose a model explaining the evolution of fungal mating systems, and argue that evolution towards heterothallism could be the central direction, a view that has been largely underestimated in the past.

Sexual reproduction in ascomycetes

Fungi have diverse sexual reproduction strategies that include homothallism, heterothallism and pseudo-homothallism. Homothallism (selfing or self-compatibility) is the process where sexual reproduction can occur without the need of a partner of opposite mating type (Casselton 2002; Coppin et al. 1997). Heterothallism (obligate outcrossing or self-incompatibility) describes a system where sexual reproduction can occur only between partners of opposite mating type (Coppin et al. 1997). In contrast, pseudo-homothallism is a process where sex can occur without the need of a second individual but including the involvement of different nuclei of opposite mating type (Merino et al. 1996; Ni et al. 2011). In addition to sexual reproduction, fungi propagate themselves very efficiently in a clonal manner by a process called asexual reproduction. The ability to reproduce efficiently both asexually and sexually sets fungi, together with some plant species and a few insects, apart from other organisms, and this state has emerged from little understood, but fascinating and controversial evolutionary processes.

Intensive studies on fungal mating systems in recent years have shed considerable light on the genetic determinants of sexual reproduction. In ascomycetes, sexual reproduction is governed by the mating-type (*MAT*) locus harbouring genes that encode transcription factors regulating the expression of pheromones, pheromone receptors and other factors involved in sexual development and mating recognition (Coppin et al. 1997; Heitman et al. 2007). In heterothallic ascomycetes, the *MAT* locus contains different alleles with dissimilar sequences and gene elements, respectively referred to as the *MAT1-1* and *MAT1-2* idiomorphs (Metzenberg and Glass 1990; Turgeon and Yoder 2000). A single haploid isolate would contain either the *MAT1-1* or *MAT1-2* idiomorph. In contrast, isolates of homothallic fungi contain both *MAT1-1* and *MAT1-2* idiomorphs in their genomes and these are usually closely linked or fused (Yun et al. 1999; Yun et al. 2000). However, in some rare cases, the two *MAT* idiomorphs in heterothallic species can be separated from each other in the genome (Galagan et al. 2005; Gioti et al. 2012; Rydholm et al. 2007; Yun et al. 1999).

Pseudohomothallic fungi resemble those that are homothallic in that they can undergo sexual reproduction without requiring a second strain of opposite mating type. However the genetic determinants of mating in pseudohomothallic fungi differ from those of homothallic species. Sexual spores as well as vegetative cells of pseudohomothallic fungi are heterokaryons, each containing two haploid nuclei of opposite mating type. Sexual reproduction occurs when these nuclei fuse (Heitman et al. 2007; Merino et al. 1996; Ni et al. 2011).

Besides these main modes of sexual reproduction mentioned above, some other rare forms of sexual reproduction also exist. One such an example is mating type switching, which is found in the well-known yeast *Saccharomyces cerevisiae* (*Saccharomycetales*). In this case, homothallic mother cells can switch mating type and cross with daughter cells in a heterothallic manner (Haber 1998; Ni et al. 2011). In other cases, same sex mating occurs where sexual reproduction can take place between partners of the same mating type (Alby et al. 2009; Lin et al. 2005). Intra-tetrad mating occurs when sexual reproduction takes place in the form of mating between spores of the same tetrad (Knop 2006). Although all of these mating strategies contribute to the diversity of reproduction in fungi, homothallism and heterothallism remain the most dominant and commonly encountered forms.

Evolution of the fungal mating system

Sexual reproduction in fungi is thought to have evolved a very long time ago due to its prevalence throughout the fungal kingdom. Although the structures of genetic determinants of sexual reproduction are very diverse between orders, genera and even species of the same genera, the location of the mating type locus is quite conserved. This suggests that sexual determinants share a common evolutionary origin in the fungi (Butler 2007; Idnurm 2011; Idnurm et al. 2008). The two most prevalent modes of reproduction, heterothallism and homothallism, are ubiquitous and play an important role in determining the fitness of fungi in changing environments. However, the question as to whether homothallism or heterothallism represents the ancestral form, and the direction in which the evolution of fungal sexual reproduction has predominantly occurred, remains a topic of considerable debate (Billiard et al. 2012; Lin and Heitman 2007).

It has commonly been suggested that homothallism arose from heterothallism and this view has relied on both empirical (Coppin et al. 1997; Fraser et al. 2007; Gioti et al. 2012; Inderbitzin et al. 2005; Nygren et al. 2011; Rydholm et al. 2007; Yun et al. 1999) as well as theoretical studies (Nauta and Hoekstra 1992). Only a few studies suggested that homothallism was the ancestral state from which heterothallism evolved (Amselem et al. 2011; Galagan et al. 2005; Geiser et al. 1998; Williams et al. 1981). Despite the fact that there is greater support for the view that homothallism arose from heterothallism, there is evidence to support both hypotheses. It is also likely that the evolution of fungal mating systems has occurred in both directions and that it differs between fungal families. In the following sections we review the main findings of studies that have been conducted on the transition of fungal mating systems. We also provide arguments that the prevalence of evolution towards heterothallism has been largely underestimated.

Transition from heterothallism to homothallism

The molecular structures of the mating type loci, in combination with molecular phylogenies have been used to predict that heterothallism is the ancestral state and that heterothallism has evolved towards homothallism. Using these approaches, evidence for the transition from heterothallism to homothallism have often been documented and in different fungal taxa such as *Cochliobolus* (*Pleosporales*) (Yun et al. 1999), *Stemphylium* (*Pleosporales*)

(Inderbitzin et al. 2005), *Crivellia* (*Pleosporales*) (Inderbitzin et al. 2006) and *Neurospora* (*Sordariales*) (Gioti et al. 2012; Nygren et al. 2011).

Yun et al (1999) suggested that homothallism evolved from heterothallism in *Cochliobolus* after examining the structure of the mating type loci in heterothallic and homothallic species. They concluded that the structure of the *MAT* locus in the homothallic *Cochliobolus luttrellii* and *Cochliobolus homomorphus* resulted from crossing over causing separated *MAT* idiomorphs from heterothallic ancestors to fuse (Yun et al. 1999). In a similar way, a single fused *MAT* locus was consistently found in all homothallic *Stemphylium* spp. (Inderbitzin et al. 2005). The authors compared the *MAT* loci from homothallic and heterothallic *Stemphylium* spp. and suggested that there had been fusion of the inverted *MAT1-1* idiomorph with the *MAT1-2* idiomorph, followed by horizontal transfer across lineages, resulted in the observed homothallism in two separated *Stemphylium* clades (Inderbitzin et al. 2005).

Neurospora provides an excellent model genus to study the evolution of sexual reproduction because it includes species with diverse sexual reproduction strategies such as heterothallism, homothallism and pseudo-homothallism. Consequently, sexual reproduction and its genetic determinants has been well characterized in these fungi (Beatty et al. 1994; Gioti et al. 2012; Metzenberg and Glass 1990; Nygren et al. 2011; Wik et al. 2008). Using a combined phylogeny of seven nuclear gene regions, Nygren et al. (2011) suggested that the common ancestor of *Neurospora* was heterothallic and that homothallism has evolved at least six times independently in the genus. However, it is necessary to note that in this study, comprehensive phylogenetic analyses suggested a homothallic ancestral state for these fungi. Despite this fact, the authors argued for a common heterothallic ancestor in *Neurospora* based on biological aspects of the fungus and the fact that predominant transition from heterothallism to homothallism has been seen in other groups of fungi (Inderbitzin et al. 2005; Yun et al. 1999) and plants (Boris Igic et al. 2008; Stebbins 1957, 1974; Takebayashi and Morrell 2001). Interestingly, the direction of evolution from heterothallism to homothallism in *Neurospora* as suggested by Nygren et al. (2011) was supported in a subsequent study by Gioti et al. (2012). These authors reviewed the structures of the *MAT* loci in a number of *Neurospora* spp., and argued in favour of a heterothallic ancestor based on the observed conservative structure of distinct heterothallic

species when compared with the variability within the loci of homothallic species. Furthermore, they proposed translocation and unequal crossover of the *MAT* idiomorphs as mechanisms to acquire the ability to self in homothallic *Neurospora* species (Gioti et al. 2012).

The unusual organization of *MAT* loci in homothallic *Cochliobolus cymbopogonis* (Yun et al. 1999), *Aspergillus nidulans* (*Eurotiales*) (Galagan et al. 2005), *Neosartorya fischeri* (*Eurotiales*) (Rydholm et al. 2007) and *Neurospora sublineolata* (Gioti et al. 2012), suggests that these loci have a heterothallic origin. Rather than having a single *MAT* locus with the fused or closely linked *MAT1-1* and *MAT1-2* idiomorphs as seen in most homothallic ascomycetes, the *MAT1-1* and *MAT1-2* idiomorphs in these fungi are unlinked, and in some cases they occur on different chromosomes. It is highly likely that these organizations represent the evolution of homothallism from heterothallic ancestors by acquiring both *MAT* idiomorphs into a single genome. Galagan et al. (2005) proposed that the observed *MAT* loci in *A. nidulans* is a result of break and translocation of homothallic *MAT* idiomorphs from a homothallic ancestor. However, this is unlikely to be the case in *N. fischeri*, a fungus closely related to *A. nidulans*, because the *MAT1* and *MAT2* loci are located in the same genome but have flanking regions of the same origin: the *MAT1* locus is flanked by *APN1* (DNA lyase) and *SLA2* (cytoskeleton assembly control) genes, while the *MAT2* locus is flanked by partial copies of these genes (Rydholm et al. 2007). The fact that the *MAT1* and *MAT2* loci are flanked by sequences having a common origin strongly suggests that the organization of *MAT1* and *MAT2* loci observed in *N. fischeri* resulted from the translocation of *MAT* idiomorphs from heterothallic ancestors. Rydholm et al. (2007) suggested that the same mechanism has led to homothallism in *A. nidulans*, and that heterothallism was thus the ancestral state in *Aspergillus* section *Fumigati*.

Transition from homothallism to heterothallism

In contrast to the suggested evolution towards selfing, the transition from homothallism to heterothallism has also been suggested. For example, Galagan et al. (2005) compared the structures of *MAT* loci in homothallic *A. nidulans* and heterothallic *A. fumigatus* and *A. oryzae* and suggested a homothallic origin. They thus proposed an evolutionary model that explains the observed structure of *MAT* loci in *A. nidulans*, *A. fumigatus* and *A. oryzae* based on a common heterothallic ancestor (Galagan et al. 2005). Their key argument was that a

fragment of the HMG gene belonging to *MAT1-2* idiomorph was found neighbouring the *A. fumigatus* *MAT1-1* idiomorph and that this would suggest the transition from homothallism to heterothallism by gene loss (Galagan et al. 2005; Paoletti et al. 2005). The presence of partial sequences of the *MAT1-1* genes on the *MAT1-2* idiomorphs or vice versa has also been seen in other fungi such as *Cordyceps takaomontana* (Yokoyama et al. 2003) and *Diaporthe* spp. (Kanematsu et al. 2007) and these would also best fit a model where there has been a transition from homothallism to heterothallism.

In a subsequent study, Rydholm et al. (2007) proposed a reversed direction of evolution, from heterothallism to homothallism for *Aspergillus* section *Fumigati* as well as other species of *Aspergillus*. This is also consistent with a suggestion by Fraser et al. (2007) that *Onygenales* and aspergilli are likely to have a common heterothallic ancestor. We support the view that there has been a transition from heterothallism to homothallism in the case of *N. fischeri* and *A. nidulans* as discussed above. However, a common heterothallic ancestor would not explain the presence of partial segments of the *MAT1-2* gene flanking the *MAT1-1* idiomorph, which appear to be a signature of incomplete deletion suggesting evolution in the opposite direction. It is possible that in *Aspergillus*, the transition between homothallism and heterothallism occurred in both directions. A common homothallic ancestor from which heterothallic lineages arose would best explain the situation for this genus. Some re-conversion to homothallism could then have occurred later through acquisition of the homothallic *MAT* idiomorphs into a single genome such as in *N. fischeri* and *A. nidulans* (as illustrated in Fig. 1).

The evidence for a transition from a homothallic to heterothallic mating system has also been documented in a comparative genomic study of the necrotrophic fungal pathogens *Sclerotinia sclerotiorum* and *Botrytis cinerea* (*Helotiales*) (Amselem et al. 2011). By comparing the genetic structures of the *MAT* locus of these two closely related species with opposite mating systems, evidence for the transition from homothallism to heterothallism was found. *Sclerotinia sclerotiorum* had the typical structure of a homothallic species with genes encoding for the alpha and HMG domains located at a single locus, whereas *B. cinerea* had the typical structure of a heterothallic species with different *MAT* dimorphs occupying the same position in the genome of different haploid isolates. Interestingly, the fragments of the *MAT1-1-1* and *MAT1-2-1* genes were respectively found on the *MAT1-2* and *MAT1-1*

idiomorphs in *B. cinerea*. This presents clear evidence supporting the evolution towards heterothallism from a homothallic ancestor by gene loss (Amselem et al. 2011).

Altering sexual compatibility through genetic manipulation of fungal mating systems

Experimental studies have shown that it is possible to convert mating systems in fungi by genetic manipulation of the *MAT* locus. By transforming the fused *MAT* locus of *C. luttrellii* into a *MAT*-deleted *C. heterostrophus* isolate, it was possible to confer selfing in this originally heterothallic species (Yun et al. 1999). This suggested that the machineries for sexual reproduction are fully available in heterothallic *C. heterostrophus* and only the exchange of *MAT* genes would be needed to alter the sexual mode of the fungus. This work also showed that it is possible for a heterothallic species to become homothallic by acquiring both *MAT* idiomorphs in a single genome.

Lu et al. (2011) showed that it was possible to convert a homothallic *C. luttrellii* strain into heterothallic strains by expressing either *MAT1-1-1* or *MAT1-2-1* from a heterothallic *C. heterostrophus* in *MAT*-deleted homothallic *C. luttrellii* isolates. The *C. luttrellii* transformants carrying opposite *MAT* genes were able to mate and produce perithecia with the same level of fertility when compared with that of a selfing wild type. Together, the studies by Yun et al. (1999) and Lu et al. (2011), provided proof that it is possible to artificially convert a heterothallic strain into one that is homothallic, or conversely to convert a homothallic strain to the heterothallic state by altering the structure of the *MAT* locus. In another similar study (Lee et al. 2003) used targeted manipulation of the *MAT* locus and converted the homothallic *Gibberella zeae* (*Hypocreales*) to produce strains of the fungus that were strictly heterothallic. Deletion of *MAT1-1* or *MAT1-2* from the homothallic strains resulted in self-sterile *MAT1-1* or *MAT1-2* deleted strains, and these strains of complementary mating type were able to cross with each other to produce perithecia with viable ascospores.

The *MAT* locus represents the “master regulator” of sexual reproduction. Thus the rearrangement of this locus will impact on sexual compatibility that can lead to the transition between reproductive modes. The possibility to switch between fungal mating systems by empirical studies also suggests that it is possible for evolution to have occurred in both directions.

The uncertainty of arguments supporting transition from heterothallism to homothallism

Although it is clear that homothallic fungi are abundant throughout the fungal kingdom, the question as to whether this reflects a favourable trait and thus whether evolution towards homothallism would be favoured in fungi remains a controversial topic. In most cases, the arguments for this direction of evolution could be biased and subjective and they usually reflect only a specific fungal lineage or they are restricted to small numbers of species.

Reconstructing ancestral states using phylogeny making possible inferences regarding the evolution of sexual reproduction has been useful in various studies (Inderbitzin et al. 2005; Inderbitzin et al. 2006; Nygren et al. 2011; Yun et al. 1999). It is, however, important to emphasize that this approach can also lead to biased conclusions for the following reasons:

(1) The advancement in phylogenetic analyses utilising multiple gene regions has improved the accuracy of phylogenetic studies. However, the suggested phylogenetic relatedness of specific groups often changes when different gene regions are used. Care should thus be taken when the direction of evolution of mating systems is predicted based solely on sister clades inferred in a phylogeny, especially where no supporting evidence is presented; (2) It is widely accepted that only a single common ancestral state is presumed at each node of a phylogeny. However, the possibility that there is more than one ancestral state at any given node being both homothallic and heterothallic cannot be excluded; (3) The most parsimonious transition that involves the least switching between reproductive modes is often used to argue for an ancestral state in a given phylogeny. However, as long as a particular reproductive mode is considered as being advanced or favourable, the transition towards it would be more likely to have multiple occurrences in the evolutionary history and therefore does not need to conform to the most parsimonious transition.

There have been assumptions that evolution toward homothallism in fungi is likely because it is in accordance with the evolution toward selfing that has been suggested for plants (Barrett 2002; Boris Igic et al. 2008; Stebbins 1974; Takebayashi and Morrell 2001). This direction of argument is not applicable because there are major biological and ecological differences between members of these two Kingdoms. Selfing in higher plants offers assurance in reproduction. Although some plant species can also produce asexually, this is at a moderate level and far less effective when compared to asexual reproduction in the fungi. In contrast, most fungal species are capable of reproducing asexually very efficiently

and this can be in addition to sexual reproduction. Therefore, reproduction in fungi is always ensured without the need to evolve towards selfing.

The other mechanism of conferring homothallism is by acquiring both *MAT* idiomorphs into a single genome at different locations. This has only been observed in *C. cymbopogonis*, *A. nidulans*, *N. fishcheri* and *N. sublineolata* (Galagan et al. 2005; Gioti et al. 2012; Rydholm et al. 2007; Yun et al. 1999). If homothallism is the favourable trait, acquiring homothallism by this mechanism should have been observed with much higher frequency throughout the fungal Kingdom, especially given the high rate of horizontal gene transfer in the fungi (Fitzpatrick 2012). Although these cases strongly suggest transition from heterothallism to homothallism, they are atypical and should not be used to make inferences on the overall evolution of fungal mating systems.

Evolution towards heterothallism

The structure and gene elements of the *MAT* locus determine the sexual compatibility in fungi and an alteration in structure can lead to a change in the reproductive strategy. From a practical point of view, it is possible for the *MAT* idiomorphs to be joined (by means of unequal cross over - giving rise to homothallism) or separated (by chromosome break or gene deletion, following by segregation - giving rise to heterothallism). While it is possible for a change at the *MAT* locus to occur either way, the important question must be which way would confer greatest benefit to the fungi. From our perspective, evolution of the fungal reproductive system is dynamic and the transition between homothallism and heterothallism could occur in either direction and this is clearly different in different fungi. Even though most studies support the view that such evolution has moved from heterothallism to homothallism (Fraser et al. 2007; Gioti et al. 2012; Inderbitzin et al. 2005; Lu et al. 2011; Nauta and Hoekstra 1992; Nygren et al. 2011; Rydholm et al. 2007; Yun et al. 1999), we believe that there are also strong reasons to favour the view that evolution has been in the direction of heterothallism.

The biological perspective

Sexual reproduction has evolved because of the advantage it confers in generating high levels of genetic variation. In this respect, heterothallism is far more effective than homothallism. With the involvement of different mating partners followed by meiosis;

heterothallism facilitates mixing and the combination of new beneficial traits into the progeny, thus increasing fitness to survive in to the environment. In contrast, homothallism, especially in organisms that have predominantly haploid lifestyles such as the fungi, facilitates inbreeding and provides no advantage over sexual reproduction in terms of generating genetic variation.

The pervasiveness of heterothallism throughout fungal kingdom emphasizes the advantages of this reproductive strategy in response to selection pressures. It is also likely that most of so-called asexual fungi have heterothallic mating systems, as has already been shown to be the case for several well-known asexual species (Horn et al. 2009; Kück and Pöggeler 2009; O’Gorman et al. 2008; Seidl et al. 2009). This would further increase the prevalence of heterothallic fungi when compared to those that are homothallic. Considering the long term evolutionary history of the fungal mating system, it is understandable that heterothallism is selected and maintained due to its advantage over homothallism.

From an ecological point of view, homothallism would benefit well adapted individuals, enabling them to reproduce and proliferate rapidly in order to occupy new environments. However, regarding adaptation to environmental changes, where high levels of diversity will be more beneficial, homothallic populations would be at a disadvantage when compared to heterothallic populations. In such cases, homothallic populations might face extinction. This is also consistent with what has been suggested in plants; that transitions towards self compatibility results in the extinction of selfing lineages (Boris Igic et al. 2008; Takebayashi and Morrell 2001). Therefore heterothallism would offer greater advantages to fungi in the e long term.

The practical perspective

The structure of the *MAT* locus in fungi is best explained by evolution from homothallism to heterothallism. Despite the differences in structure and gene contents, the *MAT* loci of closely related homothallic and heterothallic species reside at a conserved location in the genome, flanked by *SLA2* and *APN2* genes in most cases. This observation is best explained as a result of evolution from homothallism to heterothallism by gene loss. Although it is possible that unequal crossover from heterothallic predecessors could result in the co-presence of the *MAT* idiomorphs in the homothallic species as has been proposed by some studies (Gioti et al. 2012; Yun et al. 1999), such evidence has not available for most other

homothallic species. On the other hand, gene loss is observed with high frequency in the *MAT* locus and as such produces a high level of variability in the composition of genes in the *MAT* idiomorphs. The gene composition of the *MAT* idiomorphs often varies between genera (Kanematsu et al. 2007) and in some cases between species of the same genus (Butler et al. 2009; Gioti et al. 2012). Except for the mandatory *MAT1-1-1* encoding alpha domain and *MAT1-2-1* encoding HMG domain, the presence of other *MAT* genes, e.g. *MAT1-1-2* and *MAT1-1-3*, seems to be optional and variable. We thus find it difficult to explain why these genes were gained from elsewhere into the *MAT* locus, rather than that they were deleted in those lacking them. In this way, heterothallism could result from the loss of *MAT1-1-1* or *MAT1-1-2* from the *MAT* idiomorphs of homothallic ancestors. The presence of truncated ends or segments of *MAT-1* genes on the *MAT1-2* idiomorphs (Amselem et al. 2011; Seidl et al. 2009; Yokoyama et al. 2003) or vice versa (Amselem et al. 2011; Galagan et al. 2005; Paoletti et al. 2005) as has been observed in *MAT* loci of many heterothallic species residing in different genera, reinforces this possibility as this is best explained by incomplete deletion of these genes from ancestral homothallic *MAT* loci.

Concluding remarks

Whether homothallism or heterothallism presents an ancestral state represents one of the most active debates in fungal biology, yet it remains largely unanswered. Theoretically it is possible for transition to occur in both directions by the separation or joining of the *MAT* idiomorphs as has been shown in practical genetic manipulation studies. Studies on the structure of the *MAT* locus in distantly related fungal groups have also provided evidence to support these transitions occurring in nature.

Transitions between homothallism and heterothallism represent a dynamic process and it is one that could happen in both directions, and in certain cases can occur simultaneously in the same genus. Most studies on the fungal mating system have shown evidence or raised arguments supporting the view that heterothallism represents the common ancestral state. It is, however, possible, and in our opinion more likely, that the transition from homothallism to heterothallism was the major switch that shaped the evolution of mating systems in fungi.

Clearly the possibility of evolution of the mating system from homothallism to the heterothallic state has largely been underestimated. Additional and more intensive studies

considering the structures of the *MAT* locus in closely and distantly related heterothallic and homothallic species will be required to resolve this intriguing question.

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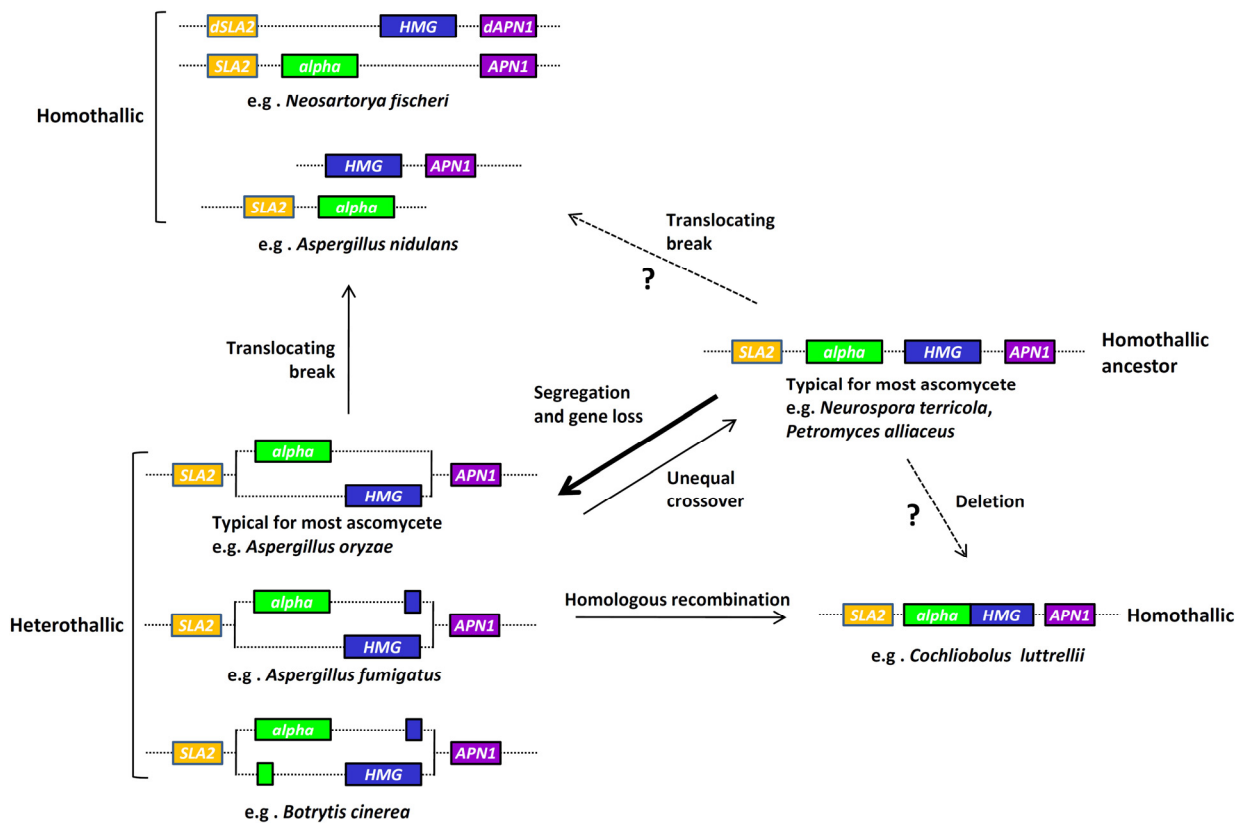


Fig. 1. The dynamic of transitions between heterothallism and homothallism occur in the fungal kingdom. The main directions suggested are presented in a thick continuous arrow. Other minor directions are marked with thin continuous arrows. Interrupted arrows with question marks indicate uncertainty but possible directions. The flanking cytoskeleton assembly control gene (*SLA2*) and DNA lyase gene (*APN1*) are presented flanking the *MAT* locus as commonly found in ascomycete, however in some atypical cases they are absent or distantly located from the *MAT* locus.

Chapter 2

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Phylogeny and taxonomy of species in the *Grosmannia serpens* complex.
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Phylogeny and taxonomy of species in the *Grosmannia serpens* complex

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Abstract: *Grosmannia serpens* was first described from pine in Italy in 1936 and it has been recorded subsequently from many countries in both the northern and southern hemispheres. The fungus is vectored primarily by root-infesting bark beetles and has been reported to contribute to pine-root diseases in Italy and South Africa. The objective of this study was to consider the identity of a global collection of isolates not previously available and using DNA sequence-based comparisons not previously applied to most of these isolates. Phylogenetic analyses of the ITS2-LSU, actin, beta-tubulin, calmodulin and translation elongation factor-1 alpha sequences revealed that these morphologically similar isolates represent a complex of five cryptic species. *Grosmannia serpens* sensu stricto thus is redefined and comprises only isolates from Italy including the ex-type isolate. The ex-type isolate of *Verticicladiella alacris* was shown to be distinct from *G. serpens*, and a new holomorphic species, *G. alacris*, is described. The teleomorph state of *G. alacris* was obtained through mating studies in the laboratory, confirming that this species is heterothallic. Most of the available isolates, including those from South Africa, USA, France, Portugal and some from Spain, represent *G. alacris*. The remaining three taxa, known only in their anamorph states, are described as the new species *Leptographium gibbsii* for isolates from the UK, *L. yamaokae* for isolates from Japan and *L. castellanum* for isolates from Spain and the Dominican Republic.

Key words: bark beetle associates, *Grosmannia alacris*, insect/fungus symbiosis, *Leptographium cas-*

tellanum, *L. gibbsii*, *L. yamaokae*, multigene phylogeny, Ophiostomatales, pine-root disease

INTRODUCTION

Species of *Leptographium* Lagerb. & Melin are anamorphic Ascomycota that are characterized generally by dark single and erect conidiophores terminating in complex conidiogenous apparatuses (Jacobs and Wingfield 2001, Kendrick 1962). *Leptographium* is well adapted for insect dispersal by producing conidia in slimy droplets at the apices of these conidiophores. More than 20 species of *Leptographium* are known to have teleomorphs accommodated in the genus *Grosmannia* Goid. (Zipfel et al. 2006), but the sexual states are not known for at least an additional 50 species.

Leptographium and *Grosmannia* have a worldwide distribution and the majority are known from conifer hosts (Grosmann 1931, Harrington 1988, Jacobs and Wingfield 2001, Paciura et al. 2010, Wingfield et al. 1988). Only a few species have been described from non-conifer hosts (Jacobs et al. 2006, Jacobs and Wingfield 2001, Paciura et al. 2010). It is widely believed that most *Leptographium* are native to the northern hemisphere where conifers are most common and that they were introduced to other parts of the world with their insect vectors (Harrington 1988, Jacobs and Wingfield 2001, Kendrick 1962, Wingfield et al. 1988, Zhou et al. 2001).

The majority of *Leptographium* species are saprophytes that cause blue stain of timber (Jacobs and Wingfield 2001, Seifert 1993). However some *Leptographium* can cause diseases of trees that result in serious economic losses (Wingfield et al. 1988). One of the best examples is black-stain root disease caused by the three varieties of *L. wageneri* (W.B. Kendr.) M.J. Wingf. (Cobb 1988, Wagener and Mielke 1961). Other *Leptographium* species, such as *L. terebrantis* S.J. Barras & T.J. Perry and *L. procerum* (W.B. Kendr.) M.J. Wingf., have been associated with disease syndromes, although they are most likely not the primary cause of disease (Alexander et al. 1988, Jacobs and Wingfield 2001, Morrison and Hunt 1988, Wingfield et al. 1988). Of note, *L. procerum* recently was introduced to China with the red turpentine beetle *Dendroctonus valens* and this association has resulted in dramatic death of trees, which is atypical of the insect or fungus in their native North America (Lu et al. 2009a, b).

Grosmannia serpens Goid. first was described associated with a root disease of pines in Italy (Goidánich 1936, Lorenzini and Gambogi 1976),

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and it has been linked more recently to pine decline in USA (Eckhardt et al. 2007). In 1980 a disease similar to the one associated with *G. serpens* in Italy (Goidánich 1936) was reported on *P. radiata* and *P. pinaster* in South Africa and the causal agent was described as *Verticicladiella alacris* M.J. Wingf. & Marasas (Wingfield and Knox-Davies 1980, Wingfield and Marasas 1980). Based on comparisons of the morphological characters of *V. alacris* with the ex-type isolate of *G. serpens*, *V. alacris* subsequently was reduced to synonymy with *L. serpens* (Goid.) Siemaszko, the anamorph of *G. serpens* (Wingfield and Marasas 1981). Apart from Italy and South Africa *L. serpens* also has been recorded in USA, France, UK, Portugal, Spain and the Dominican Republic (Eckhardt et al. 2007, Harrington 1988, Jacobs and Wingfield 2001, Masuya et al. 2009, Wingfield and Gibbs 1991).

The many reports of *L. serpens* and *L. serpens*-like isolates (including those identified as *V. alacris*) and their association with a wide variety of hosts and insects has raised questions regarding their identity. The objective of this study was to reconsider the identity of isolates originating from all the countries from which the fungus has been reported, applying comparisons of DNA sequences for five gene regions together with morphological characteristics.

MATERIALS AND METHODS

Isolates.—Those used in this study are included herein (TABLE I).

Morphology.—Anamorphs were described from malt extract agar (MEA; 2% malt extract and 2% agar, Biolab, Midrand, South Africa). Agar blocks colonized by fungal isolates were transferred to 2% water agar, and heat-sterilized pine twigs were placed on the agar surface to promote conidiophore formation. Plates were incubated at 25 C in the dark 2–3 wk.

Mating studies.—Based on the production of anamorph structures as well as on geographic locations, 10 isolates grouping in the phylogenetic analyses with the ex-type culture of *V. alacris* were selected for mating studies (TABLE I). In addition all isolates from the other four lineages were used in the mating studies. Isolates were crossed with the technique described by Grobbelaar et al. (2010) but using pine twigs instead of hardwood twigs. Plates were incubated at 25 C in the dark for up to 3 mo and examined regularly for the presence of ascospores.

Microscopy.—Conidiophores and ascospores produced on pine twigs were mounted on microscope slides in 80% lactic acid. Microscopy was done as described by Kamgan Nkuekam et al. (2011). Colors were described with the charts of Rayner (1970).

Growth studies.—Optimal temperatures of all species were determined on MEA. Agar disks were removed from the actively growing edges of 5 d old MEA plates with a 5 mm cork borer and were placed mycelium side down in the

centers of 90 mm plates containing MEA. Plates were incubated in the dark at 5–35 C at 5 C intervals. Colony diameters were measured daily at a fixed time and the measurements were discontinued when the mycelium reached the edges of the plates. Where possible three isolates per taxon with five replications at each temperature were included in the study. Mean colony diameters ($\pm$ standard deviation) were determined. Tolerance to cycloheximide was tested by transferring each isolate to MEA containing 0.5 g/L cycloheximide. Incubation was at 25 C and measurements were done as described above.

DNA extraction, PCR and DNA sequencing.—Mycelium (ca. 100 mg) grown in YM broth (2% malt extract and 0.2% yeast extract, Biolab, Midrand, South Africa) was placed in 1.5 mL Eppendorf tubes, to which 50 μ L PrepMan Ultra reagent (Applied Biosystems, Foster City, California) had been added. The tubes were heated 5 min at 100 C after which a micro pestle was used to grind the mycelium. After an additional 5 min at 100 C the mixture was centrifuged 5 min at 12000 rpm. The supernatant was collected and diluted five times with 10 mM Tris-HCl pH 8.0. Of the diluted DNA solution, 2 μ L was used as template for PCR reactions. The remainder was stored at –20 C.

Five gene regions were amplified for sequencing and phylogenetic analysis. The internal transcribed spacer region 2 and partial large subunit (ITS2-LSU) of the ribosomal DNA was amplified with primers ITS3 and LR5 (White et al. 1990). Part of the actin gene (ACT) was amplified with primers Lepact F and Lepact R (Lim et al. 2004). A portion of the β -tubulin gene (β T) was amplified with primers T10 (O'Donnell and Cigelnik 1997) and Bt2b (Glass and Donaldson 1995), and part of the translation elongation factor-1 alpha (TEF-1 α) was amplified with primers EF1-F and EF2-R (Jacobs et al. 2004). PCR with the calmodulin (CAL) primers CL1 and CL2a (O'Donnell et al. 2000) resulted in low amplicon concentrations for the isolates from Japan, while no amplification was obtained from the DNA of the other isolates. Novel CAL primers (CL2F [5'-GACAAGGAYG-GYGATGGT-3'] and CL2R [5'-TTCTGCATCATGAGYTG-SAC-3']) thus were designed based on the genome sequence of *Grosmanella clavigera* (Rob.-Jeffr. & R.W. Davidson) Zipfel et al. (GenBank accession number ACYC01000232), *Neurospora crassa* Shear & B.O. Dodge (GenBank accession number AL807366) and partial CAL sequences of *Sporothrix schenckii* Hektoen & C.F. Perkins (GenBank accession number AM117437). These primers amplified the fragment spanning from the second exon to the last exon of the CAL gene. In cases where amplification was difficult, CL2R2 (5'-CTTCTCGCCATSGAGTCAT-3') was used in combination with CL2F, but this combination provided shorter fragments.

Reaction mixtures, 25 μ L total volume, consisted of 2.5 μ L 10 $\times$ PCR reaction buffer, 2.5 mM MgCl₂, 200 μ M each dNTP, 0.2 μ M each primer (0.4 μ M each primer was used in the case of the CAL degenerate primers), 1 U FastStart Taq DNA Polymerase (Roche Applied Science, Mannheim, Germany) and 2 μ L diluted genomic DNA solution. Amplifications were performed in an Eppendorf MasterCycler[®] gradient (Eppendorf, Hamburg, Germany) under these conditions: an initial denaturation step at 95 C for

TABLE I. Fungal isolates used in this study

Species	Isolate number		Mating type	Host	Origin	GenBank accession numbers				
	^a CMW	^b Other				ITS2-LSU	ACT	βT	CAL	TEF-1 α
<i>G. alacris</i>	^c CMW 60 = CMW 2844	CBS 591.79	A	<i>Pinus pinaster</i>	South Africa	JN135313	JN135320	JN135329	JN135296	JN135304
	CMW 310		NT	<i>P. radiata</i>	South Africa		= JN135321	= JN135327	= JN135296	= JN135304
	^T CMW 621	CBS 128830	A	<i>P. pinaster</i>	Portugal		JN135318	JN135327	= JN135296	JN135305
	^T CMW 623	CBS 118621	B	<i>P. pinaster</i>	Portugal		= JN135321	= JN135327	= JN135295	JN135306
	CMW 746		A	<i>P. pinaster</i>	France		= JN135321	= JN135329	= JN135296	= JN135302
	CMW 1136	C 153	A	<i>P. taeda</i>	USA		JN135321	JN135328	JN135295	JN135302
	CMW 1137	C 169	A	<i>P. strobus</i>	USA		JN135319	= JN135327	= JN135296	JN135303
	CMW 6187		A	<i>Hylastes angustatus</i>	South Africa		= JN135321	= JN135329	= JN135296	= JN135304
	CMW 6188		A	<i>H. angustatus</i>	South Africa		= JN135321	= JN135329	= JN135296	= JN135304
	CMW 7700		NT	<i>H. angustatus</i>	South Africa		= JN135321	= JN135329	= JN135296	= JN135304
<i>G. serpens</i>	CMW 7726		NT	<i>H. angustatus</i>	South Africa		= JN135321	= JN135329	= JN135296	= JN135304
	CMW 25936		A	<i>P. pinaster</i>	Spain		= JN135318	= JN135327	= JN135295	= JN135303
	CMW 25937		A	<i>P. pinaster</i>	Spain		= JN135318	= JN135327	= JN135295	= JN135303
	CMW 191			<i>P. pinea</i>	Italy		= JN135325	= JN135334	= JN135300	= JN135307
	CMW 192			<i>P. pinea</i>	Italy		= JN135325	= JN135334	= JN135300	= JN135307
	CMW 289			<i>P. pinea</i>	Italy		= JN135325	= JN135334	= JN135300	= JN135307
	CMW 290	CBS 641.76		<i>P. pinea</i>	Italy		= JN135325	= JN135334	= JN135300	= JN135307
	^T CMW 304 = CMW 305	CBS 141.36		<i>P. sylvestris</i>	Italy	JN135314	JN135325	JN135334	JN135300	JN135307
	CMW 1988			<i>Hylurgus</i> sp.	Spain		= JN135324	= JN135333	= JN135299	= JN135310
	CMW 1989			<i>Hylurgus</i> sp.	Spain		= JN135324	= JN135333	= JN135299	= JN135310
<i>L. castellanum</i>	CMW 2320	CBS 128698		<i>P. occidentalis</i>	Dominican Republic		= JN135324	= JN135333	= JN135299	= JN135310
	^T CMW 2321	CBS 128697		<i>P. occidentalis</i>	Dominican Republic	JN135317	JN135324	JN135333	JN135299	JN135310
	CMW 853	CBS 347.90		<i>H. ater</i>	UK		= JN135330	= JN135297	= JN135312	= JN135322
	^T CMW 1376	CBS 128695		<i>H. ater</i>	UK	= JN135316	JN135322	JN135330	JN135297	JN135308
	CMW 36371			<i>H. opacus</i>	UK	= JN135322	= JN135330	= JN135297	= JN135312	= JN135322
	CMW 2079	CBS 168.93		<i>P. ponderosa</i>	USA	AY553382	JN135326	AY534930	JN135301	AY536176
	CMW 1935			<i>Pinus</i> sp.	Japan		= JN135323	JN135331	= JN135298	JN135309
	CMW 1944	CBS 128696		<i>Pinus</i> sp.	Japan		= JN135323	= JN135331	= JN135298	= JN135309
	^T CMW 4726	CBS 129732		<i>P. densiflora</i>	Japan	JN135315	JN135323	JN135332	JN135298	JN135311
	CMW 4727			<i>P. densiflora</i>	Japan		= JN135323	= JN135332	= JN135298	= JN135311
<i>L. neomexicanum</i> <i>L. yamaokae</i>	CMW 4728			<i>P. densiflora</i>	Japan		= JN135323	= JN135332	= JN135298	= JN135311
	CMW 4729			<i>P. densiflora</i>	Japan		= JN135323	= JN135332	= JN135298	= JN135311

^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 ; C = Culture Collection of TC Harrington, Department of Plant Pathology, Iowa State University, Ames, Iowa, USA.

^c Ex-type isolate of *Verticillium dactyliophila* alacris.

^T = ex-type isolates.

TABLE II. Parameters used and statistical values resulting from the different phylogenetic analyses of individual datasets

Dataset →		ITS2-LSU	ACT	βT	CAL	TEF-1α	Combined
Number of characters	Total	632	815	365	657	740	2577
	Variable	60	7	12	31	34	84
	Constant	405	798	339	614	687	2438
MP	PIC	167	10	14	12	19	55
	Number of trees	36	1	2	4	3	7
	Tree length	634	11	23	18	62	118
	CI	0.621	0.914	0.921	0.899	0.945	0.883
	HI	0.379	0.087	0.078	0.101	0.055	0.117
	RI	0.907	0.986	0.981	0.984	0.99	0.979
	RC	0.563	0.901	0.908	0.885	0.936	0.865
ML & BI	Substitution model	TrN + G	TrN + G	TrN	TrN + G	TrN + G	TrN + G
	Gamma	0.214	0.011	—	0.286	0.25	0.013
BI	Burn-in	250	300	300	300	300	500

PIC = number of parsimony informative characters; CI = consistency index; HI = homoplasy index; RI = retention index; Subst. model = best fit substitution model; Gamma = Gamma distribution shape parameter.

5 min, followed by 35 cycles of 95 °C for 30 s, 55 °C annealing for 30 s, 72 °C extension for 60 s and a final extension step at 72 °C for 8 min. The sequencing was done with both forward and reverse primers as used in PCR. Products were purified with High Pure PCR Product Purification Kit (Roche Applied Science, Mannheim, Germany) following the manufacturer's protocols, sequenced with the Big Dye® Terminator 3.1 cycle sequencing premix kit (Applied Biosystems, Foster City, California) employing the forward and reverse primers in PCR and analyzed on an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems). Consensus sequences were constructed with ContigExpress, a component of Vector NTI Advance 11 (Invitrogen, Carlsbad, California).

Phylogenetic analyses.—To examine the relatedness of *G. serpens*-like isolates with other species of *Grossmannia* and *Leptographium* ITS2-LSU sequences were compared with sequences of 66 species obtained from GenBank. For the final ITS2-LSU dataset only one isolate representing each of the *G. serpens*-like groups was included. Sequences of six species of *Ophiostoma* were used as outgroup. The ITS2 and LSU data were separated from each other and a partition homogeneity test (PHT) was conducted on the two datasets to test the congruence of the two regions with PAUP* 4.0b10 (Swofford 2003).

For the protein-coding genes (ACT, βT, CAL and TEF-1α) sequences of the *G. serpens*-like isolates were analyzed with *L. neomexicanum* M.J. Wingf., T.C. Harr. & Crous (CMW 2079) as outgroup, which was selected based on analyses of the ITS2-LSU data. Alignments were done with an online version of MAFFT 6 (Katoh and Toh 2008). To examine the possibility of combining ACT, βT, CAL and EF-1α data a PHT was conducted with PAUP* 4.0b10 (Swofford 2003). A heuristic search of 1000 replications was performed using the same software with parsimony default settings. Sequences for the protein-coding genes were analyzed separately as well as in a combined dataset. All datasets were subjected to maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI) analyses as described below.

MP analyses were performed with PAUP* 4.0b10 (Swofford 2003). One thousand random stepwise addition heuristic searches were performed with tree-bisection-reconnection

(TBR) as the branch-swapping algorithm. All characters were unordered and of equal weight. Gaps were excluded. Branches of zero length were collapsed, and all equally parsimonious trees were saved. The robustness of resultant trees was evaluated by 1000 bootstrap replications. Tree length (TL), consistency index (CI), retention index (RI), homoplasy index (HI) and rescaled consistency index (RC) were calculated for the resulting trees.

ML analyses were performed with an online version of PhyML 3.0 (Guindon and Gascuel 2003). The best fit substitution models were determined with jModelTest 0.1.1 (Posada 2008). Confidence supports were estimated with 1000 replication bootstrap analyses.

BI analyses employing a Markov chain Monte Carlo method (MCMC) were performed with MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). Evolutionary models were determined with jModelTest 0.1.1 (Posada 2008) and manually converted to MrBayes models. Four MCMC chains were run simultaneously from a random starting tree for 5 000 000 generations. Trees were sampled every 100th generation. After the runs burn-in values were determined with Tracer 1.4 (Rambaut and Drummond 2007). Trees sampled at burn-in were discarded, and posterior probabilities were calculated from a majority rule consensus tree regenerated from the remaining trees.

RESULTS

Phylogenetic analyses.—The number of characters, the substitution models used and other statistical values resulting from the different analyses of the respective datasets are presented (TABLE II). The LSU sequences of our isolates were identical, apart from the three UK isolates that differed by 1 bp. The ITS2 sequences of the UK isolates also differed by 2 bp from the other *G. serpens*-like isolates, and one of these differences was shared with isolates from Spain and the Dominican Republic. Because the PHT did not show that the nuclear rDNA datasets were incongruent ($P = 0.05$)

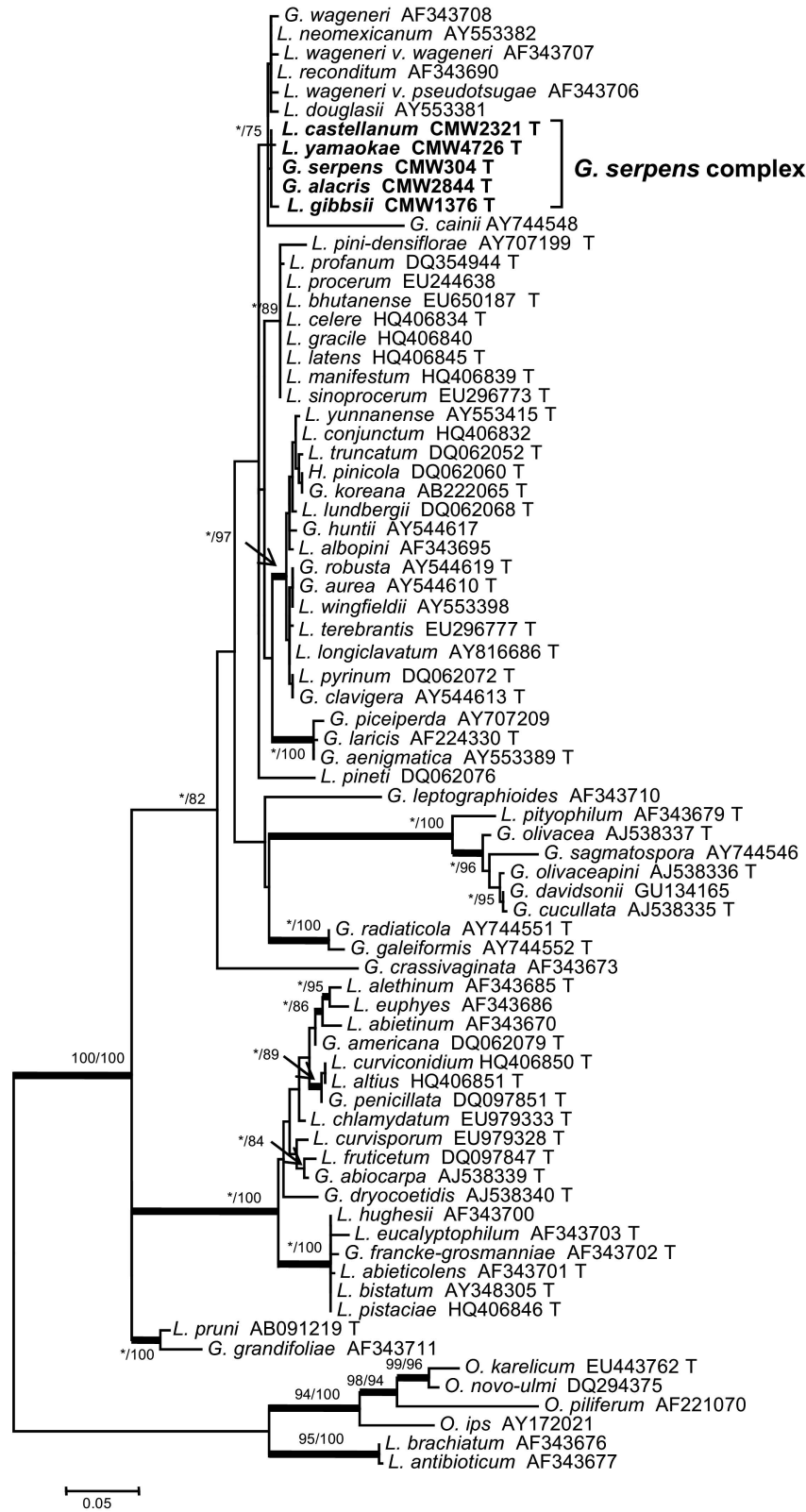


FIG. 1. ML tree derived from analysis of ITS2-LSU dataset. Bold branches have posterior probabilities ≥ 95 . Bootstrap values above 75% are indicated at nodes as ML/MP. * = bootstrap values < 75%.

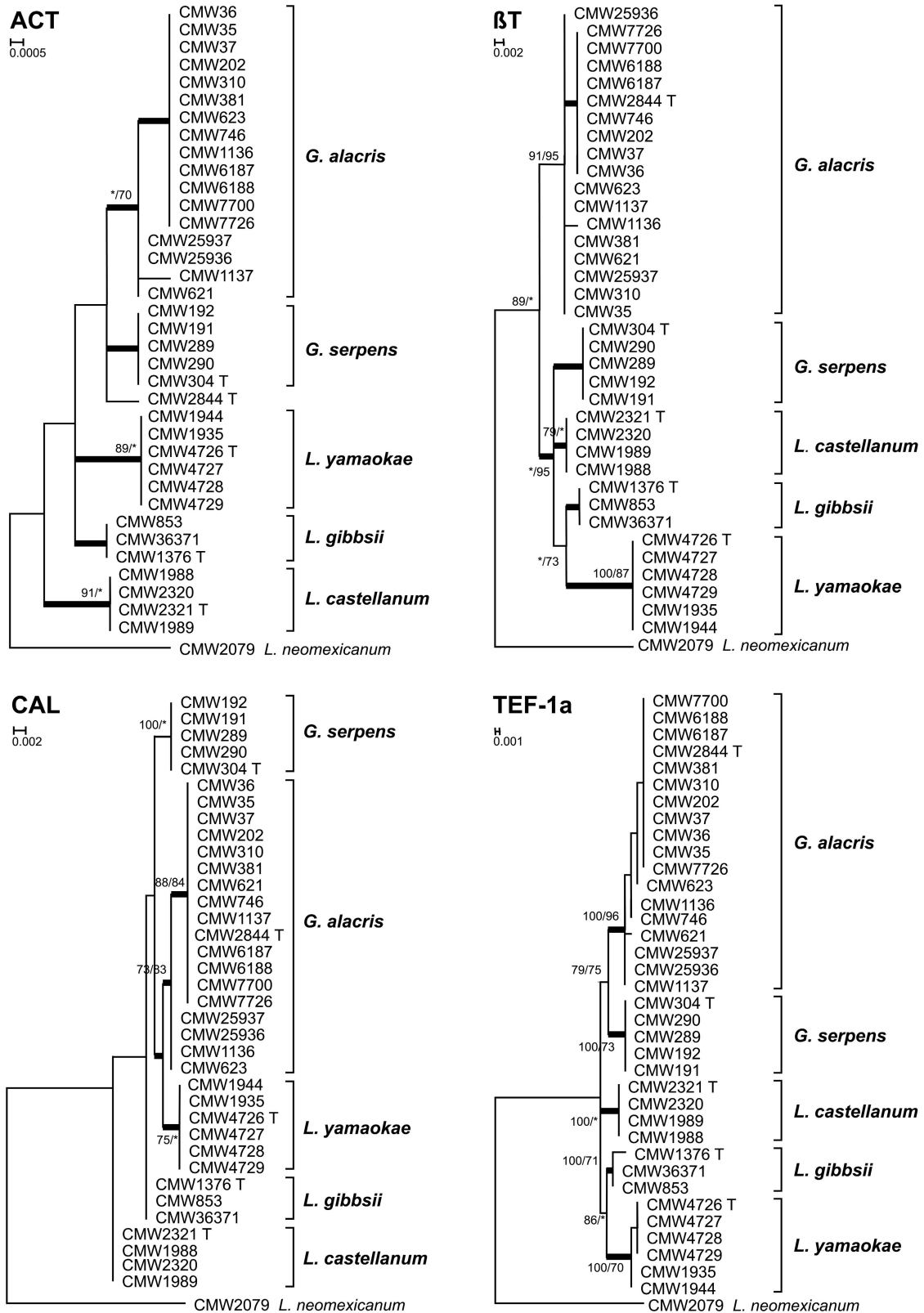


FIG. 3. ML trees derived from analyses of ACT, β T, CAL and EF-1 α datasets. Bold branches have posterior probabilities $\geq$ 90. Bootstrap values above 70% are indicated at nodes as ML/MP. * = bootstrap values < 70%.

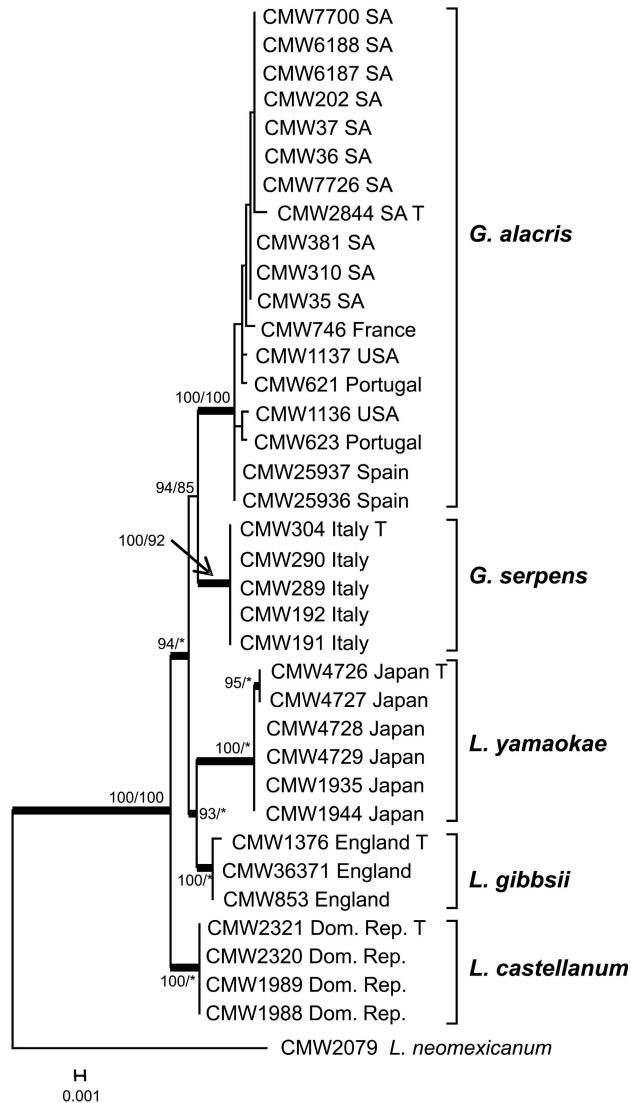


FIG. 4. ML tree derived from analysis of combined data set (ACT, β T, CAL and EF-1 α). Bold branches have posterior probabilities ≥ 99 . Bootstrap values above 75% are indicated at nodes as ML/MP. * = bootstrap values < 75%.

thus represented known taxa for which the descriptions are emended and the nomenclature is updated. The remaining three lineages represented novel taxa that we propose as new species.

Grosmannia serpens Goid., Boll. Staz. Patol. Veg. 16:27. 1936.

FIG. 5

= *Ophiostoma serpens* (Goid.) Arx, Antonie van Leeuwenhoek 18:211. 1952.

= *Ceratocystis serpens* (Goid.) C. Moreau, Rev. Mycol. (Paris), Suppl. Colon. 17:22. 1952.

Anamorph: Leptographium serpens (Goid.) Siemaszko, Planta Pol. 7:34. 1939.

= *Scopularia serpens* Goid., Boll. Staz. Patol. Veg. 16:42. 1936.

= *Verticicladiella serpens* (Goid.) W.B. Kendr., Can. J. Bot. 40:781. 1962.

= *Leptographium serpens* (Goid.) M.J. Wingf., Trans. Br. Mycol. Soc. 85:92. 1985. [superfluous combination]

Mycobank MB254673

Ascomatal bases black, globose and smooth, without ornamentation, 300–420 μ m diam, necks black, cylindrical with a slight apical taper, smooth, 400–700 μ m long, 33–65 μ m wide at the base, 21–39 μ m wide at the apex, ostiolar hyphae absent. Asci prototunicate, evanescent. Ascospores hat-shaped, hyaline, 3.3–4.8 $\times$ 1.8–2 μ m (based on Goidánich 1936, Jacobs and Wingfield 2001).

Conidiophores occurring singly, arising directly from the mycelium, erect, macronematous, mononematous, (307–)453–722(–916) μ m long, rhizoid-like structures present. Stipes dark olivaceous, not constricted, cylindrical, simple, 3–8-septate, (218–)354–623(–852) μ m long, apical cell occasionally swollen, (12–)16–24(–28) μ m wide at the apex, basal cell not swollen, (11–)14–19(–22) μ m wide at the base. Conidiogenous apparatus (57–)81–118(–146) μ m long, excluding the conidial mass, with multiple series of cylindrical branches. Primary branches dark olive, smooth, swollen at the apex, aseptate, (22–)28–45(–52) $\times$ (5.6–)9.5–19(–27) μ m, arrangement of the primary branches on the stipe-type C (more than two branches with large central branch) (Jacobs and Wingfield 2001), secondary branches light olivaceous to hyaline, occasionally swollen, aseptate, (11–)15–26(–31) $\times$ (2.5–)3.1–7.0(–13) μ m, tertiary branches hyaline, aseptate, (9.8–)12–19(–24) $\times$ (2.2–)2.4–4.2(–6.1) μ m, quaternary branches hyaline, aseptate, (7.0–)12–19(–28) $\times$ (2.0–)2.4–3.3(–4.2) μ m. Conidiogenous cells discrete, 2–3 per branch, cylindrical, tapering lightly at the apex, (10.2–)11–18(–25) $\times$ (1.4–)1.8–2.3(–2.8) μ m. Conidia hyaline, aseptate, oblong with truncate bases and rounded apices, (3.3–)3.8–5.5(–7.8) $\times$ (1.4–)1.8–2.5(–2.8) μ m.

Colonies with optimal growth at 25 C on 2% MEA, covering 90 mm plate after 4 d incubation. Little growth below 5 C, no growth above 35 C and little reduction in growth on MEA containing 0.5 g/L cycloheximide after 5 d at 25 C in the dark. Colonies dark mouse gray (15''''k). Colony margin effuse. Hyphae superficial and submerged in agar with no aerial mycelium, serpentine, septate, not constricted at septa.

Specimens examined. ITALY. From *Pinus sylvestris*, Jan 1953, *G. Goidánich* (HOLOTYPE, DAOM 34869; ex-holotype living culture CBS 141.36 = CMW 304 = CMW 305); From *P. pinea*, 1987, *P. Gambogi* and *G. Lorenzini* (CMW 289, CMW 290 = CBS 641.76 = ATCC 34322 = IMI 208636); From *P. pinea*, Nov 1986, *P. Capretii*, (CMW 191, CMW 192).

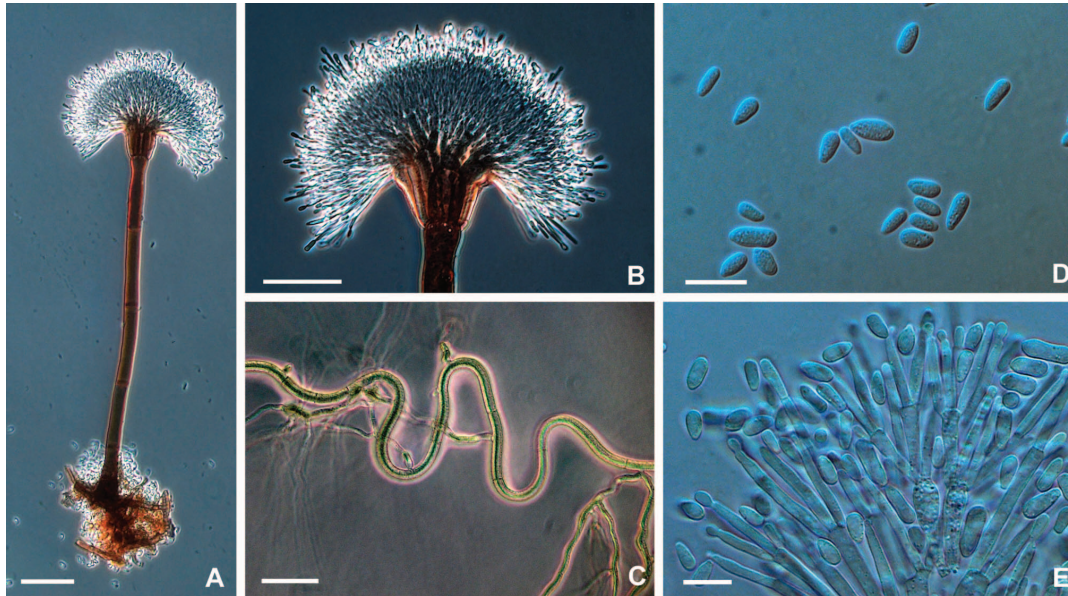


FIG. 5. *Grosmannia serpens* (CMW 290). A. Conidiophore. B. Conidiogenous apparatus. C. Conidia. D. Serpentine hyphae. E. Conidiogenous cells. Bars: 1, 2, 4 = 50 μ m; 3, 5 = 10 μ m.

Grosmannia alacris T.A. Duong, Z.W. de Beer & M.J. Wingf. sp. nov. FIG. 6

Mycobank MB561907

Anamorph: *Leptographium*.

= *Verticicladiella alacris* M.J. Wingf. & Marasas, Trans. Br. Mycol. Soc. 75:22. 1980.

= *Leptographium alacre* (M.J. Wingf. & Marasas) M. Morelet, Annales de la S.S.N.A. T.V. 40:44. 1988. [invalid, ICBN Art. 33.4]

Coloniae crescunt optime in 25 C in 2% MEA, usque ad 90 mm diam in 4 d. Margo coloniae effusa. Hyphae in agaris superficiales immersaeque, sine mycelio aereo, serpentinae septatae in septis non constrictae. Bases perithecorum nigrae globosae inornatae (195–)220–259(–283) μ m diam. Colla perithecorum nigra cylindrica apicem versus leviter angustata laeves (139–)369–667(–851) μ m sine hyphis ostiolaribus. Asci prototunicati hyalini evanescentes. Ascospores pileiformes non septatae, hyalinae non vaginatae (2.7–)3.2–3.8(–4.2) $\times$ (1.7–)2.0–2.4(–2.5) μ m. Conidiophorae singulae proxime e mycelio exorientes erectae meconematatae mononematatae (465–)653–925(–1128) μ m longae, cum structuris rhizoidiformibus. Stipae atro-olivaceae non constrictae, cylindricae simplices 4–9-plo septatae (389–)580–838(–1041) μ m longae. Conidia hyalina non septata, oblonga basibus truncatis apicibus rotundatis (4.5–)5.1–6.1(–6.8) $\times$ (1.8–)2.1–2.4(–2.6) μ m.

Ascomatal bases black, globose and smooth, without ornamentation, (195–)220–259(–283) μ m diam, necks black, cylindrical with a slightly apical taper, smooth, (139–)369–667(–851) μ m long, (31–)39–51(–58) μ m wide at base, (14–)17.2–27.7(–34.8) μ m wide at the apex, ostiolar hyphae absent. Asci prototunicate, evanescent. Ascospores hat-shaped, aseptate, hyaline, without sheath, (2.7–)3.2–3.8(–4.2) $\times$ (1.7–)2.0–2.4(–2.5) μ m.

Conidiophores occurring singly, arising directly from the mycelium, erect, macronematous, mononematous, (465–)653–925(–1128) μ m long, rhizoid-like structures present. Stipes dark olivaceous, not constricted, cylindrical, simple, 4–9-septate, (389–)580–838(–1041) μ m long, apical cell occasionally swollen, (9.6–)13–18(–22) μ m wide at the apex, basal cell not swollen, (11–)14–20(–23) μ m wide at the base. Conidiogenous apparatus (43–)61–100(–125) μ m long, excluding the conidial mass, with multiple series of cylindrical branches. Primary branches dark olive, smooth, swollen at the apex, aseptate, (16–)24–40(–51) $\times$ (5.9–)7.3–16(–27) μ m, arrangement of the primary branches on the stipe-type C (more than two branches with large central branch) (Jacobs and Wingfield 2001), secondary branches light olivaceous to hyaline, occasionally swollen, aseptate, (12–)14–24(–35) $\times$ (3.3–)4.0–7.2(–14) μ m, tertiary branches hyaline, aseptate, (12–)13–19(–22) $\times$ (2.3–)3.0–4.2(–4.9) μ m, quaternary branches hyaline, aseptate, (8.8–)11–15(–18) $\times$ (2.0–)2.4–3.0(–3.8) μ m. Conidiogenous cells discrete, 2–3 per branch, cylindrical, tapering lightly at the apex, (9.4–)11–17(–20) $\times$ (1.6–)1.7–2.1(–2.7) μ m. Conidia hyaline, aseptate, oblong with truncate bases and rounded apices, (4.5–)5.1–6.1(–6.8) $\times$ (1.8–)2.1–2.4(–2.6) μ m.

Colonies with optimal growth at 25 C on 2% MEA, covering 90 mm plate after 4 d incubation. Little growth below 5 C and no growth above 35 C. Cycloheximide tolerant with little reduction in growth on MEA containing 0.5 g/L cycloheximide after 5 d at 25 C in the dark. Colonies dark mouse gray (15''''k). Colony margin effuse. Hyphae superficial and sub-

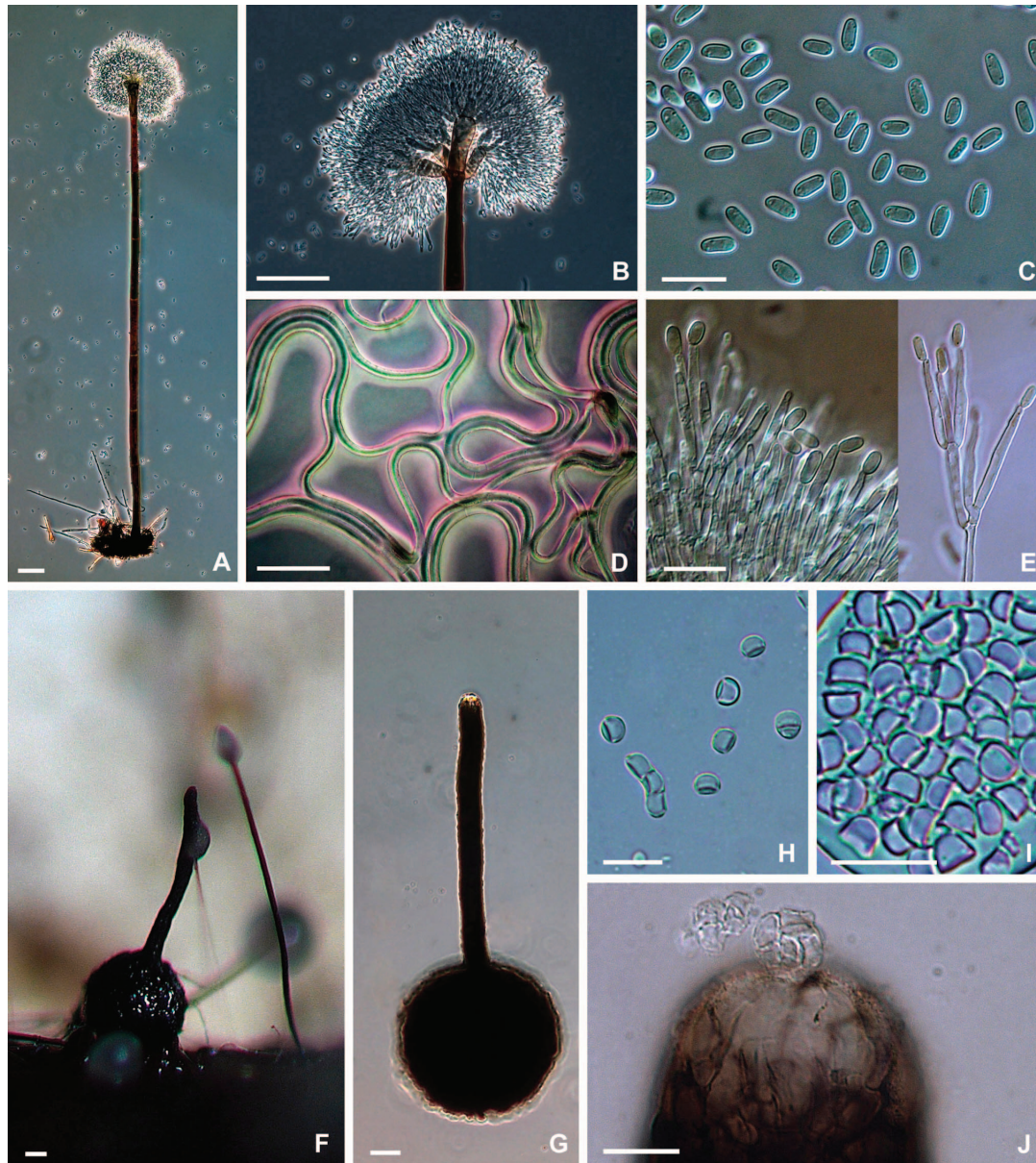


FIG. 6. *Grosmannia alacris* (CMW 6187). A. Conidiophore. B. Conidiogenous apparatus. C. Conidia. D. Serpentine hyphae. E. Conidiogenous cells. F, G. Ascoma with globose base, with conidiophore in the background (CMW 621 × CMW 623). H, I. Hat-shaped ascospores. J. Ostiole with ascospores. Bars: 1, 2, 4, 6, 7 = 50 µm; 3, 5, 8, 9, 10 = 10 µm.

merged in agar with no aerial mycelium, serpentine, septate, not constricted at septa.

Specimens examined. PORTUGAL. From *Pinus pinaster*, 1984, Maria de Fatima Moniz (HOLOTYPE, PREM 60635, dried culture obtained from cross between CMW 621 = CBS 128830 and CMW 623 = CBS 118621). SOUTH AFRICA. WESTERN CAPE: Tokai. From roots of *P. pinaster*, May 1978, M. J. Wingfield (HOLOTYPE of *V. alacris*, PREM 45483, dried culture of CMW 60 = CMW 2844 = CBS 591.79); Grabouw. From roots of *P. pinaster*, Feb 1978, M. J. Wingfield (PREM 45484); Lebanon State Forest. From roots of *P. pinaster*, Apr 1978, M. J. Wingfield (PREM 45485); Grabouw. From roots of *P. radiata*, Mar 1979, M. J. Wingfield (PREM

45486); Jonkershoek. From *P. radiata*, Apr 1984, M. J. Wingfield (PREM 56334, dried culture of CMW 310).

Commentary. Our results demonstrate that the earlier synonymy of this species with *L. serpens* (Wingfield and Marasas 1981) is incorrect. Morelet (1988) suggested that *V. alacris* represented the anamorph of *O. piceiperdum* (Rumbold) Arx [as '*piceaperdum*'], and suggested a new combination for this anamorphic species in the genus *Leptographium*. However the combination was invalid because the basionym of the species was not clearly indicated in the text (ICBN Art. 33.4, McNeill et al. 2006). Furthermore sequence data (Zipfel et al. 2006)

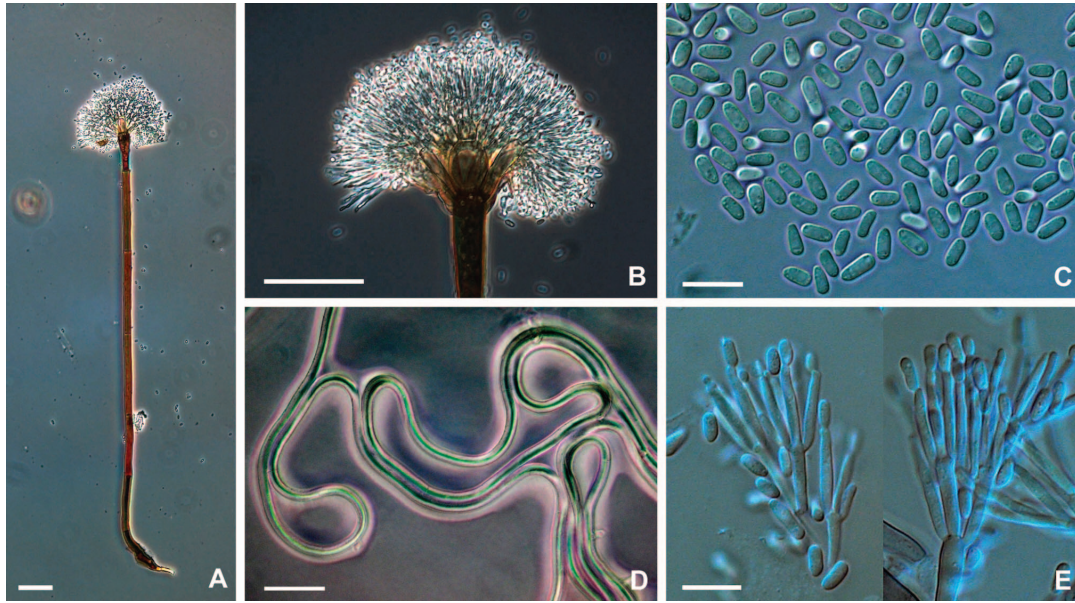


FIG. 7. *Leptographium gibbsii* (CMW 1376). A. Conidiophore. B. Conidiogenous apparatus. C. Conidia. D. Serpentine hyphae. E. Conidiogenous cells. Bars: 1, 2, 4 = 50 μ m; 3, 5 = 10 μ m.

confirmed that *G. piceiperda* represents a distinct species that is not part of the *G. serpens* complex.

Leptographium gibbsii T.A. Duong, Z.W. de Beer & M.J. Wingf. sp. nov. FIG. 7
Mycobank MB561915

Etymology. Named for Dr John Gibbs who supplied these isolates and in acknowledgement of his tremendous contribution to the discipline of forest pathology.

Coloniae crescunt optime in 25 C in 2% MEA, usque ad 90 mm diam in 4 d. Hyphae in agaro superficiales immersaeque, sine mycelio aërio, serpentinae septatae in septis non constrictae. Conidiophorae singulae proxime e mycelio exorientes erectae meconematatae mononematatae (438–)640–1067(–1424) μ m longae, cum structuris rhizoidiformibus. Stipae atro-olivaceae non constrictae, cylindricae simplices 5–12-plo septatae (367–)545–964(–1279) μ m longae. Conidia hyalina non septata, oblonga basibus truncatis apicibus rotundatis (3.8–)4.4–5.8(–6.8) $\times$ (1.9–)2.1–2.6(–3.0) μ m.

Conidiophores occurring singly, arising directly from the mycelium, erect, macronematous, mononematous, (438–)640–1067(–1424) μ m long, rhizoid-like structures present. Stipes dark olivaceous, not constricted, cylindrical, simple, 5–12-septate, (367–)545–964(–1279) μ m long, apical cell occasionally swollen, (13–)15–22(–28) μ m wide at the apex, basal cell not swollen, (10.2–)12–17(–22) μ m wide at the base. Conidiogenous apparatus (62–)74–124(–169) μ m long, excluding the conidial mass, with multiple series of cylindrical branches. Primary branches dark olive, smooth, swollen at the apex, aseptate, (16–)22–38(–49) $\times$ (4.3–)8.1–17.5(–27) μ m, arrangement of the primary branches on the stipe-type C (more than two branches with large central

branch) (Jacobs and Wingfield 2001), secondary branches light olivaceous to hyaline, occasionally swollen, aseptate, (8.5–)12–22(–32) $\times$ (3.4–)3.7–7.8(–15) μ m, tertiary branches hyaline, aseptate, (10.5–)13–17(–20) $\times$ (2.5–)3.0–4.4(–5.9) μ m, quaternary branches hyaline, aseptate, (8.9–)10.1–14(–18) $\times$ (2.3–)2.5–3.2(–3.6) μ m wide. Conidiogenous cells discrete, 2–3 per branch, cylindrical, tapering lightly at the apex, (8.9–)10.6–14(–17) $\times$ (1.4–)1.7–2.1(–2.5) μ m. Conidia hyaline, aseptate, oblong with truncate bases and rounded apices, (3.8–)4.4–5.8(–6.8) $\times$ (1.9–)2.1–2.6(–3.0) μ m.

Colonies with optimal growth at 25 C on 2% MEA, covering 90 mm plate after 4 d incubation. Little growth below 5 C, no growth above 35 C and little reduction in growth on MEA containing 0.5 g/L cycloheximide after 5 d at 25 C in the dark. Colonies dark mouse gray (15¹¹¹k). Colony margin effuse. Hyphae superficial and submerged in agar with no aerial mycelium, serpentine, septate, not constricted at septa.

Specimens examined: UNITED KINGDOM. ENGLAND: Hampshire, Yaterley Heath Wood. From *Hylastes ater* on *Pinus* sp., May 1988, J. Gibbs (HOLOTYPE, PREM 60636, dried culture of CMW 1376 = CBS 128695).

Leptographium yamaokae T.A. Duong, Z.W. de Beer & M.J. Wingf. sp. nov. FIG. 8
Mycobank MB561916

Etymology. Named for Dr Yuichi Yamaoka who first collected this species, and to acknowledge his contributions to the taxonomy of ophiostomatoid fungi and the understanding of bark beetle-fungus interactions.

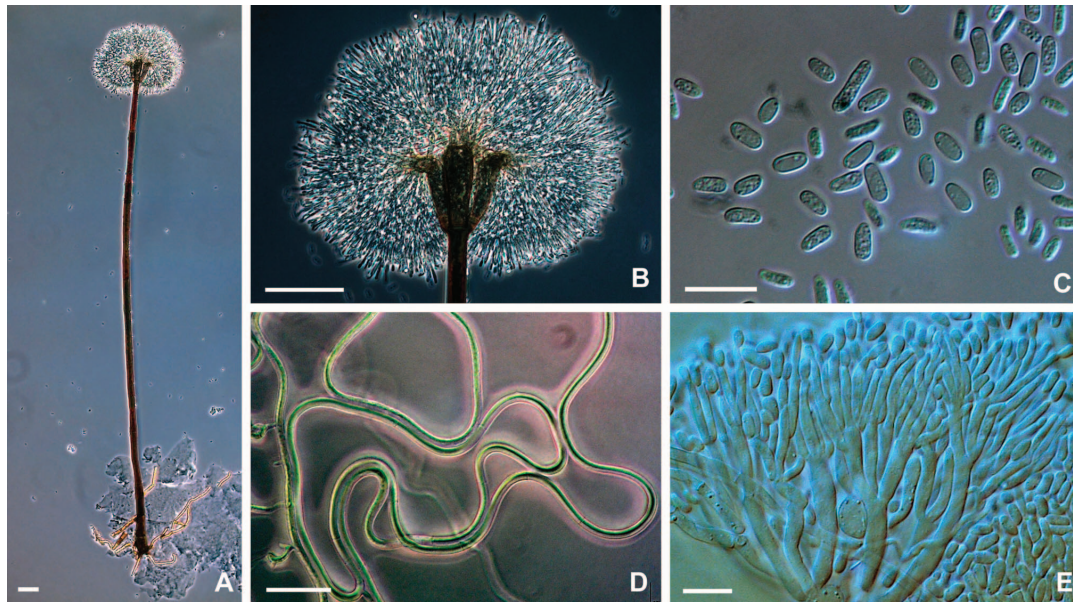


FIG. 8. *Leptographium yamaoka* (CMW 4726). A. Conidiophore. B. Conidiogenous apparatus. C. Conidia. D. Serpentine hyphae. E. Conidiogenous cells. Bars: 1, 2, 4 = 50 μ m; 3, 5 = 10 μ m.

Coloniae crescunt optime in 25 C in 2% MEA, usque ad 90 mm diam in 6 d. Hyphae in agar superficiali immersaeque, aliquando cum mycelio aereo, serpentinae septatae in septis non constrictae. Conidiophorae singulae proxime e mycelio exorientes erectae mecronematae mononematae (642–)874–1199(–1390) μ m longae, cum structuris rhizoidiformibus. Stipae atro-olivaceae non constrictae, cylindricae simplices 5–11-plo septatae (583–)779–1084(–1297) μ m longae. Conidia hyalina non septata, oblonga basibus truncatis apicibus rotundatis (3.3–)4.0–5.7(–8.3) $\times$ (1.7–)2.0–2.4(–2.8) μ m.

Conidiophores occurring singly, arising directly from the mycelium, erect, macronematous, mononematous, (642–)874–1199(–1390) μ m long, rhizoid-like structures present. Stipes dark olivaceous, not constricted, cylindrical, simple, 5–11-septate, (583–)779–1084(–1297) μ m long, apical cell occasionally swollen, (9.2–)12–18(–22) μ m wide at the apex, basal cell not swollen, (14–)16–22(–25) μ m wide at the base. Conidiogenous apparatus (60–)84–127(–156) μ m long, excluding the conidial mass, with multiple series of cylindrical branches. Primary branches dark olive, smooth, swollen at the apex, aseptate, (17–)26–54(–70) $\times$ (4.5–)6.6–14(–21) μ m, arrangement of the primary branches on the stipe-type C (more than two branches with large central branch) (Jacobs and Wingfield 2001), secondary branches light olivaceous to hyaline, occasionally swollen, aseptate, (13–)16–26(–32) $\times$ (3.1–)3.3–7.0(–13) μ m, tertiary branches hyaline, aseptate, (9.2–)12–20(–28) $\times$ (2.0–)2.4–3.8(–5.0) μ m, quaternary branches hyaline, aseptate, (9.5–)10.4–15(–19) $\times$ (1.8–)1.9–2.7(–3.7) μ m. Conidiogenous cells discrete, 2–3 per branch, cylindrical,

tapering lightly at the apex, (8.3–)10.6–14(–15.5) $\times$ (1.2–)1.6–2.1(–2.4) μ m. Conidia hyaline, aseptate, oblong with truncate bases and rounded apices, (3.3–)4.0–5.7(–8.3) $\times$ (1.7–)2.0–2.4(–2.8) μ m.

Colonies with optimal growth at 25 C on 2% MEA, covering 90 mm plate after 6 d incubation. Little growth below 5 C, and no growth above 35 C and little reduction in growth on MEA containing 0.5 g/L cycloheximide after 5 d at 25 C in the dark. Colonies isabelline (19"i) to dark mouse gray (15"m"k). Colony margin effuse. Hyphae superficial and submerged in agar with occasional aerial mycelium, serpentine, septate, not constricted at septa.

Specimens examined: JAPAN. KOFU: Yamanashi. From dead tree of *Pinus densiflora*, Jul 1996, H. Masuya (HOLOTYPE, PREM 60637, dried culture of CMW 4726 = CBS 129732); Yamanashi. From dead tree of *P. densiflora*, Jul 1996, Y. Yamaoka (PREM 60638, dried culture of CMW 1944 = CBS 128696).

Leptographium castellanum T.A. Duong, Z.W. de Beer & M.J. Wingf. sp. nov. FIG. 9
Mycobank MB561918

Etymology. Name denotes the official language (Castilian; *castellano* in Spanish) of the two countries where this fungus was first collected.

Coloniae crescunt optime in 25 C in 2% MEA, usque ad 90 mm diam in 4 d. Hyphae in agar superficiali immersaeque, sine mycelio aereo, serpentinae septatae in septis non constrictae. Conidiophorae singulae proxime e mycelio exorientes erectae mecronematae mononematae (595–)735–1002(–1277) μ m longae, cum structuris rhizoi-

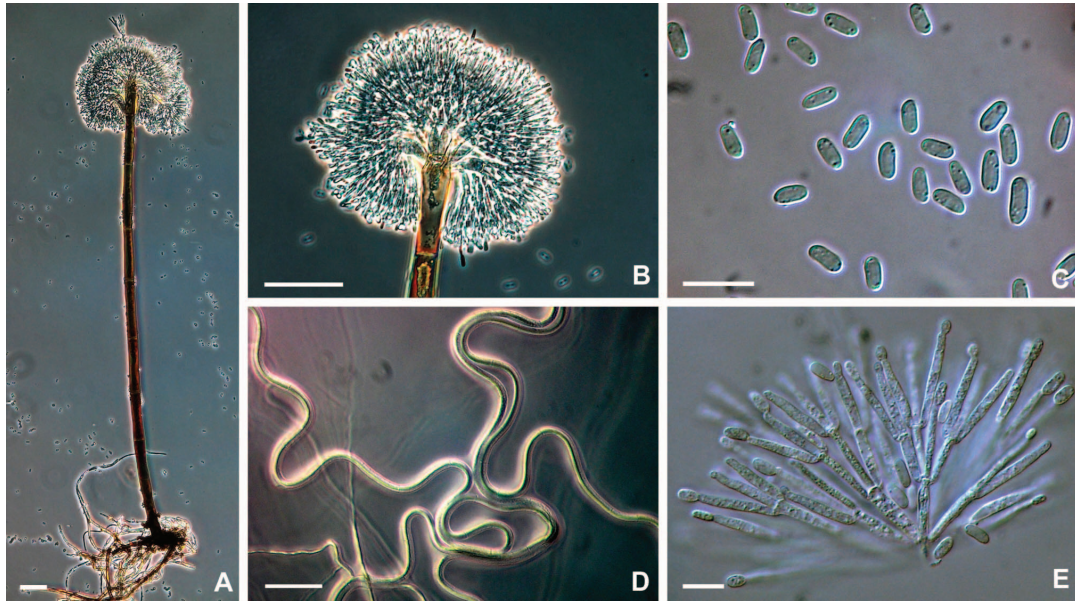


FIG. 9. *Leptographium castellanum* (CMW 2321). A. Conidiophore. B. Conidiogenous apparatus. C. Conidia. D. Serpentine hyphae. E. Conidiogenous cells. Bars: 1, 2, 4 = 50 μ m; 3, 5 = 10 μ m.

diformibus. Stipae atro-olivaceae non constrictae, cylindricae simplices 7–12-plo septatae (525–)643–900(–1187) μ m longae. Conidia hyalina non septata, oblonga basibus truncatis apicibus rotundatis (4.1–)4.6–5.4(–6.1) $\times$ (1.8–)2.0–2.4(–2.6) μ m.

Conidiophores occurring singly, arising directly from the mycelium, erect, macronematous, mononematous, (595–)735–1002(–1277) μ m long, rhizoid-like structures present. Stipes dark olivaceous, not constricted, cylindrical, simple, 7–12-septate, (525–)643–900(–1187) μ m long, apical cell occasionally swollen, (13–)14–20(–25) μ m wide at the apex, basal cell not swollen, (11–)14–20(–26) μ m wide at the base. Conidiogenous apparatus (59–)75–120(–142) μ m long, excluding the conidial mass, with multiple series of cylindrical branches. Primary branches dark olive, smooth, swollen at the apex, aseptate, (24–)30–51(–73) $\times$ (5.6–)9.5–20(–26) μ m, arrangement of the primary branches on the stipe-type C (more than two branches with large central branch) (Jacobs and Wingfield 2001), secondary branches light olivaceous to hyaline, occasionally swollen, aseptate, (9.8–)17–28(–36) $\times$ (3.3–)4.2–10.2(–16) μ m, tertiary branches hyaline, aseptate, (6.7–)12–18(–22) $\times$ (2.1–)2.7–3.8(–5.6) μ m, quaternary branches hyaline, aseptate, (9.0–)10.7–17(–21) $\times$ (1.4–)2.0–3.1(–3.7) μ m. Conidiogenous cells discrete, 2–3 per branch, cylindrical, tapering lightly at the apex, (9.8–)13–19(–25) $\times$ (1.5–)1.9–2.7(–3.3) μ m. Conidia hyaline, aseptate, oblong with truncate bases and rounded apices, (4.1–)4.6–5.4(–6.1) $\times$ (1.8–)2.0–2.4(–2.6) μ m.

Colonies with optimal growth at 25 C on 2% MEA, covering 90 mm plate after 4 d incubation. Little growth below 5 C, no growth above 35 C and little reduction in

growth on MEA containing 0.5 g/L cycloheximide after 5 d at 25 C in the dark. Colonies dark mouse gray (15''''k). Colony margin effuse. Hyphae superficial and submerged in agar with no aerial mycelium, serpentine, septate, not constricted at septa.

Specimens examined: DOMINICAN REPUBLIC. San José de las Matas. From *Pinus occidentalis*, 1991, R. Webb (HOLOTYPE PREM 60639, dried culture of CMW 2321 = CBS 128697); (PREM 60640, dried culture of CMW 2320 = CBS 128698).

DISCUSSION

Multigene sequence data generated in this study provided strong phylogenetic evidence supporting five cryptic species in *G. serpens* complex. Of the five gene regions used, only ITS2-LSU data did not distinguish among the species. This was not unexpected however because authors (Lim et al. 2004, Paciura et al. 2010) failed to gain effective resolution of species in the *Grosmannia-Leptographium* complexes using this gene region. In contrast sequence data from the four protein-coding genes strongly supported the separation of the five species. In the case of the ACT dataset the type isolate (CMW 2844) of *G. alacris* was separated from the other *G. alacris* isolates (FIG. 4) due to transition at two base pair positions (FIG. 2). The low sequence variation in this gene region allows such small changes to affect the resulting trees. However this isolate grouped in the *G. alacris* lineage for all three of the other protein-coding genes and this was also true in the combined dataset. Among the four protein-coding genes used in this study, β T and TEF-1 α

are the most variable regions and thus the most informative phylogenetically. Although the CAL gene was slightly less variable than β T and TEF-1 α , the region complemented the other gene regions well. New primers developed in this study to amplify a portion of the CAL gene for this species complex might prove useful in studies of the phylogeny of other species complexes in *Grosmannia* and *Leptographium*.

Two of the five lineages identified in this study accommodated the ex-type isolates of *G. serpens* and *V. alacris* respectively and thus represented these species. For the latter species the sexual stage was induced to form in culture through pairing of different isolates. This provided material to revive the name of the fungus including a teleomorph state as *G. alacris*. Because no sexual state could be obtained for isolates in the remaining three lineages these isolates were described as novel species of *Leptographium*.

A mating compatibility experiment also confirmed heterothallism in *G. alacris*. This was a fortuitous discovery because only one of the 10 *G. alacris* isolates used in mating study was able to cross with the other nine isolates to form sexual structures. It is likely that the other four species in the complex are also heterothallic but that the limited number of available isolates represented only one mating type and thus led to the failure of the matings. This is probably the reason why the sexual state of *G. serpens* has never been observed in culture (Goidánich 1936, Hunt 1956).

The ex-type isolate of *G. serpens* grouped in a lineage including other isolates from Italy that were collected and reported as *L. serpens* in Gambogi and Lorenzini (1977), Lorenzini and Gambogi (1976) and Wingfield et al. (1988). The morphology of these isolates corresponded with the ex-type isolate that originally was described in the same paper as the genus *Grosmannia* (Goidánich 1936). The genus was treated later as a synonym of *Ceratocystis* (Hunt 1956, Moreau 1952, Upadhyay 1981) and then as *Ophiostoma* (Harrington 1988, von Arx 1952), resulting in *G. serpens* being treated in both these genera by different authors. Zipfel et al. (2006) showed that *Grosmannia* (mostly with *Leptographium* anamorphs) and *Ophiostoma* (with *Sporothrix* and/or *Pesotum* anamorphs) are phylogenetically distinct and reinstated *Grosmannia* with *G. serpens* as one of 27 species in the genus. However sexual structures have never been observed for *G. serpens* subsequent to the first description of the species (Gambogi and Lorenzini 1977, Hunt 1956, Jacobs and Wingfield 2001, Siemazsko 1939, Upadhyay 1981, Wingfield and Marasas 1981).

The original collection of *G. serpens* was from *Pinus sylvestris*, and the fungus was described as an agent causing root disease in this tree species (Goidánich

1936). The species was discovered later also on stained *P. pinea* wood in Italy (Gambogi and Lorenzini 1977, Lorenzini and Gambogi 1976, Wingfield et al. 1988). More recently *G. serpens* was found in association with *Tomicus destruens* infesting *P. pinea* and *P. pinaster* (Sabbatini Peverieri et al. 2006) and in coarse woody debris in *P. pinea* forests in central Italy (Santini et al. 2008). In the latter two studies the fungus was identified based only on morphological characters, but it is probable that these isolates also represent *G. serpens*. Other reports of *L. serpens* based on morphology only from the Czech Republic (Kotýnková-Sychrová 1966) and Spain (Pestaña and Santolamazza-Carbone (2010) could represent any of the four cryptic species in the complex present in Europe. DNA sequence data from the original or fresh isolates will be required to confirm these reports.

Verticicladiella alacris, a species originally described in association with pine root disease in South Africa (Wingfield and Knox-Davies 1980, Wingfield and Marasas 1980), is member of a lineage in our tree (*G. alacris* in FIG. 4) that corresponds with two closely related lineages in a cluster analyses based on isozyme profiles containing the isolate of *V. alacris* (C297 = CMW 2844) and several other South African (C56, 297, 304, 306, 307, C141), USA (153, 169, 175) and Spanish (305) isolates (FIG. 1, Zambino and Harrington 1992). These two lineages were treated as *L. serpens* together with a third, more distant lineage containing two Italian isolates (C30 = CMW 304, C79 = CMW 290), shown in our study to represent *G. serpens* (FIG. 4). Our results thus suggest that a more accurate position for delineating species would be around 0.9 on the x axis in the dendrogram presented by Zambino and Harrington (1992). This also would reflect more accurately the cut-off for species in the *G. clavigera* complex further down on their cladogram as delimited in several multigene and population studies (Alamouti et al. 2011, Lee et al. 2005, Roe et al. 2010, Six et al. 2011).

Of all species in the *G. serpens*-complex the newly described *G. alacris* has the widest distribution, including isolates from France, Portugal, Spain, South Africa and USA. Of these the European isolates were from *P. pinaster* while in South Africa the species is closely associated with the non-native pine-infesting bark beetles, *Hylastes angustatus* and *Hylurgus ligniperda* found on *P. elliotii*, *P. patula*, *P. pinaster* and *P. radiata* (Wingfield and Knox-Davies 1980, Wingfield and Marasas 1980, Zhou et al. 2001). This strongly implies a European origin for the South African fungus. USA isolates of *G. alacris* were from *P. strobus* in Virginia (Lackner and Alexander 1983) and *P. taeda* in Mississippi (Zambino and Harrington 1992). There are various root-feeding bark beetles

native to USA that carry *Grosmannia*, so the fungus could be native in that country. Alternatively it could have been introduced into USA. This question has interesting and potentially important quarantine implications and will require further study.

Sequences for *G. alacris* exhibited a substantial level of variability within each of four protein coding gene regions (FIG. 4). This is most likely the consequence of widespread recombination resulting from sexual reproduction. The fact that the isolate from Portugal (CMW 623) crossed with isolates from South Africa, USA and Spain supports this hypothesis.

Grosmannia serpens has been reported (based on morphology) from native beetles on *P. taeda* and *P. palustris* from Alabama and Georgia respectively in USA (Eckhardt et al. 2007, Zanzot et al. 2010) and in low frequencies from the introduced bark beetle, *Hylurgus ligniperda*, on *P. halepensis* and *P. pinea* in California (Kim et al. 2010). Our results suggest the isolates reported as *G. serpens* in these studies might represent *G. alacris*. In the study of Kim et al. (2010) a partial ITS2-LSU sequence was produced for a single isolate. When compared with sequences generated in this study those of Kim et al. (2010) shared two unique base pairs in the LSU region with *L. castellanum*. However because sequences for this gene region do not distinguish among all five cryptic species in the complex sequence data from the protein-coding genes will be needed to identify the above-mentioned isolates from USA.

Leptographium gibbsii, one of the three novel taxa emerging from this study, was represented by only three isolates. These isolates originally were identified as *L. serpens* from *Hylastes ater* and *H. opacus* infesting billets of *P. sylvestris* in UK (Wingfield and Gibbs 1991). Neither *L. serpens* nor *V. alacris* has been reported from *H. ater* where the beetle has been examined for fungi elsewhere in the world (Harrington 1988, Jacobs and Wingfield 2001, Reay et al. 2005, Romón et al. 2007, Wingfield et al. 1988, Zhou et al. 2004). We also were unable to locate reports of fungal associates of *H. opacus* other than the study of Wingfield and Gibbs (1991), which was the source of isolates used in the present study. It thus appears that *L. gibbsii* is not a common associate of these two beetles.

Isolates of *L. yamaokae* were collected from *Pinus* in Japan (Masuya et al. 2003, 2009). In an extensive survey of ophiostomatoid fungi on *P. densiflora* its many bark beetle associates and their galleries *L. yamaokae* was isolated from only 10 of 20 *Hylastes plumbeus* galleries and from two of 48 beetles of this species (Masuya et al. 2009). Interestingly Aoshima (1965) listed several *Grosmannia* [as ‘*Ceratocystis*’] and *Leptographium* [as ‘*Verticicladiella*’] in his investigation on wood-staining fungi from Japan, but none of the descriptions of those species match that of *L. yamaokae*. Neither *L. yamaokae*

nor any fungus resembling *G. serpens* have been found in recent studies of ophiostomatoid fungi on pine in Korea (Kim et al. 2005) and China (Lu et al. 2009a, b; Paciura et al. 2010). Considering the absence of this species from these studies in the Far East and the large number of beetle species investigated by Masuya et al. (2009), the association between *L. yamaokae* and *H. plumbeus* could be quite specific.

Isolates of *L. castellanum* described in this study originated from an unknown *Hylurgus* species in Spain and from *P. occidentalis* in the Dominican Republic. The presence of this fungus in these two countries might be linked to the fact that the Dominican Republic was a colony of Spain for three centuries, which would have allowed for the easy movement of wood or wood products between them.

In 1982 *L. galleciae* Fern. Magán [as ‘*Gallaeciae*’] was described from stressed *P. pinaster* trees with root damage in Galicia, northwestern Spain (De Ana Magán 1982). Although De Ana Magán described the morphology of the holomorph, only the anamorph state was provided with a Latin binomial because they could not induce a sexual state in culture. The teleomorph they described and illustrated from wood had hat-shaped ascospores similar to those known for some members of the *G. serpens* complex. However the species description was invalid because it lacked a formal Latin diagnosis (ICBN Art. 36.1, McNeill et al. 2006). In a subsequent paper the Latin diagnosis was provided (De Ana Magán 1983), yet the name remained invalid because no reference was made in either of the two papers to a holotype specimen (ICBN Art. 37.1, McNeill et al. 2006). The morphological description of “*L. galleciae*” overlaps largely with those of the two species reported in the present study from Spain, namely *G. alacris* and *L. castellanum*, except for the fact that the ascospores of “*L. galleciae*” are wider than those of *G. alacris* and the conidia of “*L. galleciae*” are smaller than those of both *G. alacris* and *L. castellanum*. Without material linked to the original collection of De Ana Magán a neotype cannot be designated to validate “*L. galleciae*”, and no comparisons are possible between that species and those from the *G. serpens* complex described here.

Of the five species in the complex, *G. serpens* and *G. alacris* have been found associated with root diseases of pines. *Grosmannia serpens* was isolated from symptomatic *P. pinea* trees with a root disease in Italy (Lorenzini and Gambogi 1976) and *G. alacris* was isolated from diseased roots of *P. pinaster* and *P. radiata* in South Africa (Wingfield and Knox-Davies 1980, Wingfield and Marasas 1980). Studies also showed that *G. alacris* can cause lesions when inoculated in pine seedlings and mature trees (Eckhardt et al. 2004, Zhou et al. 2002). None of

these authors suggested that this fungus is a serious primary pathogen (Eckhardt et al. 2004, Matusick et al. 2008, Zhou et al. 2002). Yet among the fungi associated with conifer root-infesting bark beetles those in the *G. serpens* complex are among the most pathogenic. Because many of their vectors mature while feeding on healthy roots, these fungi might contribute to disease development in some situations. In this regard they deserve to be studied further.

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Chapter 3

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Microsatellite markers for *Grosmannia alacris* (Ophiostomataceae, Ascomycota) and other
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MICROSATELLITE MARKERS FOR *GROSMANNIA ALACRIS* (OPHIOSTOMACEAE, ASCOMYCOTA) AND OTHER SPECIES IN THE *G. SERPENS* COMPLEX¹

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- *Premise of the study:* Polymorphic microsatellite markers were developed for the pine-infecting fungus, *Grosmannia alacris*.
- *Methods and Results:* Sixteen microsatellite markers were developed by using inter-simple sequence repeat (ISSR)–PCRs and 454 sequencing methods. Seven of these markers showed polymorphisms for a South African population of *G. alacris*, and 13 markers showed polymorphism when European isolates were included. Most of the primer pairs also amplified four closely related species: *G. serpens*, *Leptographium gibbsii*, *L. castellanum*, and *L. yamaokae*.
- *Conclusions:* These new markers will be useful for population studies of *G. alacris* and other species in the *G. serpens* complex.

Key words: *Grosmannia alacris*; *Leptographium*; microsatellites; Ophiostomataceae; pine pathogen.

The pine pathogen *Grosmannia alacris* T. A. Duong, Z. W. de Beer & M. J. Wingf. is the most widely distributed species among five species currently accommodated in the *G. serpens* complex (Duong et al., 2011). Isolates of *G. alacris* have been identified from three different continents including Europe, North America, and Africa. In contrast to *G. alacris*, the other four species in the complex have much narrower distribution. *Grosmannia serpens* Goid. has been reported only from Italy; *Leptographium gibbsii* T. A. Duong, Z. W. de Beer & M. J. Wingf. only from England; *L. castellanum* T. A. Duong, Z. W. de Beer & M. J. Wingf. from Spain and the Dominican Republic; and *L. yamaokae* T. A. Duong, Z. W. de Beer & M. J. Wingf. only from Japan (Duong et al., 2011). One of these five species, *G. serpens*, causes root disease of pine in Italy, and *G. alacris* was previously implicated in root disease of pine in South Africa (Wingfield and Knox-Davies, 1980; Duong et al., 2011) and pine decline in the United States (Eckhardt et al., 2004). The global distribution of *G. alacris* raises intriguing questions regarding the diversity and movement of this species. However, there are no genetic markers available for population studies of this or other species in the *G. serpens* complex, which

precludes the ability to address such questions. In this study, we developed microsatellite markers that will facilitate population studies on *G. alacris*. The markers were also tested for their cross application with *G. serpens*, *L. gibbsii*, *L. castellanum*, and *L. yamaokae*.

METHODS AND RESULTS

The inter-simple sequence repeat (ISSR)–PCR technique (Zietkiewicz et al., 1994) was used to generate a microsatellite-enriched genomic library for *G. alacris*. Genomic DNA was extracted from freeze-dried fungal mycelia of isolate CLE 088 (Appendix 1) by using the method described by Aljanabi and Martinez (1997). The ISSR-PCRs were performed with a single primer as well as primer pairs resulting from all the possible combinations of the following primers: ISSR1 (5′-DDB(CCA)₅-3′), ISSR2 (5′-DHB(CGA)₅-3′), ISSR3 (5′-YHY(GT)₅G-3′), ISSR4 (5′-HVV(GTG)₅-3′), ISSR5 (5′-NDB(CA)₇C-3′), ISSR6 (5′-NDV(CT)₈-3′), and ISSR7 (5′-HDBD(GACA)₄-3′). The PCR reaction mixture, 50 µL total volume, consisted of 1× PCR reaction buffer (50 mM Tris-HCl, 10 mM KCl, 5 mM (NH₄)₂SO₄, 2.5 mM MgCl₂, 200 µM of each dNTP, 0.5 µM of each primer (1 µM when only one primer was used), 2 U FastStart *Taq* DNA Polymerase (Roche Applied Science, Mannheim, Germany), and 50 ng of genomic DNA. The PCR protocol was as follows: an initial denaturation at 95°C for 5 min, followed by 30 cycles of 94°C for 30 s, 52°C annealing for 30 s, and 72°C extension for 120 s, and a final extension at 72°C for 8 min. After amplification, the ISSR-PCR products were pooled and precipitated using ethanol. This served as a microsatellite-enriched genomic library. This library was submitted for 454 sequencing to Inqaba Biotech (Pretoria, South Africa), and sequences containing microsatellite repeats were screened from this library following the method described by Santana et al. (2009).

A total of approximately 3.3 Mb sequence data distributed in 15 191 single reads ranging from 40 to 340 bp in size was obtained from a quarter-plate run on a 454 GS-FLX platform. All sequence reads were assembled using ContigExpress, a component of the Vector NTI version 11.0 software package (Invitrogen, Carlsbad, California, USA), resulting in 1683 contigs and 2431 unassembled single reads. Sizes of contigs ranged from 50 bp to about 2.3 kp,

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TABLE 1. Characteristics of 16 microsatellite loci for *Grosmannia alacris*.

Locus name	Primer sequences (5′–3′) ^a	Repeat motif	Size range (bp)	PCR multiplexing	T _a (°C)	GenBank accession no.
LSM-02	F: <NED>GACACGACTGCCCATCACAC R: GTTCTCGTTGCTGTGACTATGC	(CCACGA) ₇	162–186	Plex-1	55	JN944519
LSM-11	F: <NED>CAGAAGCGAGCTGCGG R: GGACGTGAACCTTGACAAACGTG	(ACG) ₈	179–185	Plex-7	60	JN944520
LSM-14	F: <NED>ATAAGACGCCCCGTATAAGCC R: TCGCGATGTCCTCTGTTG	(TGG) ₆	192	Plex-3	55	JN944521
LSM-15	F: <VIC>TCTGCGAGGAGGGAAG R: GCCTGGTTAGTCATCTGTGGG	(GTT) ₇	291	Plex-3	55	JN944522
LSM-19	F: <PET>GCATTTCTGCCTCTGCTG R: ATTTGGTGTCCCATGCTCG	(ACGAC) ₉	281–291	Plex-5	55	JN944523
LSM-21	F: <6–FAM>TTGGGCGCAAGGGCTCA R: AACACAATCATCCCAAGACACG	(AG) ₁₀ (TG) ₈	255–259	Plex-5	55	JN944524
LSM-22	F: <6–FAM>CCGCATAGTCGCCGAAC R: ATGACCACCGCCACCTTT	(GCTGCC) ₆	453–471	Plex-1	55	JN944525
LSM-25	F: <6–FAM>GGGTGATGCGGTCTCT R: CGACGGCGCAACTGAGAC	(GGA) ₇	147–150	Plex-1	55	JN944526
LSM-26	F: <NED>GGCGTGATTATCGATGCT R: CCGAATTCACGCCAAATCA	(GCAG) ₅	244–248	Plex-2	55	JN944527
LSM-30	F: <NED>CTCCGCTGACCTGACCTGG R: GAGTACGGATCTGCCGAGGA	(CAG) ₉	287–296	Plex-2	55	JN944528
LSM-31	F: <PET>GGCAAAGTGAAAGACGTTAGG R: CCGATGCAACTACGCCAC	(GCTG) ₅	225–229	Plex-2	55	JN944529
LSM-32	F: <6–FAM>TTGGGCGCACTCGTGA R: AGCGGGCCAAAATCAGG	(CTGG) ₉ (T) _n	203–230	Plex-3	55	JN944530
LSM-33	F: <PET>GACTCAGTTTCGAGGGCGTATTT R: TCCGCGCTCGAGTGTCTT	(AG) ₁₅	359–364	Plex-2	55	JN944531
LSM-37	F: <6–FAM>CTGGTGTGTGCTGATGTTTC R: TGGCCCGACTTCAACATTG	(TGC) ₁₁	310–322	Plex-1	55	JN944532
LSM-39	F: <VIC>ACATGGCAGCAAGTCCAAGTC R: CACCATGCTCAAGTCAGCAGT	(GCA) ₁₀	361–364	Plex-2	55	JN944533
LSM-40	F: <PET>CCTCCACAGAGCAGCC R: AACCCGTCAGCACCTT	(CTG) ₆	172	Plex-5	55	JN944534

Note: T_a = optimal annealing temperature.

^aThe fluorescent dye used is presented with the primer sequences.

which correspond to the observed banding patterns of ISSR-PCR products. All contigs and sequences were searched for microsatellites using the MsaFinder interface (<http://www.genomics.ceh.ac.uk/msafinder>) (Santana et al., 2009). After filtering out sequences free of microsatellite repeats, those that were inordinately short, or those with the repeats too close to the ends, 487 microsatellite-

containing sequences were obtained that were suitable for primer design. From these, 40 sequences were selected to design primers. All primers were designed such that multiplexing in the downstream PCR and GeneScan applications was possible using the software Primer3 version 0.4.0 (Rozen and Skaletsky, 2000). In cases where the outputs from Primer3 were not appropriate, primer sites were

TABLE 2. Results of characterization of 16 identified microsatellite loci in a South African population of *Grosmannia alacris* and cross amplification with the other four species in the *G. serpens* complex. Each of the other species was represented by a single isolate.

Locus name	South African isolates (N = 46)			Cross-amplification (Allele sizes)			
	No. of observed alleles ^a	H	I	<i>G. serpens</i>	<i>L. gibbsii</i>	<i>L. castellanum</i>	<i>L. yamaokae</i>
LSM-02	2(2)	0.386	0.574	162	186	191	156
LSM-11	2(1)	0.043	0.105	179	173	170	170
LSM-14	1(0)	NA	NA	186	186	195	209
LSM-15	1(0)	NA	NA	—	—	—	296
LSM-19	3(0)	0.634	1.052	287	272	285	263
LSM-21	2(0)	0.500	0.693	—	—	—	—
LSM-22	1(2)	NA	NA	466	460	460	466
LSM-25	1(1)	NA	NA	157	147	147	147
LSM-26	2(1)	0.492	0.685	250	259	257	258
LSM-30	3(1)	0.526	0.826	286	286	281	286
LSM-31	1(1)	NA	NA	231	227	236	231
LSM-32	3(3)	0.570	0.964	200	190	188	188
LSM-33	1(1)	NA	NA	358	364	358	350
LSM-37	1(2)	NA	NA	294	331	305	312
LSM-39	1(1)	NA	NA	359	362	364	356
LSM-40	1(0)	NA	NA	177	202	226	189

Note: — = no amplification; H = gene diversity (Nei, 1973); I = information index (Lewontin, 1972); N = number of isolates screened; NA = not applicable.

^aThe numbers of additional alleles observed in five European isolates are presented in parentheses.

chosen manually. PCR for microsatellite amplification, 25 µL total volume, consisted of 1× PCR reaction buffer (50 mM Tris-HCl, 10 mM KCl, 5 mM (NH₄)₂SO₄), 2.5 mM MgCl₂, 200 µM of each dNTP, 0.2 µM of each primer, 1 U FastStart *Taq* DNA Polymerase (Roche Applied Science), and 20 ng of genomic DNA. The thermal cycling conditions for microsatellite PCRs were as follows: initial denaturation at 95°C for 5 min, followed by 35 cycles of 94°C for 30 s, 55°C (or 60°C) annealing for 30 s (Table 1), and 72°C extension for 60 s, and a final extension at 72°C for 30 min. PCR products were visualized using 1.5% agarose electrophoresis and stained with GelRed (Biotium, Hayward, California, USA). Product sizes were determined by using an ABI PRISM 3100 Genetic Analyzer with GeneScan-500 LIZ as internal size standard (Applied Biosystems, Foster City, California, USA). The number and size of alleles was determined using GENEMAPPER 4.0 (Applied Biosystems). Gene diversity and information index (Lewontin, 1972) were calculated using PopGene32 (Yeh et al., 2000).

Forty-six *G. alacris* isolates from South Africa were used to test for polymorphism of the microsatellite markers. Thirty-six of these isolates were obtained from a single location, and the remaining isolates were obtained from four other widespread locations over an extended period of time (Appendix 1). Sixteen of 40 primer pairs consistently amplified all the isolates (Table 1). Seven markers showed polymorphism within the tested population, and the remaining nine markers were monomorphic. Allelic diversity (Nei, 1973) was between 0.043 and 0.634, and information index (Lewontin, 1972) was between 0.105 and 1.052 per locus (Table 2). When five isolates from Europe were included (Duong et al., 2011), six additional markers (LSM-22, LSM-25, LSM-31, LSM-33, LSM-37, and LSM-39) showed polymorphisms between these and the South African isolates. All 16 markers were tested for cross amplification with *G. serpens*, *L. gibbsii*, *L. castellanum*, and *L. yamaokae*. Fourteen markers were amplified in *G. serpens*, *L. gibbsii*, and *L. castellanum*, and 15 in *L. yamaokae*. Allele sizes of these markers are presented in Table 1.

CONCLUSIONS

The microsatellite markers developed in this study will be useful for population studies on *G. alacris* and hopefully will elucidate its origin and pathways of global movement. Successful cross amplification of these markers in other species in the

G. serpens complex will also provide the opportunity to use them in population studies of these species.

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APPENDIX 1. Information on voucher specimens for *Grosmannia alacris* and other *Grosmannia* spp. included in the study. All specimens are represented by living cultures.

Locality/town; province/state; country	Host plant or insect vector	Isolate numbers
<i>Grosmannia alacris</i>		
Holly Springs, Ranger District; Mississippi; USA	<i>Pinus taeda</i>	¹ CLE088
La Garde Freinet, France	<i>P. pinaster</i>	CMW746
Portugal	<i>P. pinaster</i>	CMW621, CMW623
Spain	<i>P. pinaster</i>	CMW259336, CMW25937
Lebanon State Forest, Grabouw; Western Cape; South Africa	<i>P. pinaster</i>	² CMW35, CMW36, CMW202
	<i>Hylastes angustatus</i>	CMW381, CMW382
	<i>Orthotomicus erosus</i>	CMW384
	<i>Hylurgus ligniperda</i>	CMW385
Jonkershoek, Stellenbosch; Western Cape; South Africa	<i>P. radiata</i>	CMW310
Tokai State Forest, Cape Town; Western Cape; South Africa	<i>P. pinaster</i>	CMW2844
Tweefontein, Sabie; Mpumalanga; South Africa	<i>P. taeda</i>	CMW37
Jessievale; Mpumalanga; South Africa	<i>P. patula</i>	CMW6187, CMW6188, CMW7698, CMW7699, CMW7700, CMW7701, CMW7702, CMW7703, CMW7704, CMW7705, CMW7706, CMW7707, CMW7708, CMW7709, CMW7710, CMW7712, CMW7713, CMW7714, CMW7715, CMW7716, CMW7717, CMW7718, CMW7719, CMW7720, CMW7721, CMW7722, CMW7723, CMW7725, CMW7726, CMW7727, CMW7728, CMW7729, CMW7730, CMW7731, CMW7732, CMW7733
<i>Grosmannia serpens</i>		
Italy	<i>P. pinea</i>	^{3,T} CBS 141.36
<i>Leptographium gibbsii</i>		
Yaterley Heath Wood, Hampshire; England; UK	<i>Hylastes ater</i>	CBS 347.90
<i>Leptographium castellanum</i>		
San José de las Matas; Dominican Republic	<i>Pinus occidentalis</i>	CBS 128698
<i>Leptographium yamaokae</i>		
Kofu; Yamanashi; Japan	<i>Pinus densiflora</i>	^T CBS 129732

¹CLE = Culture Collection of Forest Health Dynamics Laboratory, School of Forestry and Wildlife Sciences, Auburn University, Auburn, Alabama 36849, USA.

²CMW = Culture Collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

³CBS = Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

^T = ex-holotype isolates.

Chapter 4

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

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. *Leptographium 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.

Keywords: Cryptic sex, heterothallic, homothallic, *Leptographium procerum*, *Leptogrpahium profanum*, *MAT* locus.

1. Introduction

Sexual reproduction in ascomycetes is governed by the mating type genes residing at the mating type locus (*MAT-1*) (Turgeon and Yoder 2000; Yoder et al. 1986). Although occupying 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 and Glass 1990; Turgeon and 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 (Amselem et al. 2011; Coppin et al. 1997; Kronstad and Staben 1997; Turgeon 1998; Turgeon and Yoder 2000). *MAT1-2* usually contains a single *MAT1-2-1* gene encoding a protein with a high mobility group (HMG) domain (Coppin et al. 1997; Turgeon and Yoder 2000), but in some cases a second gene (*MAT1-2-2*; *MAT1-2-3* or *MAT1-2-4*) can be found within the *MAT1-2* idiomorph (Amselem et al. 2011; Kanamori et al. 2007; 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).

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 either homothallically or heterothallically. Furthermore, such knowledge can be used to develop molecular markers to determine the mating types of isolates, replacing the laborious and time consuming process of developing and crossing mating tester strains (Brewer et al. 2011; Ramirez-Prado et al. 2008; Scherrer et al. 2005; Steenkamp et al. 2000). The ability to identify mating types using molecular markers is also useful to select isolates of opposite mating type for genetic experiments, and facilitates the discovery of sexual states of species known only by their asexual morphs.

The high level of variation in mating type genes offers considerable potential to resolve questions regarding phylogenetic relatedness in species complexes (O'Donnell et al. 2004; Turgeon 1998). 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 (Butler et al. 2009; Fraser and Heitman 2004; Nygren et al. 2011; Yun et al. 1999). 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 (Groenewald et al. 2006; Linde et al. 2003; Rau et al. 2005; Stergiopoulos et al. 2007).

Leptographium procerum and the closely related species, *L. 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 et al. 1988; Wingfield 1986). 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 and 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 et al. (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 et al. (2013) suggested that all 34 *Grosmannia* species be treated in *Leptographium sensu lato*, but to avoid unnecessary name changes, 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 from their asexual states (De Beer and 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.

2. Materials and methods

2.1. Fungal isolates

Isolates of *L. procerum* and *L. 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 October of 2009 and have been preserved in CMW.

2.2. DNA isolation

Fungal mycelium was grown in liquid medium containing 2% malt extract and 0.2% yeast extract (YM broth) for 3-5 days. Mycelium was harvested by centrifugation and lyophilized. DNA was isolated from the lyophilized mycelium using the method described by Aljanabi and Martinez (1997) with minor modifications. After the extraction, DNA was resuspended in 200 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) containing 0.02 mg/ml of RNase A. After 30 minutes 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 3M 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 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.

2.3. Cloning the mating-type idiomorphs from *L. profanum*

2.3.1. Cloning of MAT1-2 in *L. profanum*

Degenerate primers NcHMG1 and NcHMG2 (Arie et al. 1997) were used to obtain partial sequence of HMG-box of *MAT 1-2-1* in *L. profanum*. The PCR mixture, 25 µl total volume, consisted of 2.5 µl 10 × PCR reaction buffer, 2.5 mM MgCl₂, 200 µM each dNTP, 1 µM of each primer, 1 U FastStart *Taq* DNA Polymerase (Roche Diagnostics GmbH, Mannheim, Germany) and 20 to 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 sec, 55 °C annealing for 30 sec, and 72 °C extension for 60 sec, 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 *E. coli* JM109. Plasmids were extracted from the positive clones and sequenced using T6 and 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 (CMW 10550 and CMW 15002). 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 *Hind*III (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 *Hind*III digested fragments gave a signal of over 8 kb. A selection was then made to use *Hind*III-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 *Hind*III 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 and Solovyev 2000) (<http://linux1.softberry.com>). Mating type genes and other Open Reading Frames (ORFs) were identified by BLAST against the NCBI database.

2.3.2. Cloning of *MAT1-1* in *L. 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 MgCl₂, 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 10 cycles of 94 °C for 10 sec, 53 °C annealing for 30 sec, and 68 °C extension for 8 min., followed by 25 cycles of 94 °C for 15 sec, 55 °C annealing for 30 sec, and 68 °C extension for 8 min. (plus 20 sec. 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 and Solovyev 2000). *MAT* genes were identified using the predicted amino acid sequences and BLASTp against the NCBI database.

2.4. Cloning the mating-type idiomorphs of *L. 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 (CMW 45 as *MAT1-2* and CMW 216 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*.

2.5. 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 were compiled and aligned using an online version of MAFFT 6 (Kato and Toh 2008). Neighbor joining analyses were performed using MEGA 5.01 (Tamura et al. 2011) with 1000 bootstrap replicates.

2.6. Development of multiplex PCR-based mating type markers for *L. profanum* and *L. 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'-ATGCCCGATGAAGACTGCT-3') and P-MAT1-R (5'-CTTCCGAATTCCTTGGATT-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 μ l 10 $\times$ PCR reaction buffer, 2.5 mM $MgCl_2$, 200 μ M each dNTP, 0.2 μ M of each primer (both *MAT1-1* and *MAT1-2* primers), 1 U FastStart Taq DNA Polymerase (Roche) and 20 to 50 ng of genomic DNA. PCR cycling conditions were an initial denaturation at 95 $^{\circ}C$ for 5 min, followed by 35 cycles of 95 $^{\circ}C$ for 30 sec, 55 $^{\circ}C$ annealing for 30 sec, and 72 $^{\circ}C$ extension for 60 sec, with a final extension at 72 $^{\circ}C$ 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*.

2.7. 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 *MAT-1* and two *MAT-2*) and ten *L. procerum* isolates (five *MAT-1* and five *MAT-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 one year for the presence of ascomata.

2.8. Distribution of mating-type genes in a population of *L. 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 *P. 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.

3. Results

3.1. Cloning the mating-type idiomorphs in *L. profanum*

3.1.1. Cloning of *MAT1-2* in *L. profanum*

PCR with the degenerate primers NcHMG1 and NcHMG2 yielded expected bands of around 300 bps 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 *EcoRI* and *HindIII* digested fragments, respectively. A partial genomic DNA library was

constructed for *Hind*III-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 number: PENDING) consisting of a *MAT1-2-1* gene (996 pb 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 *SLA* gene flanking the *MAT1-2* idiomorph was also obtained, however neither a DNA lyase gene sequence nor any other open reading frame 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 resided 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 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 will not function fully in mating.

3.1.2. Cloning of *MAT1-1* in *L. 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 number: PENDING). 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 *O. 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 pb) and contained 5 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. procerum* is a heterothallic species.

3.2. Cloning the mating-type idiomorphs in *L. 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 number: PENDING) 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 number: PENDING) 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 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.

3.3. 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).

3.4. Development of multiplex PCR-based mating type markers for *L. profanum* and *L. 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).

3.5. 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.

3.6. Distribution of mating-type genes in a population of *L. procerum*

Both mating types were detected in a population of *L. procerum* from the USA. Surprisingly, the ratio of *MAT1-1* to *MAT1-2* was 10:10 (equally with 1:1 ratio) 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.

4. 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* (Jacobi et al. 2010; Paoletti et al. 2005), 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*.

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* (Martin et al. 2011; Yun et al. 2000), *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 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 that 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* (Unpublished sequence, GenBank number: ACXQ02000048) 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 and 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 (Kanematsu et al. 2007; Wilken et al. 2012; Yokoyama et al. 2003).

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 (Gioti et al. 2012; Nygren et al. 2011; Yun et al. 1999). Whereas, only a few others support an opposing hypothesis: that heterothallism arose from homothallism (Amselem et al. 2011; Geiser et al. 1998). The structure of the *MAT* loci in *L. procerum* and *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, unequal crossover of *MAT1-1* idiomorphs. This concept is often used to explain the rise 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 finally 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 suggested and applied in combination with other nuclear genes to resolve phylogenetic relationships and to describe new species (O'Donnell et al. 2004; Turgeon 1998). 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; Martin et al. 2011; Paoletti et al. 2006; Strandberg et al. 2010).

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 stage 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 investigate sexual reproduction of these fungi. The information of the mating type loci and PCR based mating type makers developed in this study will be valuable in the quest for sexual states and reproduction processes in *L. procerum* and *L. profanum*.

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Table 1. Fungal isolates used in this study

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

^aCMW = Culture Collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

^bCBS = Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands

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.

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

The values are presented as percent of transcript identity/percent of protein identity

- Not applicable (due to the unavailability of sequences)

* Partial sequences were compared (due to the unavailability of complete *O. quercus* MAT sequences)

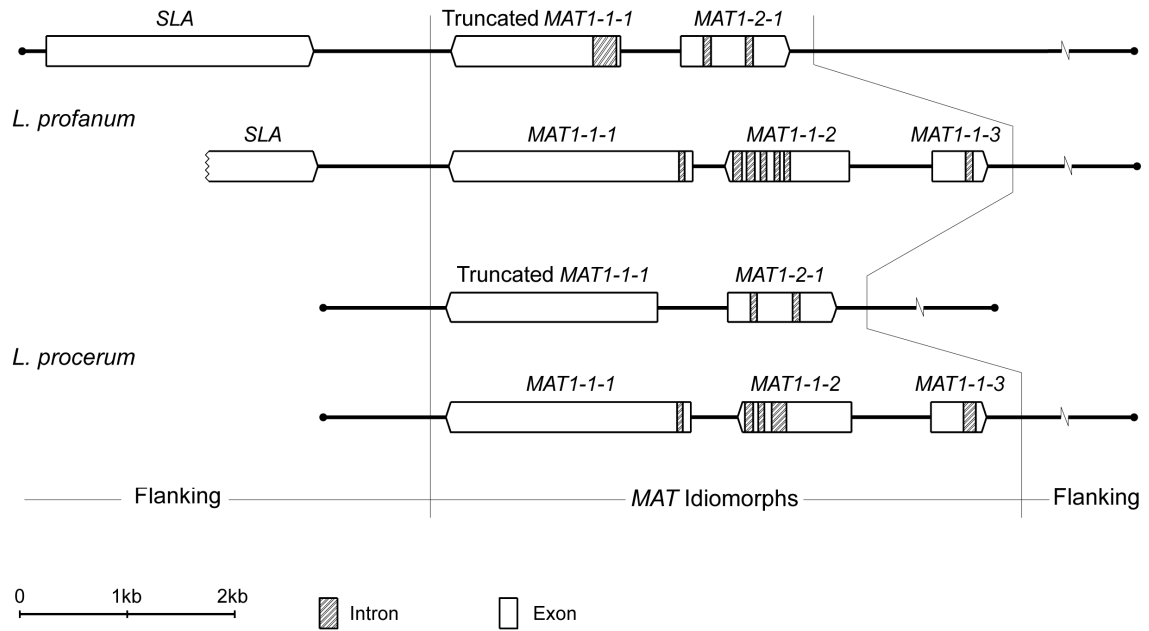


Fig. 1. Organization of the mating type loci of *L. procerum* and *L. profanum*.

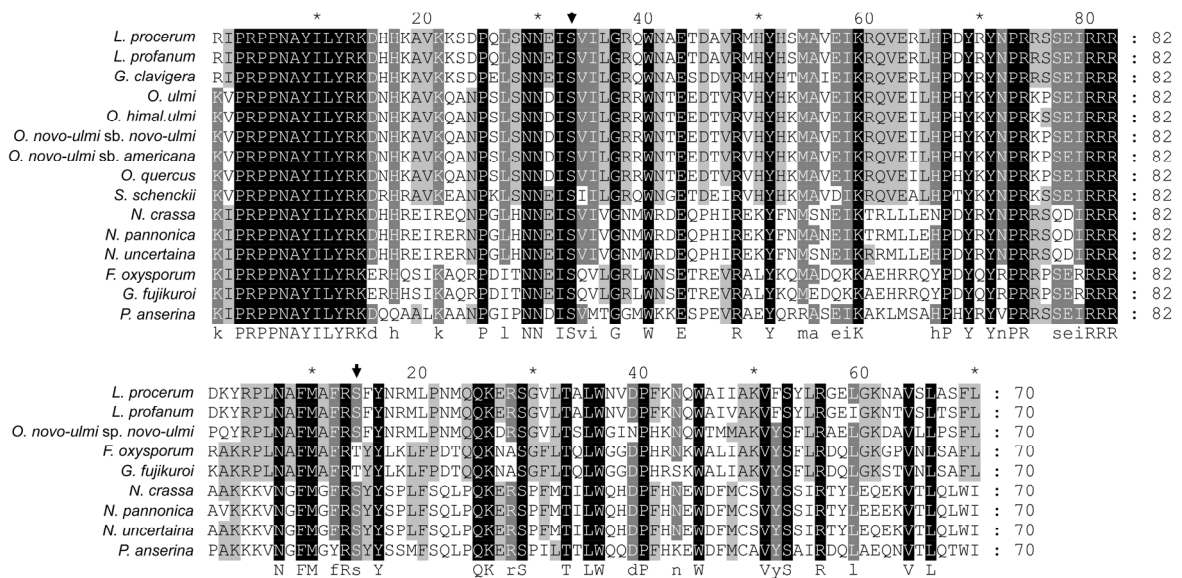


Fig. 2. Alignment of amino acid sequences of the HMG-box domain (top) and the α domain (bottom) from *L. procerum*, *L. profanum* and other ascomycetes. Black arrows indicate intron positions.



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.

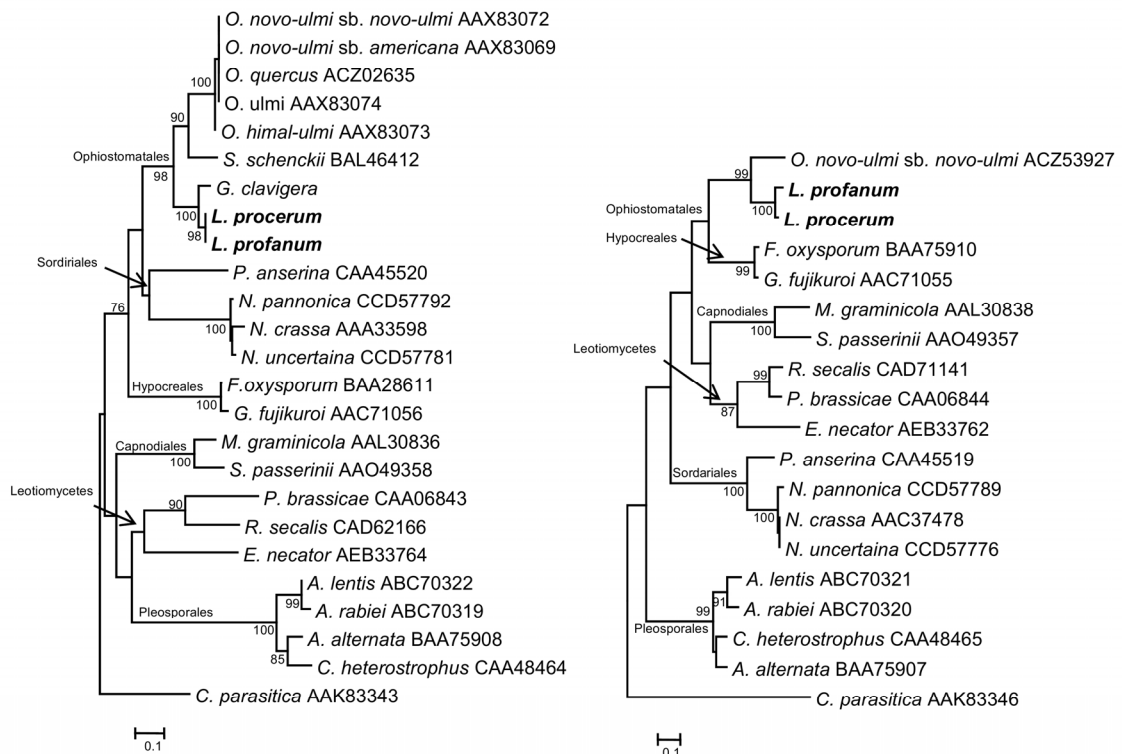


Fig. 4. Neighbour joining trees derived from analysis of the HMG-box domain (left) and α domain (right) amino acid sequences from *L. procerum*, *L. profanum* and other ascomycetes. Bootstrap values (1000 replicates) above 75% are indicated at nodes. GenBank accession numbers are presented after the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

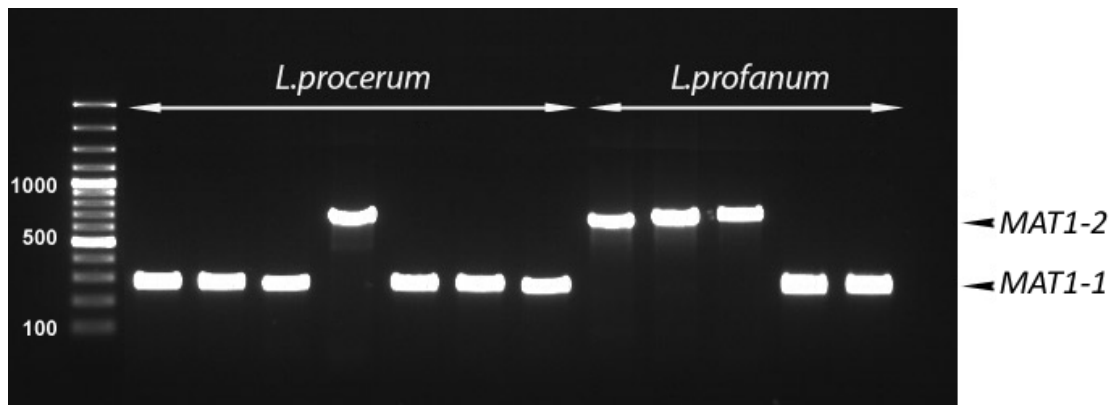


Fig. 5. Results obtained from a multiplex PCR assay to determine the mating genotype of isolates of *L. procerum* and *L. profanum*. From left to right, DNA samples from *L. procerum* CMW10; CMW12; CMW13; CMW45; CMW216; CMW10216; CMW25627; and *L. profanum* CMW10550; CMW10552; CMW10553; CMW10554; CMW10555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

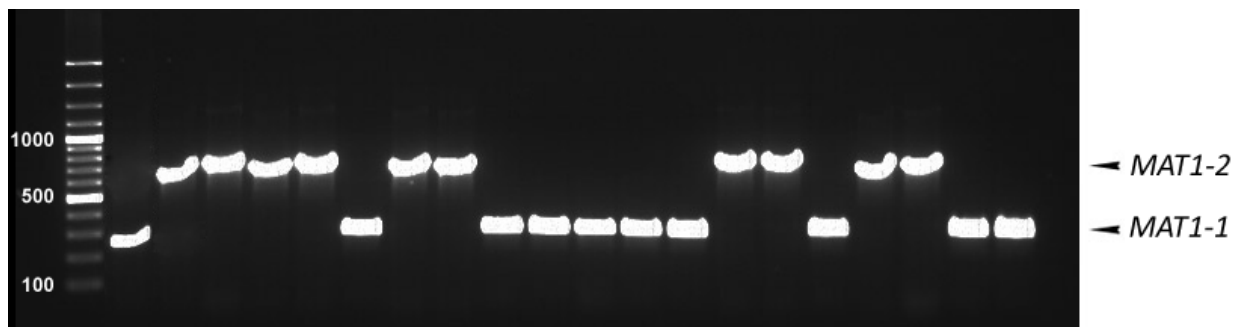


Fig. 6. PCR amplicons resulting from the multiplex PCR (using *MAT1-1* and *MAT2-1* primers) of a population of *L. procerum* isolates from the USA. The ratio of *MAT1-2* and *MAT1-2* isolates equivalent to 1:1 infers that sexual reproduction is occurring or has recently taken place within this population.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

Chapter 5

Mating type markers reveal unexpected patterns of sexual compatibility in *Leptographium sensu lato*

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 root diseases in conifers. Sexual states have been reported for more than 30 species in this group and following the dual nomenclature system, these have been treated in the teleomorph genus *Grosmannia*. For at least 59 additional species, no sexual state is known and these reside in the anamorph 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 success rate. The aim of this study was to develop markers 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 40 species and to determine thallism, in many species for the first time. Surprisingly, the results showed that heterothallic species are abundant (37 out of 40 species) in *Leptographium sensu lato*, with only three species found to be homothallic. Furthermore, a phylogeny based on *MAT* genes provided strong resolution for this group of fungi, when compared with phylogenies based on the ribosomal DNA.

Keywords: Heterothallism, homothallism, *Leptographium sensu lato*, *Grosmannia*, Mating type.

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 teleomorph 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 59 species 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 the 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 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).

The high level of sequence variability among species has led to suggestions that *MAT* gene sequences might serve as effective phylogenetic characters for species delimitation (O'Donnell et al. 2004; Turgeon 1998). In contrast to the high levels of sequence variability between species, *MAT* genes have low sequence variability within species, making them ideal where species complexes must be resolved (Barve et al. 2003). Thus, phylogenies based on *MAT* gene sequences can be concordant with those for nuclear genes (O'Donnell et al. 2004), and in some cases, have provided better resolutions for problematic species complexes (Du et al. 2005).

Mating type gene sequences and the structure of the *MAT* locus are known for only three species of *Leptographium sensu lato* (including *Grosmannia*). These include *Grosmannia clavigera* (DiGuistini et al. 2011), *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 three of these species have a truncated version of *MAT1-1-1* lacking the functional alpha domain (Duong et al. 2013). By determining the distribution of the mating type of isolates from a natural population of *L. procerum*, Duong et al. (2013) suggested that sexual recombination has occurred in this population.

Most species in *Leptographium sensu lato* are known as only mitosporic fungi. Based on the results of Duong 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. Therefore, the aims of this study were 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 species of *Leptographium sensu lato*. The possibility of using *MAT* genes for phylogenetic inference and thus to resolve species complexes in *Leptographium sensu lato* was also considered.

Material and methods

Cultures, growth condition 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. 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).

MAT primer design

The sequences of *MAT1-2-1* locus in *G. clavigera* (GenBank number: ACXQ02000048) (DiGuistini et al. 2011), *L. procerum* (GenBank pending), *L. profanum* (GenBank pending) (Duong et al. 2013), *O. ulmi* (GenBank number: AY887031) and *O. novo-ulmi* subsp. *novo-ulmi* (GenBank number: AY887029) (Paoletti et al. 2005a) were aligned and used to design *MAT1-2* primers. 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 for detection of *MAT1-1* idiomorph were thus designed from the *MAT1-1-3* gene, which appears to be commonly present in *Leptographium sensu lato* (Duong et al. 2013). The primers Oph-MAT1F1 (5'- ATGKCCRATGARGAYTGCT -3') and Oph-MAT1R2 (5'- GGCGKTKGCRTTGAYTTGTA -3') were designed based on sequences of *MAT1-1-3* gene in *L. procerum* (GenBank pending), *L. profanum* (GenBank pending) (Duong et al. 2013), *O. novo-ulmi* (GenBank number: EU163846) (Jacobi et al. 2010) to amplify part of the *MAT1-1-3* gene. In some species, where amplification with this primer combination failed, primer Oph-MAT1R1 (5'- GGCYYTRTGAAGYTTCTGTGC -3') was used in combination with Oph-MAT1F1, although this combination resulted in slightly shorter fragments.

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 MgCl₂, 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 protocols. 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 and other isolates of that same species showed null-amplification for either of the *MAT* markers, were considered to be putatively heterothallic.

Phylogenetic analyses

Sequences of ITS2-LSU of all species included in this study were obtained from GenBank. All datasets for the ITS2-LSU, *MAT1-1-3* and *MAT1-2-1* gene regions in all of the species considered in this study were compiled in MEGA5.1 (Tamura et al. 2011) and aligned using an online version of MAFFT v. 6 (Kato and Toh 2008). Bayesian inference (BI) and maximum parsimony (MP) analyses were performed for each of the datasets separately. To investigate the potential to use *MAT* gene regions as phylogenetic characters, the dataset for the

MAT1-1-3 gene of four species in the *G. serpens* complex (Duong et al 2012) together with *MAT1-1-3* sequences of *Leptographium wagneri* var. *wagneri* and *Leptographium wagneri* var. *pseudotsugae*, which were used as outgroups, were aligned and subjected to BI and MP analyses.

Bayesian inference analyses were performed using MrBayes v. 3.1.2 (Ronquist and Huelsenbeck 2003). Models for the BI analyses were adapted based on the best substitution models as determined by jModelTest v. 0.1.1 (Posada 2008). Four MCMC chains were run simultaneously for 5 million generations and tree sampling was conducted after every 100th generation. The burn-in phase was determined by plotting the likelihood scores (LnL) against the number of generations. Trees sampled in the burn-in phase were discarded. Posterior probabilities were calculated from a majority rule consensus tree regenerated from the remaining trees.

Maximum parsimony analyses were performed using PAUP v. 4.0b10 (Swofford 2002). Heuristic searches were performed with one thousand random stepwise additions and tree-bisection-reconnection (TBR) was used as the branch-swapping algorithm. All characters were unordered and of equal weight. Gaps were treated as a fifth character. All equally parsimonious trees were saved and considered. Tree length (TL), consistency index (CI), retention index (RI) were calculated. The robustness of resultant trees was evaluated by 1000 bootstrap replications.

Results

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 the case of *Grosmannia europioides*, *G. aenigmaticum* and *G. europioides*, primers Oph-MAT1R1 were successfully used in place of Oph-MAT1R2, resulting in slightly shorter PCR products. The relative primer binding positions on each of the *MAT* locus are presented in Fig. 1.

In most cases, a single PCR product was obtained from each positive reaction. The identities of PCR products were confirmed as *MAT1-2-1* or *MAT1-1-3* from sequences of all positive amplifications. All obtained sequences were deposited in GenBank and accession numbers are presented in Table 1. Portions of *MAT* genes for a total of 40 species residing in *Leptographium sensu lato* species were successfully amplified (Table 1). Based on the amplification profile, 16 species were identified as heterothallic, three species were homothallic and 21 species were tentatively assigned as being heterothallic. Examples of *MAT* PCR amplification for some of the tested isolates are presented in Fig. 2.

ITS2-LSU phylogeny

The dataset for the ITS2-LSU contained 600 characters including alignment gaps, in which 98 characters were parsimony informative. Performing jModelTest on the ITS2-LSU data resulted in TrN+G as the best fit model. Since MrBayes does not support TrN+G, the similar model GTR+G was implemented. The stationarity was reached at about 26000 generations and, therefore, 260 trees sampled in the burn-in phase were discarded. The consensus tree was computed and posterior probabilities calculated from the remaining 99480 trees. The phylogram obtained from BI analysis is presented in Fig. 3 with Bayesian posterior probabilities presented at the nodes.

Maximum parsimony analysis using ITS2-LSU dataset resulted in 20 equally parsimonious trees of 196 steps. The consistency index (CI) and retention index (RI) recorded were 0.77 and 0.917 respectively. The topologies of the MP trees were similar to that arising from BI analysis. High bootstrap supports were also found for major clades representing species complexes in the *Leptographium sensu lato* (Fig. 3).

MAT phylogenies

Phylogeny using the MAT1-2-1 gene

The *MAT1-2-1* dataset contained 34 sequences representing 30 different species. The dataset contained 212 characters including alignment gaps, in which 124 characters were parsimony informative. The model TIM2ef+I+G was suggested as the best fit model by JModelTest. Bayesian analysis implemented in this model reached stationary phase after 50000 generations and, therefore, 500 trees sampled at the burn-in phase were discarded. The consensus tree was computed and posterior probabilities were calculated from the

remaining 99000 trees. The phylogram obtained from BI analysis (Fig. 4) is presented with Bayesian posterior probabilities at the nodes. Maximum parsimony analysis using the *MAT1-2-1* dataset resulted in three equally parsimonious trees of 336 steps. The consistency index (CI) and retention index (RI) recorded were 0.637 and 0.883 respectively. Topologies of the MP trees were similar to one another for the major clades. Strong bootstrap support was evident for most of the nodes supported by BI analysis (Fig. 4). There were only two nodes that were supported in the BI analysis but not with MP analysis and one which was supported by MP analysis but not with BI analysis (Fig. 4). Most species complexes as defined by De Beer & Wingfield (2013) and supported in our ITS2-LSU phylogeny (Fig. 3), were also supported in the *MAT1-2-1* phylogeny (Fig. 4). The exception was the *L. lundbergii* complex that was not supported in by both data sets. Support for clades representing species complexes was higher in the *MAT1-2-1* phylogeny than was the case for the ITS2-LSU phylogeny (Fig. 3).

Phylogeny using the MAT1-1-3 gene

The *MAT1-1-3* dataset contained 34 sequences representing 30 different species. The dataset contained 490 characters including alignment gaps, in which 293 characters were parsimony informative. The model TPM1uf+G was suggested as the best fit model by JModelTest. Bayesian analysis implemented in this model reached the stationary phase after 10000 generations. Thus, 100 trees sampled in the burn-in phase were discarded. The consensus tree was computed and posterior probabilities were calculated from the remaining 99800 trees. The phylogram obtained from BI analysis (Fig. 5) is presented with Bayesian posterior probabilities at the nodes.

The phylogenetic tree emerging from analyses for *MAT1-1-3* dataset (Fig. 5) represented well supported clades similar to those emerging from analyses using the ITS2-LSU and *MAT1-2-1* datasets (Figs 3 & 4). Maximum parsimony analysis using the *MAT1-1-3* dataset resulted in 26 equally parsimonious trees of 860 steps. The consistency index (CI) and retention index (RI) were 0.585 and 0.824 respectively. The topologies were largely similar between the 26 parsimonious trees and congruent with that from the BI analysis. Most of the nodes supported by BI analysis were also well supported in the MP analysis with high bootstrap values (Fig. 5). There were only four nodes that were supported with BI analysis but not with MP analysis and one which was supported by MP analysis but not in the BI analysis. In the

MAT1-1-3 tree, species residing in the *L. lundbergii* complex *sensu* De Beer & Wingfield (2013) grouped together, but without statistical support, while all the other species complexes were well supported.

Phylogeny using the MAT1-1-3 gene from species in G. serpens complex

The dataset including *MAT1-1-3* sequences from an extensive group of isolates representing species in the *G. serpens* complex (Duong et al. 2012) contained 20 partial *MAT1-1-3* sequences from four species, including *Grosmannia alacris*, *G. serpens*, *Leptographium castellanum* and *L. yamaokae*. Only two isolates of *L. gibbsii*, the fifth species in the *G. serpens* complex, were available for study, and both of these contained only *MAT1-2-1* sequences.

The dataset contained 448 characters including alignment gaps, in which 29 characters were parsimony informative. The model HKY was suggested by JModelTest as the best fit model of this dataset. Bayesian analysis implementing this model reached stationary phase after 5000 generations and, therefore, 50 trees sampled in the burn-in phase were discarded. The consensus tree was computed and posterior probabilities were calculated from the remaining 9950 trees. The BI phylogram with posterior probability values at nodes is presented in Fig. 6. Three of the four species formed well supported clades including those for *G. alacris*, *L. castellanum* and *L. yamaokae*. The clade for *G. serpens* was poorly resolved but *G. serpens* isolates grouped together and formed a well supported sister clade with *G. alacris* isolates. Maximum parsimony analysis resulted in a single most parsimonious tree of 29 steps, with CI of 1 and RI of 1. The topology of MP tree was the same to that of BI tree. Levels of bootstrap support emerging from the MP analysis are presented at nodes (Fig. 6).

Discussion

Molecular markers were developed in this study making it possible to amplify and identify the mating types of 40 species residing in *Leptographium sensu lato*. Many species previously considered to be asexual were shown to be potentially heterothallic, with individual isolates containing only a single idiomorph. It will now be possible to attempt to induce sexual structures for these fungi in culture, using isolates known to represent compatible mating strains. Where this can be achieved, various genetic studies can be undertaken on these species that would otherwise not have been possible.

Prior to this study, the mating types of only three species residing in *Leptographium sensu lato* were known. It was particularly interesting that the majority of species tested were heterothallic and this was in contrast to a relatively small number (three) of homothallic species detected. This finding is intriguing but is also 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).

The results of this study are similar to those for other groups of fungi where species thought to be asexual have been shown to exist in nature as heterothallic, and sexual states have been recently discovered for some of those species (Horn et al. 2009; O’Gorman et al. 2008; Seidl et al. 2009). In native populations of these fungi where two mating types are present but sexual structures absent, it is reasonable to assume that cryptic sexual reproduction is occurring. In the case of *Leptographium sensu lato* and based on the results of this study, we believe that many species found only in the asexual form in nature probably have sexual states. However, these 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.

Duong et al. (2012) were able to show that *G. alacris* was 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 states 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 this 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 can be found. But the results clarify 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 occurred 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).

The *MAT* phylogenies generated in this study showed some levels of incongruence with one another and also with a phylogeny based on the ITS2-LSU gene region. However, in most of the cases, species residing in the same complex as defined by ITS2-LSU resided in similar groups to those emerging from analyses based on sequences for the *MAT1-1-3* and *MAT1-2-1* phylogenies. The *MAT* phylogenies provided better resolution at the terminal nodes when compared to that obtained from the ribosomal DNA sequences. Phylogenetic analyses for species in the *G. serpens* complex based on *MAT 1-1-3* sequences were also robust and concurred with those emerging from a multiple gene phylogeny produced by Duong et al. (2012). Although the *MAT* gene phylogeny for species in the *G. serpens* complex did not offer more robust resolution when compared to that for the protein coding genes (Duong et al. 2012), the *MAT* genes appear to provide an additional gene region to be used for phylogenetic analyses when studying species of *Leptographium sensu lato* and their relatives.

Species in *Leptographium sensu lato* have been assigned to different species complexes based on their relatedness in phylogeny, morphological characters, as well as their ecology (De Beer and Wingfield 2013; Linnakoski et al. 2012). As revealed by the present work, species belonging to the same species complex always share the same mode of sexual reproduction. The only three homothallic species (*G. europhioides*, *G. piceiperda* and *G. aenigmatica*) considered in this study always grouped into a single, well supported clade, known as the *G. piceiperda* complex (De Beer and Wingfield 2013; Linnakoski et al. 2012). This suggests that these species might share a common homothallic ancestor. The remaining 37 heterothallic or putatively heterothallic species reside in six different species complexes. A number of other species belonging to these six species complexes were not included in this study. However, based on the patterns observed, it is likely that they will also have heterothallic mating systems.

The unique pattern of distribution of homothallic and heterothallic species gives insight into the origin and evolution of homothallism and heterothallism in *Leptographium sensu lato*.

The pattern of distribution of sexual compatibilities has 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). Considering the pattern observed in *Leptographium sensu lato* in the present study, it is reasonable to hypothesise that homothallism in *G. piceiperda* complex has evolved once from a heterothallic ancestor. A common heterothallic ancestor would thus best explain the current pattern 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 from closely related heterothallic species such as those in the *G. clavigera* and *L. lundbergii* complexes, will be required to support this hypothesis.

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Table 1. Fungal isolates used in this study, including Genbank accession number of partial sequences of the *MAT1-2-1* and *MAT1-1-3* as well as the thallism assigned to the isolates. Abbreviations and symbols used in the table are explained in the footnote of the table.

Species	Isolate number	Insect/Host	Origin	<i>MAT1-2-1</i>	<i>MAT1-1-3</i>	Thallism
<i>G. aenigmatica</i>	CMW2199	<i>Ips typographus japonicus</i>	Japan	PENDING	PENDING(*)	HO
<i>G. alacris</i>	CMW1136	<i>Pinus taeda</i>	USA	PENDING		HE
<i>G. alacris</i>	CMW202	<i>P. pinaster</i>	South Africa		PENDING	HE
<i>G. alacris</i>	CMW310	<i>P. radiata</i>	South Africa		PENDING	HE
<i>G. alacris</i>	CMW36	<i>P. pinaster</i>	South Africa		PENDING	HE
<i>G. alacris</i>	CMW37	<i>P. pinaster</i>	South Africa		PENDING	HE
<i>G. alacris</i>	CMW621	<i>P. pinaster</i>	Portugal	PENDING		HE
<i>G. alacris</i>	CMW7726	<i>Hylastes angustatus</i>	South Africa		PENDING	HE
<i>G. americana</i>	CMW2980	<i>Larix</i> sp.	USA	PENDING		P-HE
<i>G. aurea</i>	CMW714	<i>P. contorta</i> var. <i>latifolia</i>	Canada		PENDING	P-HE
<i>G. clavigera</i>	KW1407			ACXQ02000048		HE
<i>G. europheoides</i>	CMW446	Unknown	Unknown	PENDING	PENDING(*)	HO
<i>G. koreana</i>	CMW14200	<i>P. densiflora</i>	Korea	PENDING		HE
<i>G. koreana</i>	CMW14201	<i>P. densiflora</i>	Korea		PENDING	HE
<i>G. piceiperda</i>	CMW452	<i>Pseudotsuga menziesii</i>	Unknown	PENDING		HO
<i>G. piceiperda</i>	CMW2811	<i>Picea rubens</i>	Unknown	PENDING	PENDING(*)	HO
<i>G. robusta</i>	CMW34175	<i>P. ponderosa</i>	USA		PENDING	P-HE
<i>G. robusta</i>	CMW710	Unknown	Unknown		PENDING	P-HE
<i>G. serpens</i>	CMW191	<i>P. pinea</i>	Italy		PENDING	P-HE
<i>G. serpens</i>	CMW192	<i>P. pinea</i>	Italy		PENDING	P-HE

Species	Isolate number	Insect/Host	Origin	MAT1-2-1	MAT1-1-3	Thallism
<i>G. serpens</i>	CMW289	<i>P. pinea</i>	Italy		PENDING	P-HE
<i>G. serpens</i>	CMW290	<i>P. pinea</i>	Italy		PENDING	P-HE
<i>G. serpens</i>	CMW304	<i>P. sylvestris</i>	Italy		PENDING	P-HE
<i>G. huntii</i>	CMW1006	<i>Hylurgus ligniperda</i>	Unknown	PENDING		HE
<i>G. huntii</i>	CMW1015	Unknown	Unknown		PENDING	HE
<i>G. huntii</i>	CMW2824	<i>Pinus</i> sp.	USA	PENDING		HE
<i>G. huntii</i>	CMW622	<i>P. pinaster</i>	Portugal		PENDING	HE
<i>G. huntii</i>	CMW654	<i>Pinus</i> sp.	USA	PENDING		HE
<i>G. wagneri</i>	CMW53	<i>P. ponderosa</i>	USA	PENDING		P-HE
<i>L. abieticolens</i>	CMW2866	Balsam fir	USA		PENDING	P-HE
<i>L. abietinum</i>	CMW2817	<i>Picea engelmannii</i>	USA	PENDING		P-HE
<i>L. albopini</i>	CMW2065	<i>P. strobus</i>	USA	PENDING		P-HE
<i>L. alethinum</i>	CMW3767	<i>Hylobius abietis</i>	UK		PENDING	P-HE
<i>L. aureum</i>	CMW29869	<i>P. contorta</i>	Canada	PENDING		P-HE
<i>L. bhutanense</i>	CMW18650	<i>P. wallichiana</i>	Bhutan		PENDING	HE
<i>L. bhutanense</i>	CMW18652	<i>P. wallichiana</i>	Bhutan	PENDING		HE
<i>L. castellanum</i>	CMW1988	<i>Hylurgus mickliki</i>	Spain		PENDING	P-HE
<i>L. castellanum</i>	CMW1989	<i>H. mickliki</i>	Spain		PENDING	P-HE
<i>L. castellanum</i>	CMW2320	<i>P. occidentalis</i>	Dominican Rep.		PENDING	P-HE
<i>L. castellanum</i>	CMW2321	<i>P. occidentalis</i>	Dominican Rep.		PENDING	P-HE
<i>L. celere</i>	CMW12421	<i>P. semaonensis</i>	China	PENDING		P-HE
<i>L. gibbsii</i>	CMW1376	<i>H. ater</i>	UK	PENDING		P-HE
<i>L. gibbsii</i>	CMW853	<i>H. ater</i>	UK	PENDING		P-HE

Species	Isolate number	Insect/Host	Origin	MAT1-2-1	MAT1-1-3	Thallism
<i>L. gracile</i>	CMW12316	<i>P. armandii</i>	China	PENDING		P-HE
<i>L. latens</i>	CMW12319	<i>P. armandii</i>	China	PENDING		P-HE
<i>L. longiclavatum</i>	CMW20606	<i>Picea glauca</i>	Canada	PENDING		HE
<i>L. longiclavatum</i>	CMW20607	<i>P. contorta</i>	Canada		PENDING	HE
<i>L. lundbergii</i>	CMW217	Unknown	Unknown		PENDING	HE
<i>L. lundbergii</i>	CMW2190	<i>P. sylvestris</i>	Norway	PENDING		HE
<i>L. manifestum</i>	CMW12436	<i>Larix olgensis</i>	China		PENDING	P-HE
<i>L. pineti</i>	CMW3837	<i>Pinus</i> sp.	Indonesia		PENDING	P-HE
<i>L. pini-densiflora</i>	CMW5157	<i>P. densiflora</i>	Japan		PENDING	HE
<i>L. pini-densiflora</i>	CMW5162	<i>P. densiflora</i>	Japan	PENDING		HE
<i>L. procerum</i>	CMW216	<i>P. taeda</i>	South Africa		PENDING	HE
<i>L. procerum</i>	CMW45	<i>P. sylvestris</i>	USA	PENDING		HE
<i>L. profanum</i>	CMW10552	Hickory	USA	PENDING		HE
<i>L. profanum</i>	CMW10555	<i>Nyssa sylvatica</i>	USA		PENDING	HE
<i>L. pyrinium</i>	CMW169	<i>P. resinosa</i>	USA	PENDING		HE
<i>L. pyrinium</i>	CMW3889	<i>P. jeffreyi</i>	USA		PENDING	HE
<i>L. sinoprocerum</i>	CMW26230	Unknown	China		PENDING	HE
<i>L. sinoprocerum</i>	CMW26231	Unknown	China	PENDING		HE
<i>L. terebrantis</i>	CMW30731	<i>P. sylvestris</i>	USA	PENDING		P-HE
<i>L. truncatum</i>	CMW2402	<i>P. resinosa</i>	Canada		PENDING	HE
<i>L. truncatum</i>	CMW644	<i>Hylastes</i> sp.	UK	PENDING		HE
<i>L. wagneri</i> v. <i>pseudotsugae</i>	CMW1533	<i>P. menziesii</i>	USA		PENDING	P-HE
<i>L. wagneri</i> v. <i>pseudotsugae</i>	CMW1541	<i>P. menziesii</i>	USA		PENDING	P-HE

Species	Isolate number	Insect/Host	Origin	MAT1-2-1	MAT1-1-3	Thallism
<i>L. wagneri</i> v. <i>wagneri</i>	CMW1828	<i>P. edulis</i>	USA		PENDING	P-HE
<i>L. wingfieldii</i>	CMW10221	<i>P. strobus</i>	USA	PENDING		HE
<i>L. wingfieldii</i>	CMW2096	<i>P. sylvestris</i>	France		PENDING	HE
<i>L. yamaokae</i>	CMW1935	<i>Pinus</i> sp.	Japan		PENDING	P-HE
<i>L. yamaokae</i>	CMW1944	<i>Pinus</i> sp.	Japan		PENDING	P-HE
<i>L. yamaokae</i>	CMW4726	<i>P. densiflora</i>	Japan		PENDING	P-HE
<i>L. yamaokae</i>	CMW4727	<i>P. densiflora</i>	Japan		PENDING	P-HE
<i>L. yamaokae</i>	CMW4728	<i>P. densiflora</i>	Japan		PENDING	P-HE
<i>L. yamaokae</i>	CMW4729	<i>P. densiflora</i>	Japan		PENDING	P-HE
<i>L. yunnanensis</i>	CMW5152	<i>P. yunnanensis</i>	China	PENDING		P-HE
<i>Leptographium</i> sp. X	CMW15470	<i>P. contorta</i>	Canada	PENDING		HE
<i>Leptographium</i> sp. X	CMW15493	<i>P. contorta</i>	Canada		PENDING	HE
<i>O. novo-ulmi</i>	CMW10573	<i>Picea abies</i>	Australia			HE
<i>O. novo-ulmi</i> sub. <i>novo-ulmi</i> H327					FJ858801	HE
<i>O. novo-ulmi</i> sub. <i>novo-ulmi</i> V19				AY887029		HE
<i>O. quercus</i>	CMW27847	<i>Quercus</i> sp.	UK	FJ865429		HE
<i>O. quercus</i>	CMW27845	<i>Quercus</i> sp.	Canada		JQ319596	HE

CMW = Culture Collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

HE = Heterothallic; HO = Homothallic; P-HE = Putative heterothallic; (*) = PCR amplification and sequencing was done with Oph-MAT1F1 and Oph-MAT1R1.

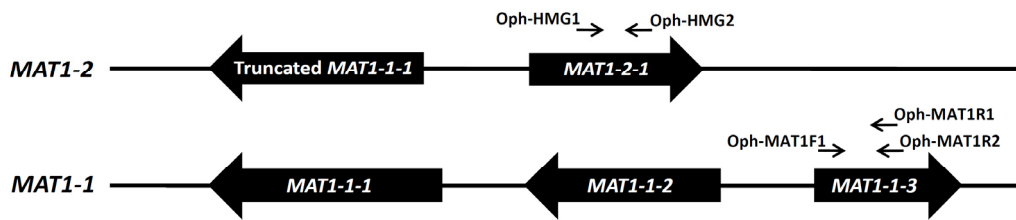


Fig. 1. Relative binding sites of primers used to amplify *MAT1-1* and *MAT1-2* in this study. The organization of *MAT* locus presented in the figure was adapted from *MAT* loci in *L. procerum* and *L. profanum*.



Fig. 2. Simultaneous agarose electrophoresis (1.5 % w/v) of *MAT1-1-3* (higher bands - around 450 bp) and *MAT1-2-1* (lower bands, about 230 pb) PCR fragments from representative *Leptographium sensu lato* species. *G. alacris*: CMW1136 (1), CMW202 (2); *G. europhioides* CMW446 (3); *G. aenigmatica* CMW2199 (4); *G. piceiperda* CMW2811 (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); *G. wagneri* CMW53 (14); *L. wagneri* var. *pseudotsugae* CMW1533 (15); *L. wagneri* var. *wagneri* CMW1828 (16). The molecular weight marker used was GeneRuler™ 100 bp Plus DNA Ladder (Fermentas).

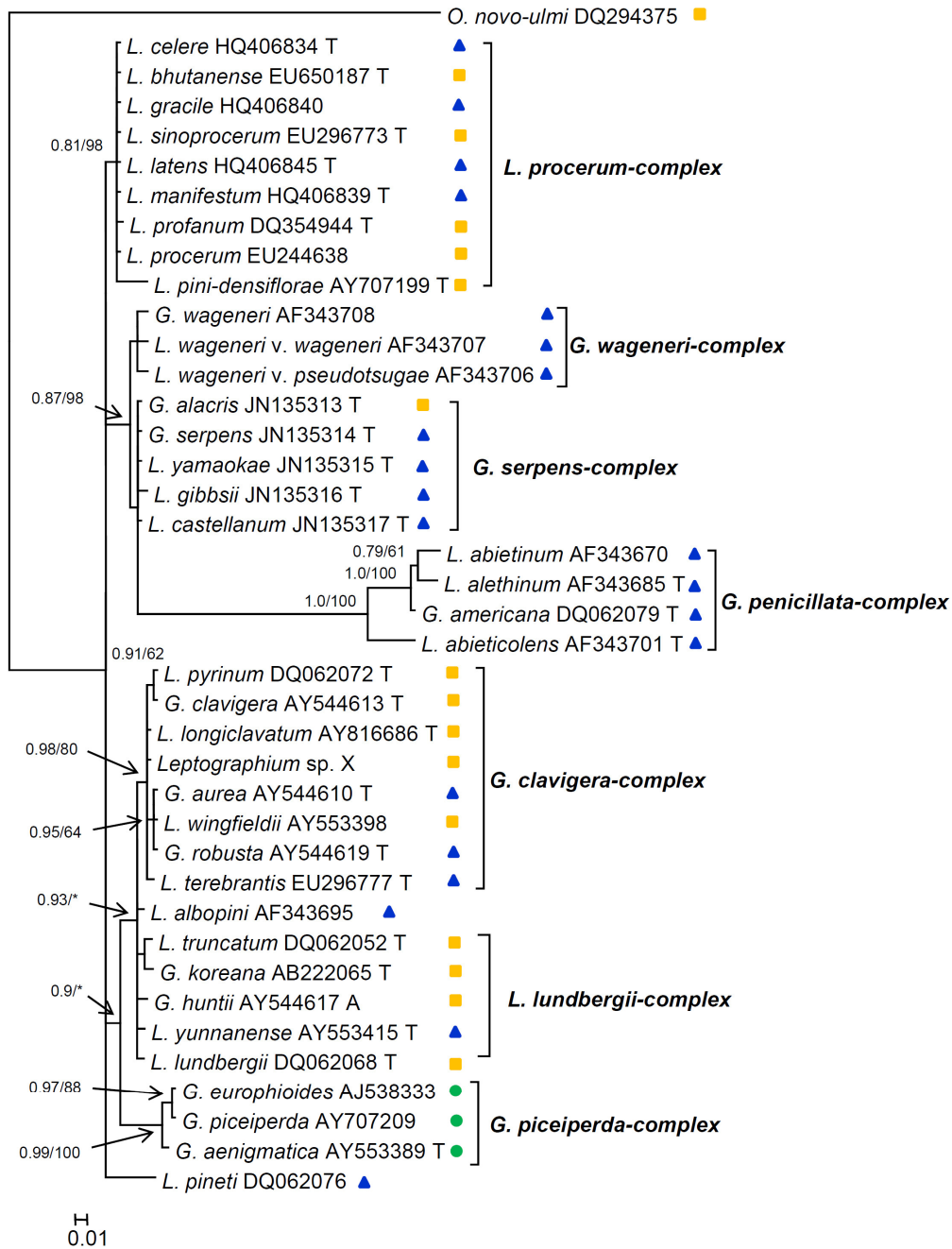


Fig. 3. Phylogram derived from Bayesian analysis of ITS2-LSU dataset. Phylogenetic support is presented at nodes as Bayesian posterior probabilities (≥ 0.8) / parsimony bootstrap ($\geq 60\%$). * = Bayesian posterior probabilities ≤ 0.8 or bootstrap values $\leq 60\%$. Homothallic species are marked with green round circles, heterothallic species are marked with yellow squares and putatively heterothallic species are marked with blue triangles. GenBank accession numbers are presented next to the species name.

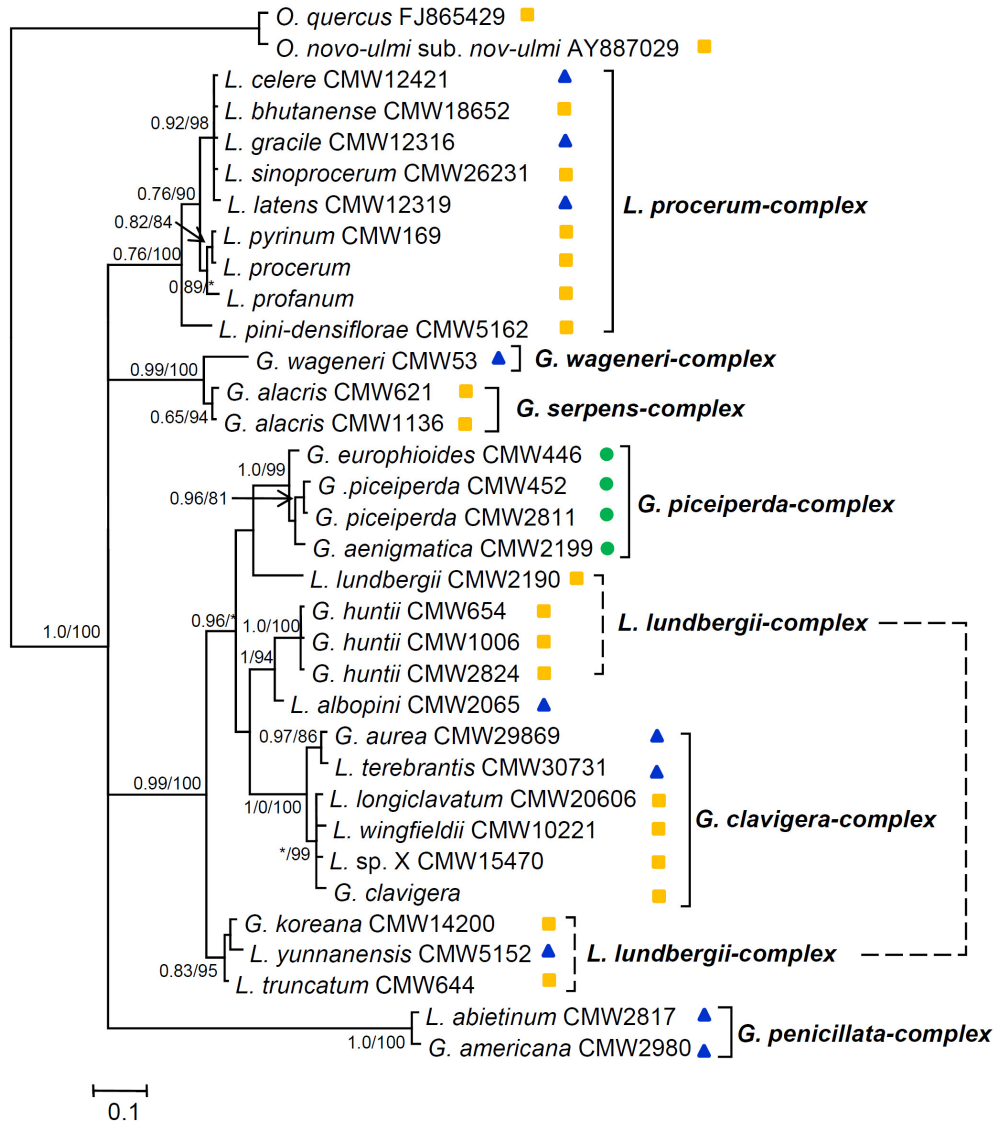


Fig. 4. Phylogram derived from Bayesian analysis of *MAT1-2-1* dataset. Phylogenetic supports are presented at nodes as Bayesian posterior probabilities (≥ 0.8) / parsimony bootstrap ($\geq 60\%$). * = Bayesian posterior probabilities ≤ 0.8 or bootstrap values $\leq 60\%$. Homothallic species are marked with green round circles, heterothallic species are marked with yellow squares and putative heterothallic species are marked with blue triangles.

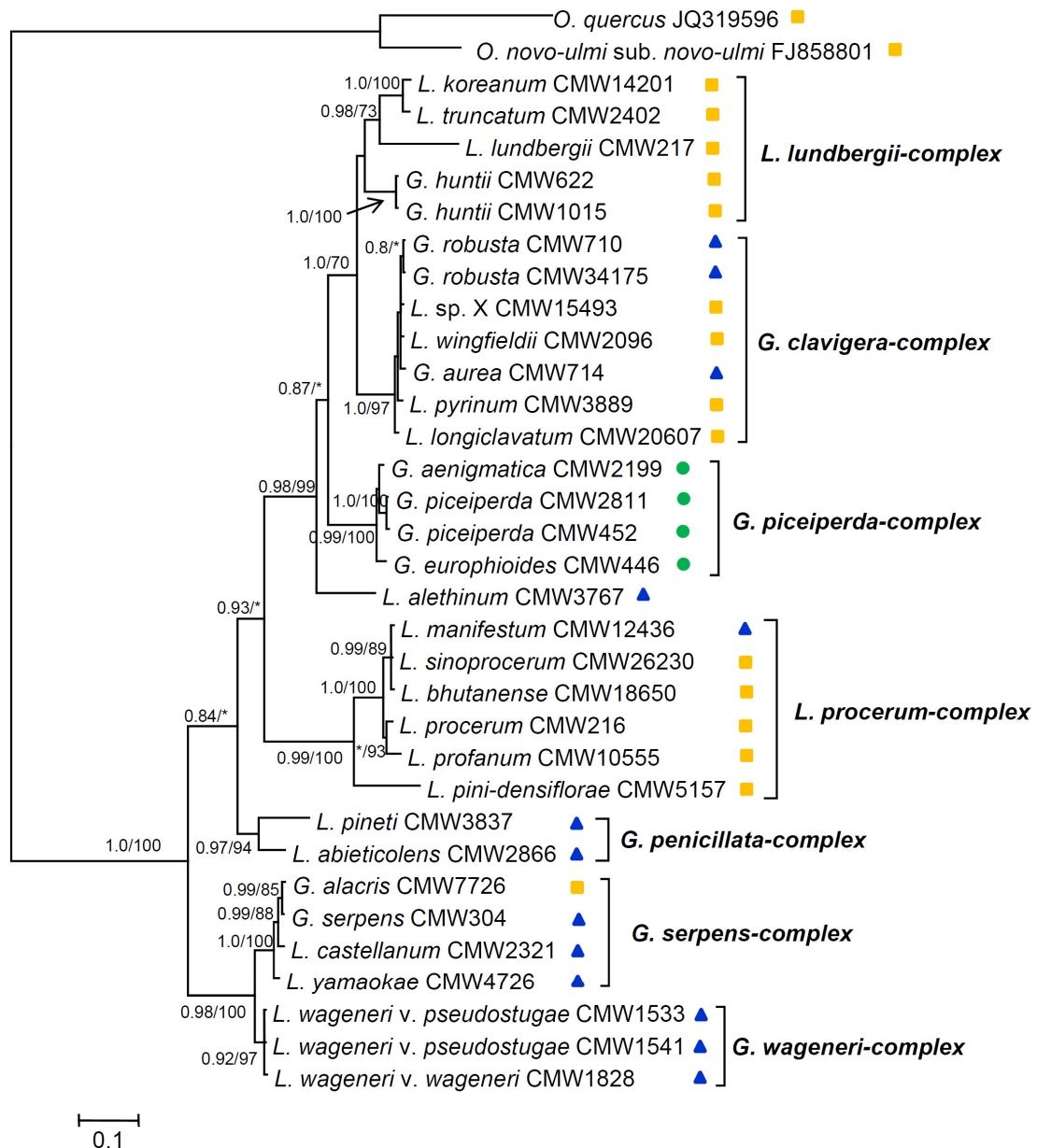


Fig. 5. Phylogram derived from Bayesian analysis of *MAT1-1-3* dataset. Phylogenetic supports are presented at nodes as Bayesian posterior probabilities (≥ 0.8) / parsimony bootstrap ($\geq 60\%$). * = Bayesian posterior probabilities ≤ 0.8 or bootstrap values $\leq 60\%$. Homothallic species are marked with green round circles, heterothallic species are marked with yellow squares and putative heterothallic species are marked with blue triangles.

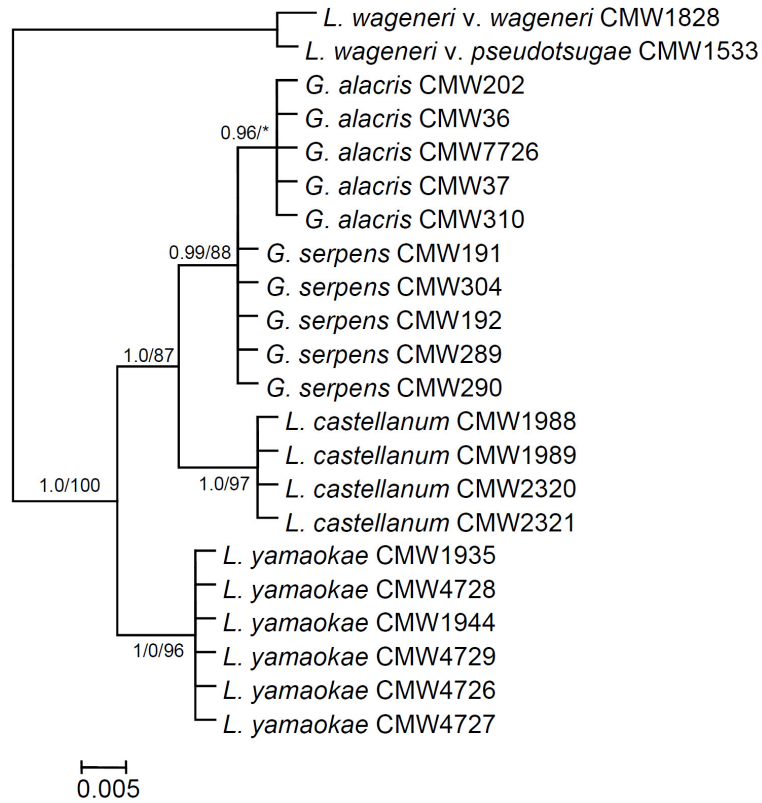


Fig. 6. Phylogram derived from Bayesian analysis of *MAT1-1-3* dataset of species in the *G. serpens* complex. Phylogenetic support is indicated at nodes as Bayesian posterior probabilities (≥ 0.95) / parsimony bootstrap ($\geq 75\%$). * = Bayesian posterior probabilities ≤ 0.95 or bootstrap values $\leq 75\%$.

Chapter 6

Microsatellite and mating-type markers reveal unexpected patterns of genetic diversity in the root-infecting fungus *Grosmannia alacris*

Abstract

Grosmannia alacris is a fungus commonly associated with root-infesting bark beetles occurring on *Pinus* spp. The fungus has been recorded from South Africa, USA, France, Portugal and Spain and importantly, has been associated with pine root diseases in South Africa and the USA. Nothing is known regarding the population genetics or origin of *G. alacris*, although its association with root-infesting beetles native to Europe suggests that it is an invasive alien in South Africa. In this study, microsatellite markers together with newly developed mating type markers were used to characterize a total of 170 isolates of *G. alacris* from South Africa and the USA. The results showed that the genotypic diversity of the South African population of *G. alacris* was very high when compared to the USA populations. Two mating types were also present in South African isolates and the *MAT1-1*/*MAT1-2* ratio did not differ from 1:1 ($\chi^2 = 1.39$, $P = 0.24$). This suggests that sexual reproduction most likely occurs in the fungus in South Africa, although a sexual state has never been seen in nature. In contrast, the large collection of USA isolates harbored only a single mating type. The results suggest that multiple introductions, followed by random mating, have influenced the population structure in South Africa. In contrast, limited introductions of a single mating type may best explain the clonality of USA populations.

Keywords: *Grosmannia alacris*, pine pathogen, population genetics, SSR markers

Introduction

Grosmannia alacris is a fungus commonly associated with root-infesting bark beetles occurring on *Pinus* spp. It was first described as *Verticicladiella alacris* associated with a pine root disease in South Africa (Wingfield and Knox-Davies 1980; Wingfield and Marasas 1980). The fungus was later reduced to synonymy with *Leptographium serpens* based on morphological similarities (Wingfield and Marasas 1981) and together with other *Leptographium* spp. has more recently been treated in *Grosmannia* as *G. serpens* (Zipfel et al. 2006). Based on molecular phylogeny, Duong et al. (2012a) split this species from *G.*

serpens and revived the name as *G. alacris*. *Grosmannia alacris* as defined by Duong et al. (2012a) has been found in South Africa, USA, France, Portugal and Spain.

In South Africa, *G. alacris* is found on the roots of *Pinus* spp. and is vectored by the root-infesting bark beetles (Coleoptera: Scolytinae) *Hylastes angustatus* and *Hylurgus ligniperda*, which are of European origin (Zhou et al. 2001). It is thus assumed that the fungus was introduced into South Africa with these insects. In the USA, *G. alacris* has been consistently isolated from *Hylastes salebrosus* and *Hylastes tenuis* as well as root tissue of *Pinus taeda* and *P. palustris* infested with these insects (Eckhardt et al. 2007; Zanzot et al. 2010). Isolates of *G. alacris* have also been collected from France, Portugal and Spain, however no thorough studies have been conducted on the insect association nor the host range of this species in those countries (Duong et al. 2012a; Jacobs and Wingfield 2001).

Various studies have suggested the correlation of *G. alacris* with symptomatic pine trees or pine decline. The fungus was originally described in association with root disease of *Pinus pinaster* and *P. radiata* in South Africa (Wingfield and Knox-Davies 1980; Wingfield and Marasas 1980). A subsequent study conducted by Zhou et al. (2002) showed that this fungus [as *L. serpens*] could produce lesions in inoculated trees in South Africa. Although the fungus was capable of causing lesions on the stem of inoculated trees, the authors concluded that this species could not be considered as a serious pathogen of pine in South Africa. In the USA, *G. alacris* [as *L. serpens*] has consistently been isolated from root tissue of symptomatic trees and from sites with severe decline symptoms and high levels of mortality (Eckhardt et al. 2007). Pathogenicity tests showed that *G. alacris* [as *L. serpens*] produced significant lesion length on inoculated seedlings and mature *P. taeda*, and in severe cases, resulted in seedling mortality (Eckhardt et al. 2004b). Although none of these studies have shown *G. alacris* to be a primary pathogen of pines, a combination of abiotic stress with intensive feeding by beetles following by the colonization of the fungus could contribute to pine decline and mortality (Eckhardt et al. 2007; Matusick et al. 2008; Wingfield et al. 1988)

Grosmannia alacris has the widest distribution of all species in the recently described *G. serpens* complex (Duong et al. 2012a). It has been associated with pine decline in various studies as well as in fungal-insect association studies in the USA and South Africa (Duong et al. 2012a; Eckhardt et al. 2004a; Eckhardt et al. 2007; Zanzot et al. 2010; Zhou et al. 2001). However, nothing is known regarding the genetic diversity of the fungus in these countries

nor the centre of origin of this species. The aim of this study was to determine the genetic diversity of populations of isolates of *G. alacris* from South Africa and the USA using microsatellite markers and to compare these. Furthermore, isolates from these populations were compared based on mating type and thus in terms of their capacity to undergo sexual reproduction.

Materials and methods

Fungal isolates

Isolates of *G. alacris* from South Africa ($N = 46$) were obtained from the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria. These included isolates collected from Jessievale and Tweefontein, Mpumalanga, South Africa ($N=37$), and other isolates collected from four locations within a 50 km radius from Cape Town, Western Cape, South Africa ($N=9$). Isolates from the USA ($N = 124$) were collected from three locations including Fort Benning, Georgia ($N = 40$), Holly Springs Ranger District, Mississippi ($N = 26$) and Oakmulgee Ranger District, Alabama ($N = 58$). Fungal isolations were made on 2 % malt extract agar (MEA). The isolate used to clone the mating type gene (CMW1136) was obtained from the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria. In all cases, isolates used in this study were purified from single germinating conidia or single hyphal tips.

DNA extraction and ISSR amplification and allele determination of SSR loci

Single conidial or single hyphal tip cultures were grown on YM broth (2% malt extract and 0.2% yeast extract) for 3-5 days. The mycelium was harvested by centrifugation and lyophilized in 1.5 mL Eppendorf tubes. The tubes were submerged into liquid nitrogen and mycelium was ground into a fine powder using a pipette tip. DNA was extracted from the powder using PrepMan™ Ultra reagent (Applied Biosystems, California, USA). About 10 mg of lyophilized mycelial powder was added to 1.5 mL Eppendorf tubes, to which 100 μ l PrepMan™ Ultra reagent was added and mixed. The tubes were heated at 96 °C for 5 minutes, followed by centrifugation at 10.000 g for 10 minutes. DNA solution obtained was diluted five times with 10 mM Tris-HCl, pH 8.0 and stored at -20 °C until further use. Two microliters of diluted DNA solution was used as template in a SSR-PCR reaction. SSR-PCR was performed using 16 microsatellite markers developed by Duong et al. (2012b). SSR-PCR

reaction components, PCR cycles and allele assignment was done as described in Duong et al. (2012b).

Gene and genotypic diversity

Allele frequency at each locus was calculated using the program POPGENE (Yeh et al. 1999). Nei's genetic diversity (Nei 1973) was calculated for each population using the same program. Genotypic diversity (G) was estimated following the equation $G = \frac{1}{\sum P_i^2}$ where P_i is the observed frequency of the i^{th} genotype (Stoddart and Taylor 1988). Maximum percentage genotype diversity $G^* = (G/N) \times 100$, where N = number of isolate, was calculated to avoid sample size bias. Chi-square (χ^2) test for differences in allele frequency were calculated for each locus across all populations. The obtained χ^2 values were compared using the contingency table at $P = 0.001$ to determine the significance of the allele frequency differences at each locus. Where the observed values were higher than those on the contingency table, the gene diversities across loci were considered significantly different to one another.

Population structure and differentiation

The computer program STRUCTURE 2.3 was used to infer and assign population structure of all isolates (Pritchard et al. 2000). The Monte Carlo Markov Chain (MCMC) was run with 100 000 replicates with initial burn-in of 20 000 for K ranging from 1 to 15 at 20 iterations for each cycle. Clusters were assigned with cluster identity above 75%.

Population differentiation was calculated between populations using the program Multilocus (Agapow and Burt 2000) with an estimate of Wright's F_{st} as $\theta = (Q - 1)/(1 - q)$ where Q is the probability that two alleles from the same population are the same and q is the probability that two alleles from different populations are the same (Weir 1997). When $\theta = 0$, allele frequencies are the same between two populations, inferring that two populations are identical. In contrast, when $\theta = 1$, there are no alleles shared between two populations and, therefore, the two populations are completely isolated. The statistical significance of θ was determined by comparing the observed value to that of 1000 randomized datasets. The null-hypothesis was that there is no difference between two populations. Gene flow (M)

between populations was also calculated following the formula $M = [(1/\theta) - 1]/2$ (Cockerham and Weir 1993).

Linkage disequilibrium

The possibility of random mating within different populations was evaluated based on linkage disequilibrium analyses. The index of association (I_A) and r^2D were calculated using the program Multilocus (Agapow and Burt 2000). The observed value of I_A and r^2D were compared to that of 1000 time randomized datasets. The null-hypothesis was that the alleles are randomly associated and the population is expected to freely undergo recombination. If the observed I_A and r^2D were significantly different from those obtained from the randomized datasets ($P \leq 0.05$), the null-hypothesis was rejected. In contrast, where the observed I_A and r^2D were not significantly different from those obtained from the randomized datasets, the null-hypothesis of random association of alleles would be supported, showing that the populations undergo random mating.

Development of mating type markers for *G. alacris*

The mating type gene *MAT1-2-1* in *G. alacris* isolate CMW1136 was cloned using the method described in detail in Duong et al. (2013). Degenerate primers NcHMG1 and NcHMG2 (Arie et al. 1997) were used to obtain a part of the HMG box of the *MAT1-2-1* gene. The PCR reaction ingredients and cycling conditions were the same with that described in Duong et al. (2013). The PCR product was cloned into pGEM[®]-T (Promega, Wisconsin, USA) and transformed into *E. coli* JM109. Plasmids were extracted, confirmed by sequencing using T6 and SP7 primers. Plasmid containing HMG box sequences was used as template to synthesize DIG-labeled probe using a PCR DIG probe synthesis kit (Roche Diagnostics GmbH, Mannheim, Germany) with NcHMG1 and NcHMG2 as primers. Southern hybridization of DNA samples, digested to completion with *Eco*RI, *Hind*III and *Bam*HI, were conducted. Based on the results obtained from Southern analysis, the *Hind*III digested fragments with size ranges corresponding to that of the hybridized signal were recovered from agarose gel, ligated into the pBluescript II KS (+) phagemid vector (Stratagene, California, USA) and transformed into *E. coli* JM109 to create a partial genomic library. Colony hybridization using the same probe as used in Southern analysis of genomic DNA was conducted to screen for *MAT1-2-1* gene. Plasmids from positive clones were prepared and sequenced using primer walking. Gene was

predicted using FGENESH+ (Salamov and Solovyev 2000) and identified as *MAT1-2-1* by BLAST against the NCBI database. The sequence of *MAT1-2-1* obtained was used to design *MAT1-2* specific primers, LA-Mat2F (5'- CGACGGTGAAGTATGTCATTGA -3') and LA-MAT2R (5'- TTCGGTCTCGTCGCCAGT -3'). For detection of *MAT1-1*, primers Oph-MAT1F1 (5'- ATGKCCRATGARGAYTGCT -3') and Oph-MAT1R2 (5'- GGCGKTKGCRTTGAYTTGTA -3'), were developed based on sequences of *MAT1-1-3* genes from *Leptographium procerum* (GenBank pending), *Leptographium profanum* (GenBank pending) and *O. novo-ulmi* sub. *novo-ulmi* (GenBank number: FJ858801).

The *MAT1-1* and *MAT1-2* primers were used in separate PCRs (with either *MAT1-1* or *MAT1-2* primers) as well as combined in a multiplex PCR (with both *MAT1-1* and *MAT1-2* primers) to test the ability to identify the mating type of *G. alacris* isolates.

The PCR mixture consisted of 2.5 µl 10 × PCR reaction buffer, 2.5 mM MgCl₂, 200 µM each dNTP, 0.2 µM of each primer, 1 U FastStart *Taq* DNA Polymerase (Roche Diagnostics GmbH, Mannheim, Germany) and 20 to 50 ng of genomic DNA. PCR cycle used 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. PCR products were examined using agarose gel electrophoresis, stained with GelRed (Biotium, Hayward, California, USA) and examined under UV light.

Determination of mating type ratio in populations

DNA samples from all isolates from South Africa and the USA populations were used in multiplex PCRs (containing both *MAT1-1* and *MAT1-2* primers) to determine the mating type and calculate mating type ratio in populations. PCR reaction ingredients and cycling conditions used were as above. Mating type of isolates was assigned based on the size of resulting PCR products. The PCR primers were designed such that the *MAT1-1* amplicon was 479 bp and the *MAT1-2* amplicon was 623 bp. The mating type ratio was noted as number of *MAT1-1* isolates per number of *MAT1-2* isolates for each population.

Results

Gene and genotypic diversity

All 16 primer pairs consistently amplified microsatellite loci from all isolates considered in this study. Six markers namely LSM-14, LSM15, LSM-22, LSM-25, LSM-39 and LSM-40 were

monomorphic across all isolates and were excluded in further analyses. Ten markers were polymorphic and produced 35 alleles across all isolates from South Africa and the USA (Table 1). Of these, seven were polymorphic for the South African population, seven for Georgia, five for Alabama and all ten for Mississippi populations of isolates. Only three alleles were shared in all populations of isolates.

The number of alleles per locus ranged from two to five across all 170 isolates. There were 20 alleles for the South African, 23 for Georgia, 20 for Mississippi and 16 for Alabama populations. There were seven unique alleles in the South African population and 15 alleles were unique for three populations from the USA. Most of the 28 alleles were shared between different populations from the USA, in which only the population from Mississippi had three unique alleles that were not found in any other population. Seven of the total of 35 alleles were shared between all four populations including those from the USA and South Africa (Table 1).

A total of 32 multilocus haplotypes (*MLH*) per 46 isolates were observed for the South African population, of which many *MLHs* were represented by only a single isolate. The number was much lower for populations from the USA where there were nine *MLHs* per 40 isolates for Georgia, five *MLHs* per 26 isolates for Mississippi and 13 *MLHs* per 58 isolates for Alabama. No *MLH* was shared between the South African and the USA populations. Common *MLH* were found in isolates from Georgia and Alabama, Georgia and Mississippi but not between Alabama and Mississippi. The genotype diversity was high for the South African population ($G = 25.8$) and much lower for the three populations from USA ($G = 4.2$ for Georgia, $G = 3.6$ for Mississippi and $G = 4.5$ for Alabama) (Table 1). The maximum percentage diversity (G^*) was highest for the South African population (56.1%), followed by Mississippi (13.7%), Georgia (10.5%) and Alabama (7.7%). Mean genetic diversity across all loci (H) ranged from 0.126 to 0.515 for all populations (Table 2). Chi-square values obtained from the clone-corrected population indicated significant differences ($P = 0.001$) for gene diversity at all loci across all populations (Table 2).

Population differentiation and gene flow

The results (θ) indicated a significant distance between South African and USA populations (at $P < 0.001$). In contrast, the three populations from USA were closer to one another and in which Georgia and Mississippi and Georgia and Alabama were not significantly different to

each other ($\theta = 0.02$ and 0.08 respectively). Among the USA populations, there was a moderate distance between the Mississippi and Alabama populations ($\theta = 0.3$) (Table 3). All isolates from South Africa resided in a single group as suggested by Structure analysis (Fig. 1). The isolates from USA fell into two groups, in which one included those from Georgia and Mississippi and the other included isolates from Georgia, Mississippi and Alabama (Fig. 1). There was very little evidence of gene flow (M) between South African and USA populations (M value was between 0.4 and 0.57) (Table 3). In contrast, the levels of gene flow were much higher between populations from USA, in which the highest value was observed between Georgia and Mississippi ($M = 24.5$) and the lowest was between Mississippi and Alabama ($M = 1.17$) (Table 3).

Linkage disequilibrium

The observed I_A and r^2D values from each population with corresponding P values are presented in Table 4. The observed r^2D values for the South African and Alabama populations fell within the 1000 randomized dataset (Fig. 2 A and D) with P values of 0.373 and 0.936 respectively. The observed r^2D values for the Georgia and Mississippi populations fell beyond the ranges obtained from the 1000 randomized datasets ($P < 0.001$) (Fig. 2 B and C). Similar results were obtained for the I_A values. The null-hypothesis, that the alleles are randomly associated and the population is expected to freely undergo recombination, were supported for the South African and Alabama populations and rejected for the Georgia and Mississippi populations.

Development of mating type markers for G. alacris

PCR using NcHMG1 and NcHMG2 primers and *G. alacris* isolate CMW1136 DNA as template resulted in a product of about 300 bps in size. Cloning of this PCR product into pGEM[®]-T and sequenced the recombinant vector using T6 and SP7 primers confirmed that it was the HMG domain of the *MAT1-2-1* gene. BLAST analysis showed that HMG domain in *G. alacris* shared high homology with those in other closely related Ophiostomatoid fungi such as *L. procerum*, *L. profanum*, and *G. clavigera*. Southern analysis of *Hind*III digested DNA sample produced a hybridized signal of about 2.5 kb in size. A partial genomic library was successfully constructed from *Hind*III digested fragments of corresponding size (around 2.5 fraction) and screened this library by colony hybridization resulted in one positive clone out of about 700

colonies. The cloned insert [2.47 kb] was sequenced (Genbank pending) and FGENESH+ was used for annotation. Additional analyses using BLAST against the NCBI database confirmed the presence of a complete *MAT1-2-1* gene (CDS 948 bp in size, 828 bp as mRNA). The *MAT1-2* specific primers (LA-Mat2F and LA-MAT2R) were designed from sequence of *MAT1-2-1* and consistently amplified a 623-bp portion of *MAT1-2-1* gene from *MAT1-2* isolates of *G. alacris* (Fig. 3). The *MAT1-1* primers (Oph-MAT1F1 and Oph-MAT1R2) were used to amplify a 479-bp portion of *MAT1-1-3* from *MAT1-1* isolates (Fig. 3). Both *MAT1-1* and *MAT1-2* primers could be used in a single multiplex PCR to amplify portions of *MAT1-2-1* gene and *MAT1-1-3* gene from *MAT1-2* and *MAT1-1* isolates respectively (Fig. 3). The amplified fragments were easily differentiated based on the size difference (Fig. 3).

Determination of mating type ratio in populations

By using the multiplex PCR assay developed, the mating type of isolates from South Africa and USA were determined (Fig. 4). There were 19 *MAT1-1* and 27 *MAT1-2* isolates in a total of 46 South African isolates and Chi-square (χ^2) test showed that this did not differ from an expected 1:1 ratio of a random mating population ($\chi^2 = 1.39$, $P = 0.24$). All isolates from USA were of the *MAT1-2* type (Table 4).

Discussions

Microsatellite markers applied in this study to populations of *G. alacris* from South Africa provided the surprising result that the fungus is genetically diverse in this country. This result was unexpected given that it is well-recognized that the fungus is vectored by a non-native alien invasive insect in South Africa (Zhou et al. 2001), and is presumably also not native. Mating type analyses also showed that the South African isolates include representatives of both the *MAT1-1* and the *MAT1-2* types. The fungus is thus heterothallic and possibly undergoing sexual recombination. In contrast, a population of *G. alacris* from the USA was essentially clonal and represented by a single mating type. This suggests that the fungus was most likely introduced into that country from an as of yet unknown source.

The South African population of *G. alacris* had a very high level of genetic and genotypic diversity ($G^* = 56.1\%$), which suggests that random mating must have occurred within this population. The test of random association of alleles supported this hypothesis with the observed I_A and r^2D values falling within the randomized datasets. Moreover, the mating

type distribution of 19 *MAT1-1* and 27 *MAT1-2* isolates and a chi-square statistic showed no significant difference from the expected 1:1 ratio of a randomly mating population ($\chi^2 = 1.39$, $P = 0.24$). This suggests strongly that random mating would account for the high genetic and genotypic diversity observed in the South African population as has been true in other studies (Barve et al. 2003; Chérif et al. 2006; Groenewald et al. 2006; Linde et al. 2003; Rau et al. 2005; Stergiopoulos et al. 2007).

Grosmannia alacris is believed to have been introduced into South Africa. This view is based on the fact that it is intimately associated with non-native bark beetles that infest *Pinus* spp. that are also non-native and were first introduced into the country in the early 1700's (Wingfield and Knox-Davies 1980; Wingfield and Marasas 1980; Zhou et al. 2001). The high level of genetic diversity in *G. alacris* revealed in this study is surprising for an introduced organism which might more likely be reflected by a clonal population associated with a founder effect (Brasier 1996; Linde 2010; Milgroom et al. 2008; Prospero et al. 2007). The broad genetic diversity could, however, be explained by the presence of two mating types that would facilitate random mating and the expansion of genetic diversity. While a sexual state has never been seen for *G. alacris* in nature, ascomata of the fungus have previously been produced in crosses with the fungus in laboratory tests (Duong et al. 2012a). More intensive inspection of galleries of *Hylastes angustatus*, the primary vector of *G. alacris* in South Africa will most likely reveal the presence of sexual structures of the fungus.

The mating ratio tests showed that only *MAT1-2* gene was present in all of the isolates from the USA. Unlike the situation in South Africa, this implies that the fungus can reproduce only asexually in the United States. This explained the low level of genotypic diversity observed in all populations from the USA. Although linkage disequilibrium tests suggested non-random recombination of alleles from Georgia and Mississippi, the alleles are found randomly recombined for the Alabama population. However, with the presence of only one mating type, the possibility for sexual reproduction as the source of random recombination of alleles observed in Alabama population is eliminated. It is possible that this random combination of alleles reflects sexually derived genotypes that were initially introduced into this population. The test for random association of alleles using I_A and r^2D values has been used to make inference about sexual reproduction in various studies (Bihon et al. 2012; Geiser et al. 1994; Groenewald et al. 2008; Morgan et al. 2007). Although this is a good

indirect reference of sexual reproduction, the results of this study as in the case of Alabama population emphasized that the use of these values need to be supported by additional genetic data such as genetic and genotypic diversity and mating type distribution.

The lack of shared *MLHs* together with a very low level gene flow between South African population and USA populations suggests a long period of separation between these two sources of isolates. This was also well supported with the structure analysis that showed South African isolates residing in a single group, separate from those from the USA. The results of the population differentiation analyses (θ) also suggest that the South African population is strongly separated from the USA population, with strong statistical support. The most tangible explanation here is that the South African and USA populations are most likely linked to different sources and times of introduction. In contrast, the number of shared *MLHs* as well as gene flow was high between populations from the USA, and given the low genotypic diversity observed, these populations are most likely linked to a single or a small number of introductions.

Duong et al. (2012a) suggested an European origin of *G. alacris* but also stated the possibility of being native to the USA based on the fact that it is associated in that country with native insects infesting native pine species. The results of the present study strongly support the view that *G. alacris* has most likely been introduced into the USA, in which case, it has established a novel association with native root-infesting bark beetles. Such novel associations between fungi associated with introduced bark beetles and native insects are not unusual and are well known for the Dutch Elm disease fungi (Anderson and Holliday 2003; Strobel and Lanier 1981) and more recently for at least two pine-infesting bark beetles (Jacobs et al. 2004; Lu et al. 2009).

Unlike the South African situation, it is not possible to identify an insect with which *G. alacris* might have entered the United States. However, at least one non-native pine root infesting bark beetle is known in the area in which *G. alacris* is found (Thompson 2011). This *Hylastes* species (*H. opacus*) would be a good candidate for further study of its fungal associates, both in the USA as well as in its area of origin.

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Table 1. Allele frequencies and genotype diversity by population.

Locus	Allele	South Africa	USA		
			Georgia	Mississippi	Alabama
LSM-02	1	0.739	0.400	0.654	0.069
	2	0.261	—	—	—
	3	—	0.600	0.346	0.931
LSM-11	1	0.978	0.400	0.654	—
	2	0.022	0.600	0.346	1.000
LSM-19	1	0.478	—	—	—
	2	0.283	0.400	0.654	—
	3	0.239	0.600	0.346	1.000
LSM-21	1	0.500	—	—	—
	2	0.500	1.000	0.539	1.000
	3	—	—	0.462	—
LSM-26	1	0.565	1.000	0.539	1.000
	2	—	—	0.462	—
	3	0.435	—	—	—
LSM-30	1	0.565	—	—	—
	2	—	0.400	0.615	—
	3	0.391	0.075	—	0.224
	4	0.044	0.525	0.385	0.776
LSM-31	1	1.000	1.000	0.539	1.000
	2	—	—	0.462	—
LSM-32	1	—	0.050	0.385	—
	2	—	0.350	0.269	—
	3	—	0.025	—	0.069
	4	0.587	0.500	—	0.931
	5	0.217	—	—	—
	6	—	0.075	0.346	—
	7	0.196	—	—	—
LSM-33	1	1.000	0.400	0.654	—
	2	—	0.550	0.231	0.638
	3	—	—	0.115	0.052
	4	—	0.050	—	0.310
LSM-37	1	—	0.025	—	0.086
	2	—	0.575	0.346	0.914
	3	—	0.075	0.385	—
	4	1.000	0.325	0.269	—
<i>G</i>		25.805	4.211	3.558	4.450
<i>G*</i>		56.098	10.526	13.684	7.672
<i>MLH</i>		32	9	5	13

G = Genotype diversity (Stoddart and Taylor 1988), *G** = percentage maximum diversity,
MLH = Multilocus haplotype

Table 2. Gene diversity (H) and chi-square value for differences in allele frequencies for 10 SSR loci across clone-corrected populations.

Locus	South Africa	USA			Chi-square (χ^2)	df
		Georgia	Mississippi	Alabama		
LSM-02	0.386	0.480	0.453	0.128	36.287*	6
LSM-11	0.043	0.480	0.453	0.000	41.474*	3
LSM-19	0.634	0.480	0.453	0.000	28.757*	6
LSM-21	0.500	0.000	0.497	0.000	39.861*	6
LSM-26	0.492	0.000	0.497	0.000	38.376*	6
LSM-30	0.526	0.559	0.473	0.348	48.509*	9
LSM-31	0.000	0.000	0.497	0.000	22.358*	3
LSM-32	0.570	0.619	0.660	0.128	66.749*	18
LSM-33	0.000	0.535	0.506	0.494	59.936*	9
LSM-37	0.000	0.558	0.660	0.158	58.553*	9
Mean	0.315	0.371	0.515	0.126		

* Indicate significant difference at $P = 0.001$, df = degree of freedom.

Table 3. Population differentiations (θ) (above the diagonal) and gene flow (M) (below the diagonal) between populations.

	South Africa	Georgia	Mississippi	Alabama
South Africa	—	0.40*	0.41*	0.57*
Georgia	0.75	—	0.02 ^{NS}	0.08 ^{NS}
Mississippi	0.72	24.50	—	0.3*
Alabama	0.38	5.75	1.17	—

* Indicate significant difference at $P < 0.001$ and ^{NS} Indicate no significant difference (at $P < 0.05$).

Table 4. Observed I_A , r^2D values with corresponding significant level (P) and mating type ratio of each populations.

Population	I_A	r^2D	P	MAT1-1/MAT1-2 ratio
South Africa	0.014	0.002	0.373	19/27
Georgia	3.629	0.609	< 0.001	0/40
Mississippi	2.912	0.325	< 0.001	0/26
Alabama	-0.185	-0.046	0.936	0/58

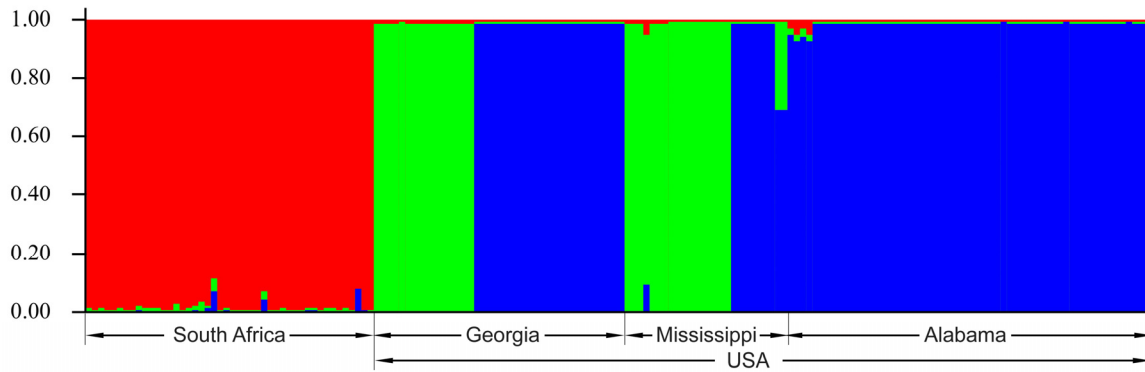


Fig. 1. Affiliation of individual genotypes of *G. alacris* as suggested by Structure 2.3.4. Different colour within a vertical bar indicates population affiliation of individual genotypes. The sources of isolates are given underneath the bar.

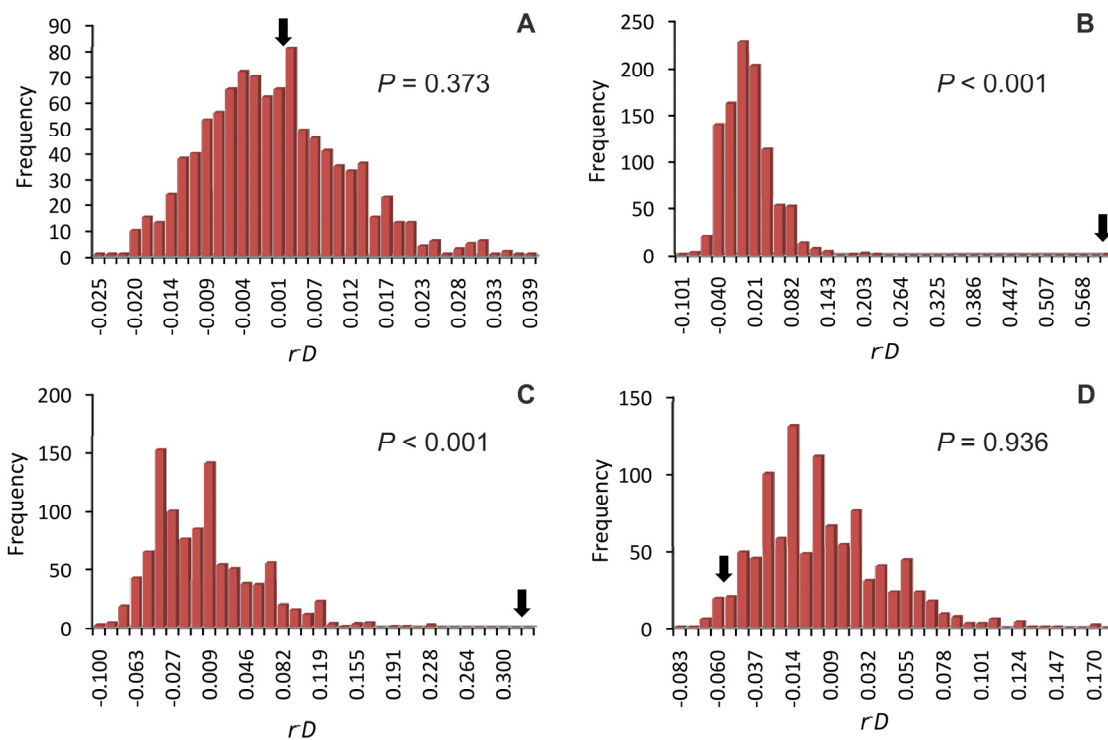


Fig. 2. Histograms constructed from rD values obtained from 1000 time randomized datasets of South African (A) and USA populations from Georgia (B), Mississippi (C) and Alabama (D). Arrows indicated the observed rD values of each population.

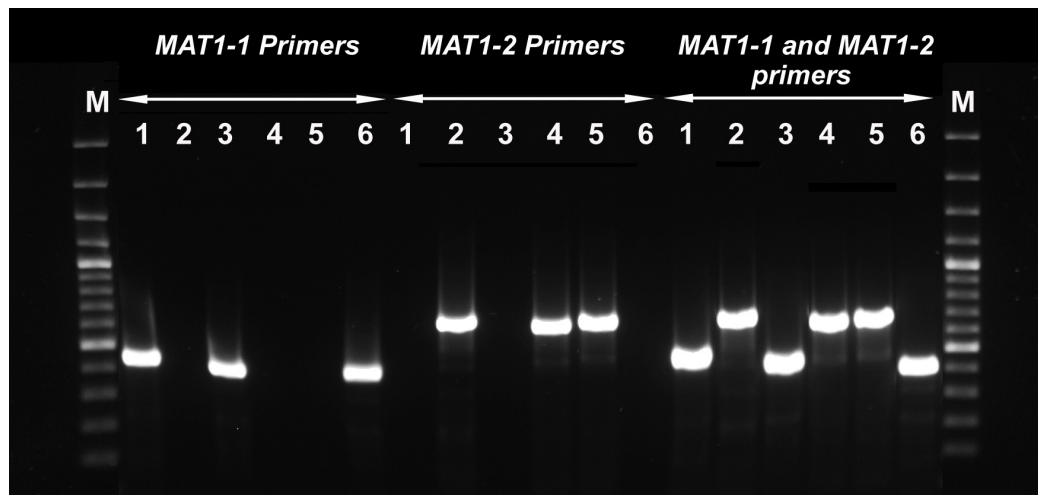


Fig. 3. Agarose gel [1.5%] showing the amplicons obtained from PCR and multiplex PCR assays using mating type primers on *G. alacris* isolates: CMW7701 (1), CMW7702 (2), CMW7703 (3), CMW7705 (4), CMW7706 (5), CMW7707 (6). The molecular weight marker used was GeneRuler™ 100 bp Plus DNA Ladder (Fermentas). The 500bp and 1000bp marker is more concentrated than the other bands.

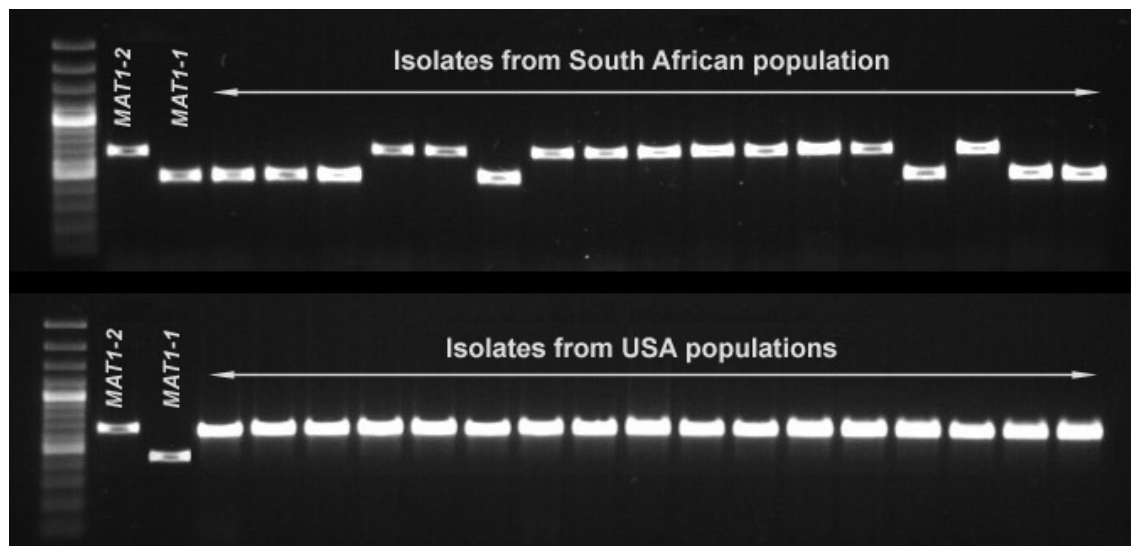


Fig. 4. Agarose gel [1.5%] showing the amplicons obtained from a multiplex PCR assay to determine mating type of some representative isolates from South African and USA populations. The molecular weight marker used was GeneRuler™ 100 bp Plus DNA Ladder (Fermentas). The 500bp and 1000bp marker is more concentrated than the other bands.

Summary

Summary

Leptographium sensu lato is an ascomycete genus with species well adapted for insect dispersal. Most species in *Leptographium s.l.* are saprophytes that cause blue stain in timber, but some species are capable of causing disease in their host trees. Many species in the genus have a global distribution, but little is known regarding their pattern of dispersal or centers of origin. Although the genus (in the broad sense) is comprised of both sexual (*Grosmannia*) and asexual (*Leptographium*) species, limited information is available regarding the mating type genes and sexual compatibility of these species. The main aims of this thesis were to revise the taxonomy, characterize the mating type locus, and to study the sexual compatibility and population biology of selected species of *Leptographium s.l.* A multigene phylogeny including five genes has led to the delineation of five species in a newly defined *Grosmannia serpens* complex, in which three species were described as new. This study presents the first comprehensive multigene phylogeny for any of the species complexes defined in *Leptographium s.l.* The mating type genes in *Leptographium procerum* and *L. profanum* were characterized. These fungi were shown to have a heterothallic mating system, suggesting that they have cryptic sexual states, even though they have been known exclusively as being asexual. Mating type markers were developed for *L. procerum*, *L. profanum* and many other species in *Leptographium s.l.* The primers were used to amplified portions of the mating type genes of 40 different species and the thallism was confirmed in many species for the first time and showing that the majority of species are heterothallic. Microsatellite makers were developed for *G. alacris* and these markers were used together with the mating type markers, to elucidate the population biology and mode of sexual reproduction in natural populations. Other than the specific results emerging from studies in this thesis, molecular tools that were developed provide opportunities to study the taxonomy, sexual compatibility and to aid in the discovery of sexual states of many species in the *Leptographium sensu lato*, other genera in the Ophiostomatales, as well as in other Ascomycetes.