UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Identification and application of mating type gene sequences in *Ophiostoma*

by

Pieter Marthinus Wilken

Submitted in partial fulfilment of the requirements for the degree

MAGISTER SCIENTIAE

In the faculty of Natural & Agricultural Science

University of Pretoria

Pretoria

May 2009

Study Leaders:

Prof. Brenda D. Wingfield

Mr. Z. Wilhelm de Beer

Prof. Michael J. Wingfield

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Declaration

I, the undersigned, hereby declare that the thesis submitted for the degree *Magister Scientiae* to the University of Pretoria contains my own independent work. This work has hitherto not been submitted for any degree at any other University.

_____

Markus Wilken

May 2009

TABLE OF CONTENTS

Acknowledgments **1**

Preface **2**

CHAPTER 1

Mating type (*MAT*) genes in Ascomycetes with special reference to *Ophiostoma* species **4**

1. Introduction 5
2. Sexual reproduction and mating in Ascomycetes 6
   - 2.1 Aspects of sexual reproduction 7
   - 2.2 Nomenclature relating to mating genes 8
3. Mating type loci and idiomorphs: Selected Ascomycete case studies 10

   Yeasts

   3.1 Saccharomyces cerevisiae 10

   Filamentous Ascomycetes

   3.2 Neurospora crassa 12

   3.3 Podospora anserina 14

   3.4 Cochliobolus heterostrophus 15
4. Characteristics of the *MAT* locus 17
5. *Ophiostoma* ecology and taxonomy 18
   - 5.1 The mating type genes in *Ophiostoma* 20
6. Conclusions 21
7. References 22

CHAPTER 2

Presence of both *MAT1-1* and *MAT1-2* mating idiomorph sequences in single isolates of the heterothallic fungus, *Ophiostoma quercus* **39**

Abstract 40

Introduction 41

Materials and Methods 43

Results 45

Discussion 47

References 50

CHAPTER 3

A *MAT1-2-1* phylogeny for *Ophiostoma* spp. related to *O. quercus* **64**

Abstract 65

Introduction 66

Materials and Methods 68

Results 70

Discussion 71

References 75

Summary **87**

ACKNOWLEDGEMENTS

I would like to express my sincere thanks and appreciation to the following:

My supervisors, Mr. Wilhelm de Beer, Prof. Brenda Wingfield and Prof. Mike Wingfield, for their continued support and guidance throughout the course of this study.

My colleagues and friends at FABI, especially Magriet van der Nest, Juanita Nel and Francois Boshoff for their continued support and encouragement during this degree.

The University of Pretoria (UP), National Research Foundation (NRF), members of the Tree Protection Co-operative Programme (TPCP) and the DST-NRF Centre of Excellence (CoE) in Tree Health Biotechnology for financial assistance.

My mentorship students, Melissa Simpson and Mathews Sebonego for all their effort and help in the lab.

My family for their encouragement and prayers, especially my brother Hardus for help with work in the lab.

My loving wife, Ricky, for your support, encouragement, understanding and help in all I have done for this accomplishment.

To my Father and Lord who guides me through all I have done.

PREFACE

Fungi display various modes of sexual reproduction and these provide valuable information regarding their taxonomy and population biology. Although sex in fungi has been extensively studied, there is little or no information regarding the mating strategy in many important groups of fungi. This is true for *Ophiostoma* spp. where detailed information regarding the mating type (*MAT*) idiomorphs is available for a very small number of species.

The aim of the studies presented in this thesis was to expand knowledge of the *MAT* genes to more species of the genus *Ophiostoma*. The thesis is comprised of three chapters including a review of the literature relating to mating in Ascomycetes, as well as the first report of *MAT-1* and *MAT-2* idiomorph sequences in *O. quercus* and a *MAT-2* phylogeny for the genus *Ophiostoma*. The chapters are presented as independent entities and this has resulted in some degree of duplication between them.

The first chapter of the thesis provides an overview of sexual reproduction in fungi. The process of mating, mating terminology, and associated aspects provides the basis for a detailed discussion on the genetic aspects of mating. To highlight these aspects, the mating genes of selected well-studied model Ascomycetes are described. The final part of the review focuses on *Ophiostoma*, which is the genus of interest in the studies making up this thesis.

Chapter 2 deals with mating type genes in a representative of *Ophiostoma*, *O. quercus*. This species has a heterothallic mating system where only one of the two mating genes is usually found in a single isolate. Using both previously and newly designed primers, the presence of both mating genes in a heterothallic species is illustrated. These results were unexpected as each isolate of a heterothallic species normally contains only one of the two *MAT* genes.

In chapter 3, the use of *MAT-2* primers designed as part of the study making up chapter 2 is evaluated for a larger selection of *Ophiostoma* species. Although these primers were designed and tested for use in *O. quercus*, they were used successfully to amplify a large fragment of the *MAT-2* idiomorph across 17 species of *Ophiostoma*. The availability of the *MAT-2* data allowed for the evaluation of the *MAT-2* open reading frame for phylogenetic use. The low level of sequence variation observed between species in this region precludes the use of the region in phylogenetics of closely related *Ophiostoma* species.

The genus *Ophiostoma* is a large and complex genus that has been intensively studied for many years. The availability of *MAT* sequence data for a limited number of species of the genus allowed an opportunity to study the genus from a perspective different to that previously considered. The results have provided some intriguing insights that will form the basis of future studies relating to mating type genes in *Ophiostoma*.

CHAPTER 1

Mating type (*MAT*) genes in Ascomycetes with special reference to *Ophiostoma* species

1. Introduction

The process of mating plays an important role in the life cycle of many fungi (Coppin *et al*. 1997). Long before the first fungal mating type (*MAT*) genes were cloned, there was intense interest in the genetic mechanisms that control mating. Although the microscopic and morphological characteristics of mating have been investigated over a relatively long period of time, studies on the genetic aspects of mating are still in their infancy. Cloning of the *Saccharomyces cerevisiae* mating locus (Astell *et al*. 1981) was the first major step towards unravelling the molecular aspects of mating for fungi. Later, Glass *et al*. (1988) cloned and analysed the *Neurospora crassa MAT* locus and provided the first detailed information regarding mating in the filamentous Ascomycetes. Since that time, the number of *MAT* genes for which DNA sequence is available has increased steadily.

Studies on mating and the *MAT* locus in fungi have made it clear that two genes found in opposite idiomorphs essentially control all aspects of mating including recognition, fertilization and spore formation (Coppin *et al*. 1997, Glass & Nelson 1994). Although a large amount of sequence similarity exists within these genes, very little is known regarding the manner in which mating is controlled outside of the model fungal species such as *S. cerevisiae* and *N. crassa*. This is surprising considering that these genes are crucial to the life cycle and survival of species. The *MAT* genes are central to both life and reproduction and are under unique selection pressures (Turgeon 1998). An insight into the functioning and role of these genes would thus provide a better understanding of the biology and ecology of an organism.

The genus *Ophiostoma* provides an example in fungi where mating type genes are not well understood. This is despite the fact that two major pandemics killing Elm trees in the Northern Hemisphere during the last century has focused research on *Ophiostoma* spp. (Brasier 1990). The research on this group has also resulted in the first molecular data for *MAT* genes in this complex genus (Paoletti *et al.* 2005, 2006). A fragment of 2200 bp has been amplified from *MAT-2* isolates of *O. ulmi*, *O. novo-ulmi* and *O. himal-ulmi* (Paoletti *et al*. 2005). The predicted protein contained a characteristic HMG domain usually found in the *MAT-2* mating protein (Arie *et al*. 1997, Coppin *et al*. 1997, Glass & Nelson 1994). A follow-up study identified 200 bp of DNA unique to the *MAT-1* idiomorph of *Ophiostoma* (Paoletti *et al.* 2006). These are the only data available on molecular aspects of the *MAT* genes in *Ophiostoma* and there is clearly a wide scope for further work on the mating genes in species of this genus.

The focus of this review is on the mating type genes controlling sexual reproduction in Ascomycetes. In essence, the mating type genes show very little variation in function or organisation. To highlight the main points regarding mating, the review focuses on the first three species for which these genes were cloned and studied. This review also considers the genus *Ophiostoma* as a whole and the mating type information available for this complex genus.

2. Sexual reproduction and mating in Ascomycetes

Mating and sexual reproduction in most Ascomycetes are controlled by a single locus, the *MAT* locus, containing one of two possible versions of a mating type gene, thus a bipolar mating system (Glass & Nelson 1994, Nelson 1996). For successful mating to occur, both these versions are needed. The way in which these genes are brought together leads to a range of different sexual systems (Nelson 1996). In order to appreciate the functioning of these systems, a basic knowledge of the sexual cycle of filamentous fungi is required.

The life cycle of the fungus *Podospora anserina* (Fig. 1) can be considered representative of most sexually reproducing Ascomycetes (Coppin *et al*. 1997, Glass & Nelson 1994). Many Ascomycetes can reproduce both sexually and asexually (Kendrick 2000). The sexual reproduction route is initiated only under specific conditions such as nutrient starvation, temperature change or high nutrient reserve levels (Coppin *et al*. 1997, Kendrick 2000). Under these conditions, the haploid vegetative hyphae begin to form multicellular, coiled ascogonia (a female element), as well as unicellular microconidia (a donor or male element). Attraction between the vegetative hyphae of two mating isolates occurs and is controlled by mating type specific pheromones and pheromone receptors (Coppin *et al.* 1997, Pöggeler & Kück 2001).

When the vegetative hyphae from the two different mating strains of a fungal species come into contact, sexual reproduction is initiated (Coppin *et al*. 1997). Fusion occurs between the ascogonia of one of these strains and the microconidia of the other strain. A specialised structure known as a trichogyne facilitates the movement of the donor nucleus from the microconidium to the ascogonium, representing fertilization and triggering fruiting body formation (Coppin *et al*. 1997).

After fertilization, the nuclei of the different mating type strains do not fuse immediately. They rather undergo mitosis to populate the developing fruiting body with a large number of

multinucleated cells that make up the dikaryon. During the final stages of the process, the pairs of opposite nuclei migrate into specialised cells called ascogenous hyphae. These cells then form a specialised structure involved in mating, the crozier or hook cell. A single round of mitosis divides the crozier into two uninucleate cells (the basal and lateral cells, respectively) and a single binucleated cell. This binucleated cell once again undergoes specialisation to form the ascus cell in which karyogamy finally takes place. Directly following karyogamy, the nucleus undergoes meiosis, followed by mitoses to form the ascospores that are released. These ascospores now carry the parental mating type genes at the mating type locus. The basal and lateral cells that were formed earlier can once again fuse and re-divide to form a new crozier, thus repeating the process and forming more ascospores. This is a process that ensures that hundreds of asci and ascospores can be produced from a single fertilization event, thereby optimising the sexual encounter. The sexual cycle provides a means for a fungus to reap the benefits of sexual recombination, while producing a viable long-term survival structure in the form of the ascospore (Fig. 1) (Coppin *et al*. 1997, Elliot 1994).

The life cycle in most heterothallic fungal species follow the generalised example described above. Most stages can be observed under a microscope and the process is easily characterised even though the genes controlling these processes may not be known. In the genus *Ophiostoma,* the mating type genes controlling the life cycle and sexual reproduction have not been widely studied. The use of information about mating and mating type genes from other filamentous Ascomycetes has served as a guide to facilitate gene discovery in the Dutch Elm disease pathogens, *O. ulmi and O. novo-ulmi* (Paoletti *et al.* 2005).

2.1 Aspects of sexual reproduction

An important aspect to bear in mind when studying mating types in fungi is the application of the terms male and female in the fungal life cycle. From the typical heterothallic life cycle discussed above, one can see that both “male” and “female” organs can be formed by each isolate. In a mating interaction, the term “male” is generally applied to the isolate acting as the donor of genetic material while “female” is defined as the receiver of this material (Coppin *et al*. 1997). The so-called “male” and “female” forms are thus linked to neither mating type, *MAT* genes, or for that matter any other sex genes. In essence, fungi are hermaphroditic with respect to their sexual organs, but they can initiate the sexual cycle in a range of different ways.

Three main forms of sexual reproduction characterise mating in filamentous Ascomycetes (Nelson 1996, Pöggeler 2001). The difference between these lies in the way the opposite mating types are brought together. This can lead to a range of reproductive strategies being present in a single genus of fungi. The three main strategies are as follows:

Homothallism: The sexual cycle can be initiated and completed by a single mycelium without the need for an opposite mating type. Mycelia of a homothallic isolate contain both mating types within a single haploid genome. During homothallic mating, self-compatible ascogonia and microconidia are formed, either on the same or on different mycelia. The microconidia act as the donor elements while the ascogonia act as the receiving elements. The sexual process proceeds through all the stages discussed earlier. All ascospores formed contain a single haploid genome containing both mating types (Nelson 1996, Pöggeler 2001).

Heterothallism: Functions in the opposite manner to homothallism. For completion of the sexual cycle, a favourable interaction between two mycelia of opposite mating type is needed. Each of the isolates contains a haploid genome with only one of the two possible mating types. Mating brings these together to complete the sexual cycle. A microconidium in one isolate donates its haploid genome to the ascogonium of the second isolate. Again mating proceeds in the standard fashion. The ascospores formed contain a single genome with either of the two mating types (Nelson 1996, Pöggeler 2001).

Pseudohomothallism: This type of interaction initially appears as homothallism in that the isolate can complete the sexual cycle without a partner of opposite mating type. Ascospores in pseudohomothallic species do not contain a single genome such as is present in homothallic ascospores, but they have two haploid genomes. Each genome contains only a single mating type resulting in each ascospore having both mating types in separate genomes. These species thus act homothallically, but are not truly homothallic (Nelson 1996, Pöggeler 2001).

2.2 Nomenclature relating to mating genes

Mating type genes fulfill an indispensable role in the Ascomycete life cycle and feature prominently in both the sexual and asexual phases. In 1981, the first mating sequences were published and these were for the yeast *S. cerevisiae* (Astell *et al.* 1981). Mating type genes have since been widely studied in the filamentous Ascomycetes particularly with exploration of the *MAT* genes in *N. crassa* (Glass *et al.* 1988), *P. anserina* (Picard *et al.* 1991) and

Cochliobolus heterostrophus (Turgeon *et al*. 1993). Currently, DNA sequences of the *MAT* genes are available for a large number of species and therefore a system of naming these genes were required to facilitate communication between research groups (Turgeon & Yoder 2000).

The most important nomenclature issue to resolve was that regarding the two versions of the *MAT* locus conferring the different mating specificities. Previously, these versions had been termed “alleles” as they represent alternative forms of the genes at the mating type locus (Glass *et al*. 1988). Later, molecular comparisons of these mating type alleles showed differences in size, sequence and gene content. Based on this, the term “idiomorphs” (Metzenberg & Glass 1990) was suggested to describe these mating type “alleles”. Glass *et al.* (1990) defined an idiomorph as “sequences that occupy the same locus in different strains but are not related in sequence or (probably) common descent.” This term has been accepted in recent literature (Arie *et al*. 1997, Arnaise *et al*. 2001, Paoletti *et al*. 2005, 2006, Pöggeler *et al*. 2006) and will be the preferred term for the purpose of this thesis.

In 2000, Turgeon & Yoder made a formal proposal for a defined system of naming mating type idiomorphs. Before the advent of molecular techniques, researchers describing a new mating type idiomorph assigned names at random (i.e. *MAT-1* and *MAT-2*). This led to much confusion as idiomorphs with similar characteristics could have opposite names purely by chance. The proposed nomenclatural system of Turgeon & Yoder (2000) is based on the commonly seen motifs found in the proteins coded by genes in each idiomorph. Firstly, the locus at which the mating type genes are found is termed the *MAT-1* locus. In turn, the two idiomorphs are named *MAT-1-1* and *MAT-1-2*, respectively. The *MAT-1-1* idiomorph is identified by the presence of a protein having an α-box motif, while *MAT-1-2* in turn contains an Open Reading Frame (ORF) encoding a protein with an HMG (High Motility Group) motif. The genes in the *MAT-1-1* idiomorph are termed *MAT-1-1-1, MAT-1-1-2, MAT-1-1-3* etc. In the same manner, the genes at the *MAT-1-2* idiomorph are named *MAT-1-2-1, MAT-1-2-2,* etc. The authors added that these names could be shortened: *MAT* for the locus and *MAT-1* and *MAT-2* for the idiomorphs. Gene 1 at *MAT-1* would be *MAT-1-1* and gene 1 at *MAT-2* would be *MAT-2-1*. In cases where more than one *MAT* locus is present, the original *MAT* terminology should be used to avoid confusion, *MAT-1* for locus 1 (with idiomorphs *MAT-1-1* and *MAT-1-2*) and *MAT-2* for locus 2 (with idiomorphs *MAT-2-1* and *MAT-2-2*).

3. Mating type loci and idiomorphs: Selected Ascomycete case studies

Yeasts

3.1 Saccharomyces cerevisiae

One of the best-studied organisms in terms of *MAT* genes is the yeast, *S. cerevisiae*. This single-celled Ascomycete is fast growing and genetically well characterised, making it an ideal model organism for studying mating-related processes. Any general characteristics seen in this well studied species might provide clues to the same sexual processes in more complex Ascomycetes.

The life cycle

The life cycle of a yeast differs from those of filamentous Ascomycetes. *Saccharomyces cerevisiae* is a haploid fungus that switches to a true diploid phase during the sexual cycle. Haploid cells of opposite mating types (**a** and α) fuse and form a zygote. Cell fusion is followed by nuclear fusion yielding a true diploid phase. When induced by environmental conditions, the cell forms an ascus that produces haploid spores from a meiotic event. Germination of these haploid spores initiates the repeat of the life cycle (Elliott 1994, Nelson 1996).

The *MAT* locus

The *MAT* locus controls all the processes associated with sexual reproduction. In yeasts, the *MAT* idiomorph at the *MAT* locus is referred to as a cassette (Fig. 2). Region Y of the cassette is a region of dissimilarity between *MAT**a*** and *MATα*. The region is 642 base pairs (bp) in length for *MAT**a*** and 747 bp in the case of *MATα*. Two flanking regions are found on either side of the dissimilar region. W/X to the left is 1427 bp in size and Z1/Z2 to the right is 327 bp (Astell *et al.* 1981). However, both the *MAT**a*** and *MATα* cassettes are found as silent copies elsewhere in the *S. cerevisiae* genome, named HML and HMR (Rine *et al*. 1979). The fundamental structure of each cassette is the same, although these regions have no direct effect on the sexual cycle of the isolate. These extra cassettes are involved in a process that

allows *S. cerevisiae* to switch from one mating type (***a***) to the other (*α*) (Strathern & Herskowitz 1979).

Functioning of the *MAT* locus

The dissimilar *MAT**a*** and *MATα* coding regions in *S. cerevisiae* encode transcripts involved in the mating process. The first transcript of *MATα*, referred to as *α1*, controls expression of α-specific genes through a 15 amino acid (aa) region that is a conserved DNA-binding motif. This conserved domain forms part of a heterodimeric protein (together with ***a1***) involved in regulation of mating function (Astell *et al.* 1981). Another protein making up the *MATα* idiomorph, *α2* inhibits the constitutive expression of *MAT**a*** related genes, ensuring that the cell mating type represents *MATα*. Constitutive expression of *MAT**a*** mating type genes results in a *MAT**a*** mating specificity for any haploid cell not containing an expressed α cassette. In the diploid phase, the protein product of the *MAT**a*** locus forms a heterodimer (Dranginis 1990) with protein product *α2* to regulate diploid functions necessary to complete the sexual cycle.

The switching process

An interesting characteristic of *S. cerevisiae* is that an isolate that originates from a single cell can enter the diploid phase and produce haploid ascospores in media after successive generations (Elliot 1994). In order to produce the diploid phase, it is necessary to have cells of both mating types present. To achieve this, progeny from the single original isolate need to change mating type sometime during the life cycle. The process involved here is known as bi-directional mating type switching (Strathern & Herskowitz 1979).

For mating type switching to take place, the genetic information for both mating types need to be present in the genome of a yeast strain (Strathern & Herskowitz 1979). This is achieved by the silent cassettes for *MAT**a*** and *MATα* being present in the cell together with the true *MAT* locus, which is responsible for the phenotype of the isolate (Fig. 2). A single gene controls the switching process, the homothallic or *HO* gene. In the presence of the Ho endonuclease, a double stranded break initiates the process whereby the *MAT* cassette is switched with the silent cassette of the opposite mating type through a complex process (reviewed in Herskowitz 1988). This allows the isolate to assume the opposite mating type and allows for mating with isolates that have maintained the original mating type phenotype (Fig. 3). Through this process, a single isolate can reproduce sexually.

Filamentous Ascomycetes

Data for the *MAT* idiomorphs of various filamentous Ascomycetes are available. The organisation and structure of the *MAT* idiomorphs in different species is in essence the same, especially the conserved regions in the proteins encoded by these idiomorphs (Coppin *et al*. 1997, Glass & Nelson 1994). The *MAT* idiomorph structure and function for the first three species that were studied, is discussed below (Fig. 4).

3.2 Neurospora crassa

The life cycle

Neurospora crassa is a heterothallic, filamentous Ascomycete. It is a colonist of natural environments that have been subjected to burning and it is regarded as a genetic model for the filamentous Ascomycetes (Perkins 1992). *Neurospora crassa* follows the typical life cycle discussed above, with the opposite mating type idiomorphs named ***a*** and *A* (*Mta* and *MtA*). The two idiomorphs of *N. crassa* were cloned and sequenced by Glass *et al.* (1988) (Fig. 4).

The *MAT* loci

To clone the *MAT* idiomorph successfully, the close positional proximity of the *MAT* locus to the temperature sensitive marker *un-3* was exploited (Glass *et al.* 1988). Phenotypic identification of a cosmid containing the temperature sensitive mutant also contained the *mtA* idiomorph. Complementation using this cosmid and a *mtA* sterile mating type strain confirmed the presence of the mating information on this cosmid.

A *mta*-specific library was probed using *mtA*-specific mating type DNA. The sequences flanking the *mtA* region were homologous to the same region in the *mta* idiomorph. A region was identified that could convert a *mtA*-specific mating strain to a *mta*-specific strain. Further analysis showed that the *MAT* information is present only in a single, stable copy with no evidence for mating type switching, as is seen in *S. cerevisiae*. The *N. crassa MAT* idiomorphs are exceptionally large because the mating genes of this species are also involved in vegetative incompatibility (Glass *et al.* 1988). This is the only known example where vegetative incompatibility is combined with the *MAT* idiomorph.

Comparison of the *mtA* and *mta* idiomorphs revealed the size of the *mtA* idiomorph as 5301 bp (Glass *et al*. 1990) and the *mta* idiomorph as 3235 bp (Staben & Yanofsky 1990). Regions flanking the idiomorphs showed very high similarity between *mtA* and *mta*. There is an abrupt transition from flanking sequence to idiomorph-specific sequence for both regions.

Functioning of the *MAT* loci

Primary analysis identified a single ORF at the *mtA* idiomorph, *matA-1* (Glass *et al.* 1990). A 288 aa putative peptide is encoded by the *matA-1* ORF with a sub-region of the protein showing similarity with the *α1* polypeptide from *S. cerevisiae*. The highest level of conservation is found in a 15 aa region, the α-box motif, indicating that *matA-1* is involved in the regulation of mating actions (Glass *et al.* 1990). Regulation might be via the formation of a heterodimer as is seen in the yeast *mta/α*-heterodimer (Dranginis 1990). The C-terminal part of the peptide is highly acidic and is thought to control functions not directly related to fertilization (Coppin *et al.* 1997).

Ferreira *et al*. (1996) identified two additional ORFs in the *mtA* idiomorph through RNA analysis. The first of these is the mat*A-2* ORF that encodes a protein 373 aa in length. Comparison with databases showed no conserved regions except for similarity to the *P. anserina SMR1* gene. It has been hypothesised that a 20 aa region could potentially be a new DNA binding motif in the form of an amphipathic α-helix (Ferreira *et al.* 1996, Shiu & Glass 2000).

The third ORF, *matA-3* is transcribed in the opposite direction to that of *matA-2*. The 324 aa peptide encoded by the matA-3 gene has an HMG-domain similar to the characteristic motif seen in the *MAT-2* idiomorph. This gene could therefore also regulate gene expression through DNA-binding (Grosschedl *et al.* 1994). What has been shown in mutational studies is that *matA-1* and *mata-1* (see below) are essential for the mating and fertilization, while *matA-2* and *matA-3* are required to increase the efficiency of mating (Ferreira *et al*. 1996).

DNA from the *mta* idiomorph region was also sequenced and analysed (Staben & Yanofsky 1990). A single ORF was identified and termed *mata*-1. The predicted peptide is 382 aa long and contains an HMG DNA-binding sub-region. The remaining part of the *mata-1* transcript controls vegetative incompatibility. Mutational changes targeting this region interfere with vegetative incompatibility leaving fertilization functions intact (Staben & Yanofsky 1990).

3.3 Podospora anserina

Life cycle

Once the mating type genes for *N. crassa* had been identified, it was possible to isolate the *MAT* locus from the closely related fungus, *P. anserina* (Debuchy & Coppin 1992, Picard *et al.* 1991). Both *Neurospora* and *Podospora* belong to the Ascomycete family, Sordariaceae. *Podospora anserina* is a coprophilous fungus that does not follow the exact mating cycle discussed earlier (Esser 1974). It is a pseudohomothallic species that has both mating types compartmentalised in a single ascospore, but in different nuclei. This results in a type of homothallism, although mating still only occurs between isolates of opposite mating type (Esser 1974). Picard *et al*. (1991) isolated the mating type idiomorphs mat- and mat+ (Fig.4).

The *MAT* locus

A probe of the *N. crassa mtA* mating region hybridised to cosmids containing *P. anserina mat-* fragments (Picard *et al*. 1991), confirming sequence similarity between *N. crassa mtA* and *P. anserina mat-*. However, an opposite mating type probe using *N. crassa mta* failed to bind the *mat+* idiomorph. The *mat+* idiomorph was identified using homology to the opposite *mat-*idiomorph. The sizes of the idiomorphs were 4.7 kb for the *mat-* idiomorph and 3.8 kb for the *mat+* idiomorph and were flanked by conserved regions. Similar to the *MAT* loci of *N. crassa,* it was found that the mating type information of *P. anserina* was present as a single copy and it showed no potential for mating type switching.

Functioning of the *MAT* locus

A single ORF was identified in the *mat+* idiomorph and three coding regions were identified in the *mat-* idiomorph (Debuchy & Coppin 1992, Debuchy *et al*. 1993). The gene *FMR1,* which is found in the *mat-* idiomorph, encodes for a 305 aa peptide. Comparison of the amino acid sequence with the *matA-1* peptide of *N. crassa* resulted in the identification of an α-domain motif, resembling that observed in the *S. cerevisiae MAT*α1 gene (Debuchy & Coppin 1992). This similarity suggests a role for *FMR1* in mating gene regulation, similar to that in *S. cerevisiae* and *N. crassa*.

In a later study, two more genes in the *mat-* region were identified, *SMR1* and *SMR2* (Debuchy *et al*. 1993). Functional analysis of the 356 aa *SMR1* protein revealed a

characteristic fungal mating motif first seen in *N. crassa matA-2*. The proposed peptide from this gene could also form an amphipathic α-helix, characteristic of transcriptional activators (Giniger & Ptashne 1987). This might suggest a role for the gene product in regulating *MAT* functions.

The *SMR2* protein is 288 aa in length. Analysis showed the protein to have an HMG-box similar to that of *N. crassa matA-3* (Debuchy *et al*. 1993). This strongly implicates the peptide in gene regulatory functions (Grosschedl *et al*. 1994).

Sequence analysis for the opposite *mat+* idiomorph identified a single ORF, *FPR1* encoding a 402 aa polypeptide. This protein contains the conserved DNA-binding motif HMG indicative of a role in gene regulation (Grosschedl *et al*. 1994). This gene is homologous to the mat***a****-1* gene from *N. crassa* and could have a shared function between these species. The presence of this HMG gene is characteristic of the *MAT-2* idiomorph in both *N. crassa* and *P. anserina* (Turgeon & Yoder 2000).

Only two genes are necessary for fertilization in *P. anserina* (Coppin *et al*. 1997). These genes are the major regulators of the sexual cycle and are *FMR1* in *mat-* and *FPR1* from the *mat+* idiomorph (Debuchy & Coppin 1992, Picard *et al*. 1991). The other two genes present at the *mat-* idiomorph are needed for post-fertilization events and are involved in mating, although not directly in fertilization (Debuchy *et al*. 1993).

3.4 Cochliobolus heterostrophus

Life cycle

Cochliobolus heterostrophus is the third filamentous Ascomycete for which the *MAT* genes were cloned and analysed. This fungus is a maize pathogen (Yoder *et al*. 1986) with a life cycle similar to that of *N. crassa* (Guzman *et al*. 1982). It is also heterothallic and has a single mating locus (Yoder *et al.* 1986). Unlike the case for *P. anserina,* nothing was known about the *MAT* genes for any relatives of *Cochliobolus*. Turgeon *et al*. (1993) characterised the mating idiomorphs, *MAT-1* and *MAT-2* (Fig. 4).

The *MAT* locus

Turgeon *et al.* (1993) cloned and undertook functional analysis of the mating type genes of *C. heterostrophus*. To clone the genes, the authors used functional complementation. A *MAT-2* strain was transformed with cosmid libraries containing *MAT-1* DNA. The cosmid transforming the strain into a homothallic strain (screened by mating studies) was thus identified as containing the *MAT-1* DNA information. *MAT-2* was identified after probing with *MAT-1*-specific sequence.

Southern blot analysis showed the presence of a single copy of each *MAT* idiomorph (Turgeon *et al*. 1993), indicating that mating type switching would not be possible. *MAT-1* is 1.3 kb in size and *MAT-2* is 1.2 kb in size. Regions flanking these idiomorphs show a high degree of conservation between the idiomorphs.

Functioning of the *MAT* locus

The *MAT-1* idiomorph contains a single ORF, *MAT-1* that encodes a peptide 343 aa in length. A comparison of this peptide with those in databases produced expected similarity to the *N. crassa matA-1* and *P. anserina FMR1* products. A conserved domain similar to the yeast α-box is found within the protein and confirms the involvement of this protein in gene regulation (Turgeon *et al*. 1993).

A 343 aa translation product can be found from the *MAT-2* gene, the only gene encoded by the *MAT-2* idiomorph. An HMG-box conserved domain was found in the *MAT-2* translation product that showed high similarity to the translation products of *N. crassa mata-1,* as well as the *P. anserina FPR1*. No similarity was observed between the *MAT* proteins of *C. heterostrophus* and that of *N. crassa* or *P. anserina* outside the regions of the conserved domains. From this analysis, it is clear that the conserved domains of the proteins are involved in DNA binding and adds credence to the idea that these proteins act as transcriptional regulators (Turgeon *et al*. 1993).

Cochliobolus heterostrophus differs from the species for which *MAT* genes were isolated previously, in that only a single functional gene is present at both mating type loci. These genes correspond to the genes needed for fertilization. The genes needed for post-fertilization events are thus not closely linked with the *MAT* locus in this species.

4. Characteristics of the *MAT* locus

From the above-mentioned and other studies on mating types, some general characteristics of the *MAT* locus become clear. *MAT* contains the mating type idiomorphs responsible for mating responses in filamentous Ascomycetes. Depending on the idiomorph present at this locus, an isolate can be either *MAT-1* or *MAT-2*. Although these idiomorphs are different, similarities exist between them (Fig. 4). Some of these are as follows:

- The flanking regions of *MAT-1* and *MAT-2* (within a species) share a high percentage of similarity ranging from 98% to as high as 100% (Glass *et al.* 1990, McGuire *et al*. 2001, Turgeon 1998). This conservation has been exploited in species identification (Paoletti *et al*. 2006), as well as in PCR determination of mating type specificity (Dyer *et al*. 2001, Steenkamp *et al.* 2000).

- A single gene in each idiomorph is needed for a successful fertilization event. In *MAT-1,* it is the α-box containing gene and for *MAT-2* the HMG-box gene. Other genes might be present at the idiomorphs but are thought to control processes not directly involved in mating. The minimum requirement to constitute a pair of mating type idiomorphs is seen in *C. heterostrophus* (Turgeon *et al*. 1993).

- The conserved domains of the peptides encoded by these genes define the idiomorphs. An idiomorph that contains an α-box motif is characterised as *MAT-1*. Although genes at this idiomorph may also contain other functional domains (HMG-box or amphipathic helix), the presence of the α-box is characteristic. For the *MAT-2* idiomorph, the presence of the conserved HMG-box is the defining characteristic (Turgeon & Yoder 2000).

- In both the idiomorphs, an intron interrupts the gene in the conserved region. Strikingly, all *MAT* genes containing the HMG DNA binding motif, share an intron of size 54-68 bp placed at a conserved serine position (Arie *et al*. 1997, Coppin *et al.* 1997, Turgeon & Yoder 2000). This can be used to confirm that an HMG box that was cloned and sequenced belongs to the mating type genes. A conserved intron also interrupts the α-box motif in all *MAT-1* idiomorphs (Coppin *et al*. 1997, Turgeon & Yoder 2000). This can also confirm the nature of a gene thought to be a *MAT-1* gene.

- No evidence for bi-directional mating type switching has been found in any filamentous Ascomycetes studied to date and is confirmed by the absence of multiple copies of the *MAT* locus. This is in contrast to what is seen in *S. cerevisiae,* where a single isolate can switch its mating type under favourable conditions. However, there are reports of unidirectional mating type switching in some filamentous Ascomycetes (Harrington & McNew 1997, Perkins 1987, Witthuhn *et al.* 2000).

These important points can guide the characterisation and study of *MAT* genes in fungi. Suggestions have been made that flanking conservation could be used to locate the opposite mating type, once the flanking region of one of the mating types is known (Arie *et al.* 1997). Genome walking, using degenerate primers targeting these conserved regions, has also successfully been used (Arie *et al.* 1997, Paoletti *et al.* 2005) and could help with the isolation of the *MAT* idiomorphs.

5. Ophiostoma *ecology and taxonomy*

The genus *Ophiostoma* forms part of an economically important group of fungi commonly referred to as the ophiostomatoid fungi (Wingfield *et al*. 1993). The majority of species in this group are insect-associated fungi that inhabit wood. They are characterised by morphological adaptations to facilitate insect dispersal. These include ascomata with spherical bases and long necks giving rise to sticky masses of ascospores, as well as anamorph structures (classified in genera such as *Leptographium* and *Pesotum*) with sticky drops of conidia (Wingfield *et al*. 1993). These spore drops attach to the bodies of wood-infesting bark- and ambrosia beetles (Malloch & Blackwell 1993) and even hyperphoretic mites (Bridges & Moser 1983).

Various types of symbiotic relationships, some of which are not fully understood, exist between the arthropods and these fungi (Six 2003). What is known is that most of these fungi have the ability to colonize freshly exposed sapwood of trees (Gibbs 1993). Many of the species are pigmented and cause an undesirable staining of the wood leading to a reduction in commercial value of timber (Seifert 1993). Some species, including the causal agents of Dutch Elm Disease (DED) are serious pathogens that kill trees (Brasier 1991, Harrington 1993).

Since the early 1990's, a substantial number of publications have dealt with the phylogenetic classification of *Ophiostoma* and related genera (Aghayeva *et al.* 2004, De Beer *et al*. 2003,

Hausner *et al*. 1993, Harrington *et al.* 2001, Lim *et al*. 2004, Spatafora & Blackwell 1994). One of the most comprehensive recent publications was by Zipfel *et al.* (2006) in which a large sample of isolates was used in a multigene phylogenetic study. Using data from the β-tubulin and nuclear large ribosomal subunit gene, 50 *Ophiostoma* species were analysed. The description for *Ophiostoma* was amended to accommodate species with *Sporothrix*, *Pesotum* and *Hyalorhinocladiella* anamorphs. Furthermore, the teleomorph genera *Grosmannia* and *Ceratocystiopsis* were reinstated for two monophyletic lineages alongside *Ophiostoma* within the Ophiostomatales.

Zipfel *et al*. (2006) not only produced a useful phylogeny of the three genera within the Ophiostomatales but also examined the relationships of species within *Ophiostoma sensu stricto* (Fig. 5). Although the focus of the study was not on the subgroups within *Ophiostoma*, the results revealed a clear grouping of different species into well-defined clades. One of these clades showed the relatedness of the DED fungi to the bluestain fungus, *O. quercus*. This species has been isolated from *Quercus* and *Fagus* species in the Northern Hemisphere (Brasier & Kirk 1993) and on various hardwood species as well as on commercial *Pinus* spp. in the Southern Hemisphere (De Beer *et al*. 2003). The fungus apparently does not cause tree diseases, but is associated with bluestain of timber after felling and, therefore, is economically important (De Beer *et al*. 2003). Other species grouping closely in the DED - *O. quercus* clade include *O. auracariae,* as well as the conifer-infesting species *O. canum*, *O. piceae, O. flexuosum* and *O. distortum*. However, the relationships within and between these and other species groups in *Ophiostoma* remain largely unresolved. Sequences of additional gene regions are necessary for further clarification.

Conventionally, DNA regions used in phylogenetic analysis for fungi included the internal transcribed spacer (ITS) regions of the ribosomal DNA operon (Aghayeva *et al.* 2004, Gorton *et al.* 2004, Marin *et al.* 2003, Zipfel *et al.* 2006), β-tubulin gene (Aghayeva *et al.* 2004, Gorton *et al.* 2004, Marin *et al.* 2003, Zipfel *et al.* 2006), glyceraldehyde-3-phosphate dehydrogenase (GPD) gene (Guerber *et al.* 2003, Inderbitzin *et al.* 2005) and the elongation factor 1-α (EF1-α) gene (Inderbitzin *et al.* 2005). More recently, mating type genes (Inderbitzin *et al.* 2005, Marin *et al.* 2003, 2005) have also begun to emerge as candidates for use in Ascomycete phylogenetics, and they are worth considering for this purpose in the Ophiostomatales.

Turgeon (1998) suggested that the HMG-box of the *MAT-2* idiomorph might offer equal or superior phylogenetic resolution compared to more commonly employed genes such as ITS and GPD sequences. It was shown that high interspecies variation between *MAT-2* genes was the reason for this (Turgeon 1998). Du *et al.* (2005) showed that the mating type genes proved useful in improving phylogenetic resolution of species in the *Collectrichum* complex. These researchers concluded that *MAT-2* HMG sequence is a powerful tool for resolving species within these species complexes. However, phylogenetic analysis using *MAT* sequences is limited to a comparison using only the dataset from one mating specificity due to the sequence differences inherent in the *MAT* genes. Another important aspect relating to the use of *MAT* genes in phylogenetics rests on obtaining sequence data for comparison relatively easily and quickly.

5.1 The mating type genes in Ophiostoma

Due to the notoriety of the DED pathogens, various aspects of the biology and genetics of *O. ulmi*, *O. novo-ulmi* and *O. himal-ulmi* have been intensely studied (Brasier 1990, 1991, Brasier & Kirk 1993, 2001, Brasier & Mehrotra 1995, Paoletti *et al*. 2005, 2006). In 2005, the first studies of mating type in *Ophiostoma* were made using these fungi (Paoletti *et al*. 2005). These researchers used degenerate primers (Arie *et al*. 1997) to amplify the HMG box from a *MAT-2* isolate of *O. novo-ulmi* subsp. *americana*. Using genome walking the complete *MAT-2* idiomorph was characterised. *MAT-2* contains a single ORF encoding a 202 aa peptide (Fig. 6). This protein contains the characteristic HMG DNA binding domain. The gene also contains a single intron of 57 bp that is found at the position shown to be conserved in all the filamentous Ascomycetes studied to date (Arie *et al.* 1997, Coppin *et al.* 1997, Turgeon & Yoder 2000).

Paoletti *et al*. (2006) found evidence for the introgression of DNA from resident *O. ulmi* populations into invading *O. novo-ulmi* populations at fronts where both these pathogens are found. They were able to amplify and sequence part of the 3'-flanking region as well as 200 bp of *MAT-1* specific sequence (Fig. 6). This allowed for the comparison of the *MAT-1* and *MAT-2* flanking regions, confirming the expected conservation at the 3' flanking position.

Studies on the DED fungi provided the first mating type sequence data for the genus *Ophiostoma*. The presence of the HMG-box in *MAT-2* and conservation of the 3' flanking region suggests that the organisation of the *MAT* genes in this genus will correspond to what has been seen in other species.

6.0 Conclusions

Studies investigating the genes that govern mating are crucial to understanding the biology and genetics of fungi. Therefore, it is surprising that sequence data for the *MAT* idiomorphs are available for only a small number of species. This situation is beginning to change and in future, information on these genes will certainly contributed more deeply to phylogenetic and other studies.

The genes controlling the mating process in filamentous Ascomycetes show a large amount of conservation in structure and function, although substantial differences occur in the organisation and sequence of the gene regions. Mating is controlled by two dissimilar idiomorphs whose protein products contain regions involved in DNA binding. This allows for regulation of genes downstream from the mating type genes to control the physiological changes associated with mating. Although the mating process is broadly understood, many details relating to this process remain to be investigated.

The mating genes for several fungal species have been characterised. In terms of *Ophiostoma*, some mating type gene information is available, but only for three closely related species. This is barely representative of a hugely diverse order such as the Ophiostomatales, including more than 230 species. The full *MAT-2* idiomorph has been characterised for *O. ulmi*, *O. novo-ulmi* and *O. himal-ulmi*, but only a small amount of 200 bp has been sequenced for the *MAT-1* idiomorph. Based on the large morphological and ecological variation in the Ophiostomatales, exploration of mating type genes of other species in the group is necessary. This will allow for a clearer understanding of the effect of mating types on the biology of species in this important group.

7.0 References

Aghayeva, D.N., Wingfield, M.J., De Beer, Z.W. & Kirisits, T. (2004) Two new *Ophiostoma* species with *Sporothrix* anamorphs from Austria and Azerbaijan. *Mycologia* **96**: 866-878

Arie, T., Christiansen, S.K., Yoder, O.C. & Turgeon, B.G. (1997) Efficient cloning of Ascomycete mating type genes by PCR amplification of the conserved *MAT* HMG box. *Fungal Genetics and Biology* **21**: 118-130

Arnaise, S., Zickler, D., Le Bilcot, S., Poisier, C. & Debuchy, R. (2001) Mutations in mating type genes of the heterothallic fungus *Podospora anserina* lead to self-fertility. *Genetics* **159**: 545-556

Astell, C.R., Ahlstrom-Jonasson, L., Smith, M., Tatchell, K., Nasmyth, K.A. & Hall, B.D. (1981) The sequence of the DNAs coding for the mating type loci of *Saccharomyces cerevisiae*. *Cell* **27**: 15-23

Brasier, C.M. (1990) China and the origins of Dutch Elm disease: An appraisal. *Plant Pathology* **39**: 5-16

Brasier, C.M. (1991) *Ophiostoma novo-ulmi* sp. nov., causative agent of current Dutch Elm disease pandemics. *Mycopathologia* **115**: 151-161

Brasier, C.M. & Kirk, S.A. (1993) Sibling species within *Ophiostoma piceae*. *Mycological Research* **97**: 811-816

Brasier, C.M. & Kirk, S.A. (2001) Designation of the EAN and NAN races of *Ophiostoma novo-ulmi* as subspecies. *Mycological Research* **105**: 547-554

Brasier, C.M. & Mehrotra, M.D. (1995) *Ophiostoma himal-ulmi* sp. nov., a new species of Dutch Elm disease fungus endemic to the Himalayas. *Mycological Research* **99**: 205-215

Bridges, R.J. & Moser, J.C. (1983) Role of two phoretic mites in transmission of bluestain fungus, *Ceratocystis minor*. *Ecological Entomology* **8**: 9-12

Coppin, E., Debuchy, R., Arnaise, S. & Picard, M. (1997) Mating types and sexual development in filamentous Ascomycetes. *Microbiology and Molecular Biology Reviews* **61**: 411-428

De Beer, Z.W., Wingfield, B.D. & Wingfield, M.J. (2003) The *Ophiostoma piceae* complex in the Southern Hemisphere: A phylogenetic study. *Mycological Research* **107**: 469-476

Debuchy, R. & Coppin, E. (1992) The mating types of *Podospora anserina*: Functional analysis and sequence of the fertilization domains. *Molecular and General Genetics* **233**: 113-121

Debuchy, R., Arnaise, S. & Lecellier, G. (1993) The *mat*- allele of *Podospora anserina* contains three regulatory genes required for the development of fertilized female organs. *Molecular Genetics and Genomics* **241**: 667-673

Dranginis, A.M. (1990) Binding of yeast a1 and alpha 2 as a heterodimer to the operator DNA of a haploid-specific gene. *Nature* **347**: 682-685

Du, M., Schardl, C.L., Nuckles, E.M. & Vaillancourt, L.J. (2005) Using mating type gene sequences for improved phylogenetic resolution of *Collectotrichum* species complexes. *Mycologia* **97**: 641-658

Dyer, P.S., Furneaux, P.A., Douhan, G. & Murray, T.D. (2001) A multiplex PCR test for determination of mating type applied to the plant pathogens *Tapesia yallundae* and *Tapesia acuformis*. *Fungal Genetics and Biology* **33**: 173-180

Elliott, C.G. (1994) *Reproduction in fungi: Genetical and physiological aspects*. Chapman & Hall, London, New York

Esser, K. (1974) *Podospora anserina*. In *Handbook of Genetics* (R.C. King, ed.), Plenum Press, New York/London: 531-551

Ferreira, A.V., Saupe, S. & Glass, N.L. (1996) Transcriptional analysis of the mta idiomorph of *Neurospora crassa* identifies two genes in addition to mta-1. *Molecular and General Genetics* **250**: 767-774

Gibbs, J.N. (1993) The biology of Ophiostomatoid fungi causing sapstain in trees and freshly cut logs. In *Ceratocystis and Ophiostoma: Taxonomy, Ecology and Pathogenicity* (M.J. Wingfield, K.A. Seifert & J. Webber, Eds), APS Press, St. Paul, Minnesota: 153-160

Giniger, E. & Ptashne, M. (1987) Transcription in yeast activated by a putative amphipathic alpha helix linked to a DNA binding unit. *Nature* **330**: 670-672

Glass, N.L. & Nelson, M.A. (1994) Mating type genes in mycelial Ascomycetes. In *The Mycota I: Growth, Differentiation and Sexuality* (J.G.H. Wessels & F. Meinhardt, Eds), Springer-Verlag, Berlin, Germany: 295-306

Glass, N.L., Grotelueschen, J. & Metzenberg, R.L. (1990) *Neurospora crassa* A mating type region. *Proceedings of the National Academy of Sciences of the United States of America* **87**: 4912-4916

Glass, N.L., Vollmer, S.J., Staben, C., Grotelueschen, J., Metzenberg, R.L. & Yanofsky, C. (1988) DNAs of the two mating-type alleles of *Neurospora crassa* are highly dissimilar. *Science* **241**: 570-573

Gorton, C., Kim, S.H., Henricot, B., Webber, J. & Breuil, C. (2004) Phylogenetic analysis of the bluestain fungus *Ophiostoma minus* based on partial ITS rDNA and β-tubulin gene sequences. *Mycological Research* **108**: 759-765

Grosschedl, R., Giese, K. & Pagel, J. (1994) HMG domain proteins: architectural elements in the assembly of nucleoprotein structures. *Trends in Genetics* **10**: 94-100

Guerber, J.C., Liu, B., Correll, J.C. & Johnston, P.R. (2003) Characterization of diversity in *Colletotrichum acutatum* sensu lato by sequence analysis of two gene introns, mtDNA and intron RFLPs, and mating compatibility. *Mycologia* **95**: 872-895

Guzman, D., Garber, R.C. & Yoder, O.C. (1982) Cytology of meiosis-I and chromosome-number of *Cochliobolus heterostrophus* (Ascomycetes). *Canadian Journal of Botany* **60**: 1138-1141

Harrington, T.C. (1993) Diseases of conifers caused by species of *Ophiostoma* and *Leptographium*. In *Ceratocystis and Ophiostoma: Taxonomy, Ecology and Pathogenicity* (M.J. Wingfield, K.A. Seifert & J. Webber, Eds), APS Press, St. Paul, Minnesota: 161-172

Harrington, T.C. & McNew, D.L. (1997) Self-fertility and uni-directional mating type switching in *Ceratocystis coerulescens*, a filamentous Ascomycete. *Current Genetics* **32**: 52-59

Harrington, T.C., McNew, D., Steimel, J., Hofstra, D. & Farrell, R. (2001) Phylogeny and taxonomy of the *Ophiostoma piceae* complex and the Dutch Elm disease fungi. *Mycologia* **93**: 111-136

Hausner, G., Reid, J. & Klassen, G.R. (1993) On the phylogeny of *Ophiostoma*, *Ceratocystis* s.s., and *Microascus*, and relationships within *Ophiostoma* based on partial ribosomal DNA sequences. *Canadian Journal of Botany* **71**: 1249-1265

Herskowitz, I. (1988) Life cycle of the budding yeast *Saccharomyces cerevisiae*. *Microbiological Reviews* **52**: 536-553

Inderbitzin, P., Harkness, J., Turgeon, B.G. & Berbee, M.L. (2005) Lateral transfer of mating system in *Stemphylium*. *Proceedings of the National Academy of Sciences of the United States of America* **102**: 11390-11395

Kendrick, B. (2000) *The Fifth Kingdom*. Mycologue Publications, Sydney, B.C.

Lim, Y.W., Alamouti, S.M., Kim, J.-J., Lee, S. & Breuil, C. (2004) Multigene phylogenies of *Ophiostoma clavigerum* and closely related species from bark beetle-attacked *Pinus* in North America. *FEMS Microbiology Letters* **237**: 89-96

Malloch, D.W. & Blackwell, M. (1993) Dispersal biology of the Ophiostomatoid fungi. In *Ceratocystis and Ophiostoma: Taxonomy, Ecology and Pathogenicity* (M.J. Wingfield, K.A. Seifert & J. Webber, Eds), APS Press, St. Paul, Minnesota: 195-206

Marin, M., Castro, B., Gaitan, A., Preisig, O., Wingfield, B.D. & Wingfield, M.J. (2003) Relationships of *Ceratocystis fimbriata* isolates from Colombian coffee-growing regions based on molecular data and pathogenicity. *Journal of Phytopathology* **151**: 395-405

Marin, M., Preisig, O., Wingfield, B.D., Kirisits, T., Yamaoka, Y. & Wingfield, M.J. (2005) Phenotypic and DNA sequence data comparisons reveal three discrete species in the *Ceratocystis polonica* species complex. *Mycological Research* **109**: 1137-1148

McGuire, I.C., Marra, R.E., Turgeon, B.G. & Milgroom, M.G. (2001) Analysis of mating-type genes in the Chestnut Blight fungus, *Cryphonectria parasitica*. *Fungal Genetics and Biology* **34**: 131-144

Metzenberg, R.L. & Glass, N.L. (1990) Mating type and mating strategies in *Neurospora*. *Bioessays* **12**: 53-59

Nelson, M.A. (1996) Mating systems in Ascomycetes: a romp in the sac. *Trends in Genetics* **12**: 69-74

Paoletti, M., Buck, K.W. & Brasier, C.M. (2005) Cloning and sequence analysis of the *MAT-B* (*MAT-2*) genes from the three Dutch Elm disease pathogens, *Ophiostoma ulmi*, *O. novo-ulmi*, and *O. himal-ulmi*. *Mycological Research* **109**: 983-991

Paoletti, M., Buck, K.W. & Brasier, C.M. (2006) Selective acquisition of novel mating type and vegetative incompatibility genes via interspecies gene transfer in the globally invading eukaryote *Ophiostoma novo-ulmi*. *Molecular Ecology* **15**: 249-262

Perkins, D.D. (1987) Mating type switching in filamentous Ascomycetes. *Genetics* **115**: 215-216

Perkins, D.D. (1992) *Neurospora*: the organism behind the molecular revolution. *Genetics* **130**: 687-701

Picard, M., Debuchy, R. & Coppin, E. (1991) Cloning the mating types of the heterothallic fungus *Podospora anserina*: developmental features of haploid transformants carrying both mating types. *Genetics* **128**: 539-547

Pöggeler, S. (2001) Mating type genes for classical strain improvements of Ascomycetes. *Applied Microbiology and Biotechnology* **56**: 589-601

Pöggeler, S. & Kück, U. (2001) Identification of transcriptionally expressed pheromone receptor genes in filamentous Ascomycetes. *Gene* **280**: 9-17

Pöggeler, S., Nowrousian, M., Ringelberg, C., Loros, J.J., Dunlap, J.C. & Kück, U. (2006) Microarray and real-time PCR analyses reveal mating type-dependent gene expression in a homothallic fungus. *Molecular Genetics and Genomics* **275**: 492-50

Rine, J., Strathern, J.N., Hicks, J.B. & Herskowitz, I. (1979) A suppressor of mating type locus mutations in *Saccharomyces cerevisiae*: evidence for and identification of cryptic mating type loci. *Genetics* **93**: 877-901

Seifert, K.A. (1993) Sapstain of commercial lumber by species of *Ophiostoma* and *Ceratocystis*. In *Ceratocystis and Ophiostoma: Taxonomy, Ecology and Pathogenicity*

(M.J. Wingfield, K.A. Seifert & J. Webber, Eds), APS Press, St. Paul, Minnesota: 141-151

Shiu, P.K. & Glass, N.L. (2000) Cell and nuclear recognition mechanisms mediated by mating type in filamentous Ascomycetes. *Current Opinion in Microbiology* **3**: 183-188

Six, D.L. (2003) Bark beetle-fungus symbiosis. In *Insect Symbiosis* (K. Bourtzis & T.A. Miller Eds), CRC Press, Washington, D.C.: 97-114

Spatafora, J.W. & Blackwell, M. (1994) The polyphyletic origins of Ophiostomatoid fungi. *Mycological Research* **98**: 1-9

Staben, C. & Yanofsky, C. (1990) *Neurospora crassa* a mating type region. *Proceedings of the National Academy of Sciences of the United States of America* **87**: 4917-4921

Steenkamp, E.T., Wingfield, B.D., Coutinho, T.A., Zeller, K.A., Wingfield, M.J., Marasas, W.F. & Leslie, J.F. (2000) PCR-based identification of *MAT-1* and *MAT-2* in the G*ibberella fujikuroi* species complex. *Applied and Environmental Microbiology* **66**: 4378-4382

Strathern, J.N. & Herskowitz, I. (1979) Asymmetry and directionality in production of new cell types during clonal growth: the switching pattern of homothallic yeast. *Cell* **17**: 371-381

Turgeon, B.G. (1998) Application of mating type gene technology to problems in fungal biology. *Annual Review of Phytopathology* **36**: 115-137

Turgeon, B.G. & Yoder, O.C. (2000) Proposed nomenclature for mating type genes of filamentous Ascomycetes. *Fungal Genetics and Biology* **31**: 1-5

Turgeon, B.G., Bohlmann, H., Ciuffetti, L.M., Christiansen, S.K., Yang, G., Schafer, W. & Yoder, O.C. (1993) Cloning and analysis of the mating type genes from *Cochliobolus heterostrophus*. *Molecular and General Genetics* **238**: 270-284

Wingfield, M.J., Seifert, K.A. & Webber, J.F. (1993) *Ceratocystis and Ophiostoma: Taxonomy, ecology, and pathogenicity*. APS Press, St. Paul, Minnesota

Witthuhn, R.C., Harrington, T.C., Wingfield, B.D., Steimel, J.P. & Wingfield, M.J. (2000) Deletion of the *MAT-2* mating type gene during uni-directional mating type switching in C*eratocystis*. *Current Genetics* **38**: 48-52

Yoder, O.C., Valent, B. & Chumley, F. (1986) Genetic nomenclature and practice for plant pathogenic fungi. *Phytopathology* **76**: 383-385

Zipfel, R.D., De Beer, Z.W., Jacobs, K., Wingfield, B.D. & Wingfield, M.J. (2006) Multi-gene phylogenies define *Ceratocystiopsis* and *Grosmannia* distinct from *Ophiostoma*. *Studies in Mycology* **55**: 75-97

Fig. 1. Life cycle of the fungus, *P. anserina* (from Coppin *et al.* 1997).

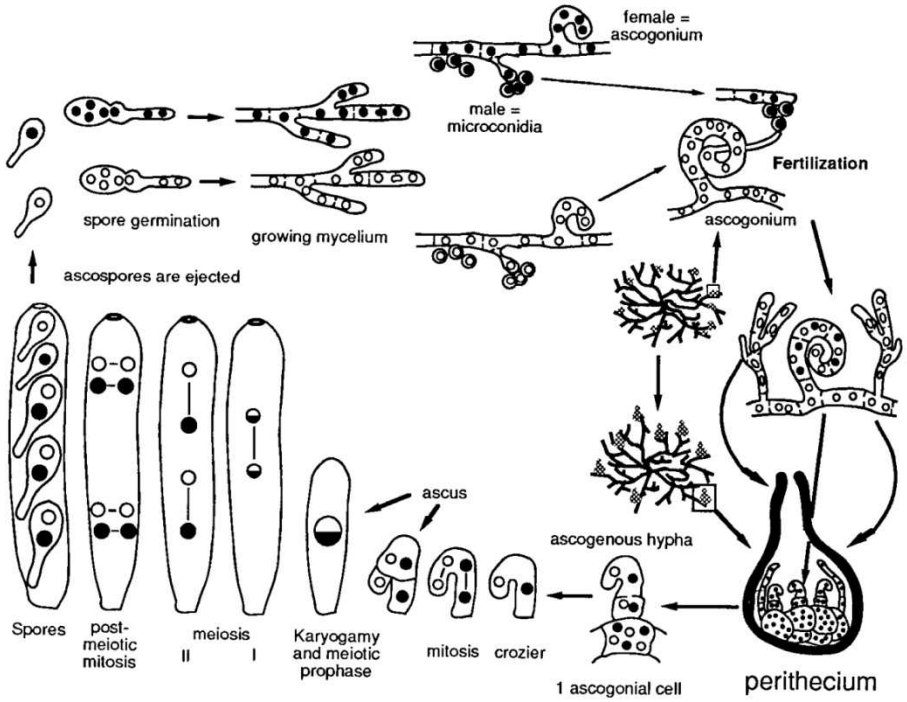

female =
ascogonium
male =
microconidia
Fertilization
ascogonium
spore germination
growing mycelium
ascospores are ejected
ascus
ascogenous hypha
Spores
post-
meiotic
mitosis
meiosis
II
I
Karyogamy
and meiotic
prophase
mitosis
crozier
1 ascogonial cell
perithecium

Fig. 2. The organisation of the *MAT* cassettes in *S. cerevisiae*. *MAT* indicates the true *MAT* loci. HML and HMR indicate the extra cassettes present to facilitate mating type switching. W/X is the left boundary and Z1/Z2 is the right boundary. The Y region is the part of the cassette that contains the mating information (from Elliott 1994).

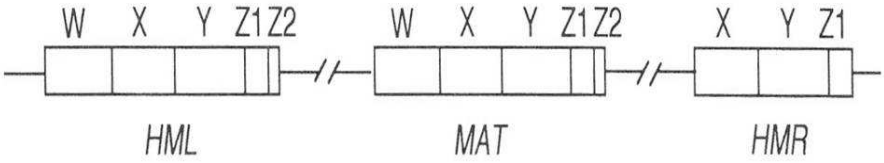

W X Y Z1 Z2
W X Y Z1 Z2
X Y Z1
HML
MAT
HMR

Fig. 3. The mating switch in yeast. A single mother cell, S (mating type α) gives rise to two daughter cells, D1 (α) and D2 (**a**). Daughter cell D1 gives rise to another daughter cell D1-1 (α). In the fourth generation, eight cells were formed from the single mother cell S, four of these **a** mating type and four of these α mating type (from Strathern & Herskowitz 1979)

.

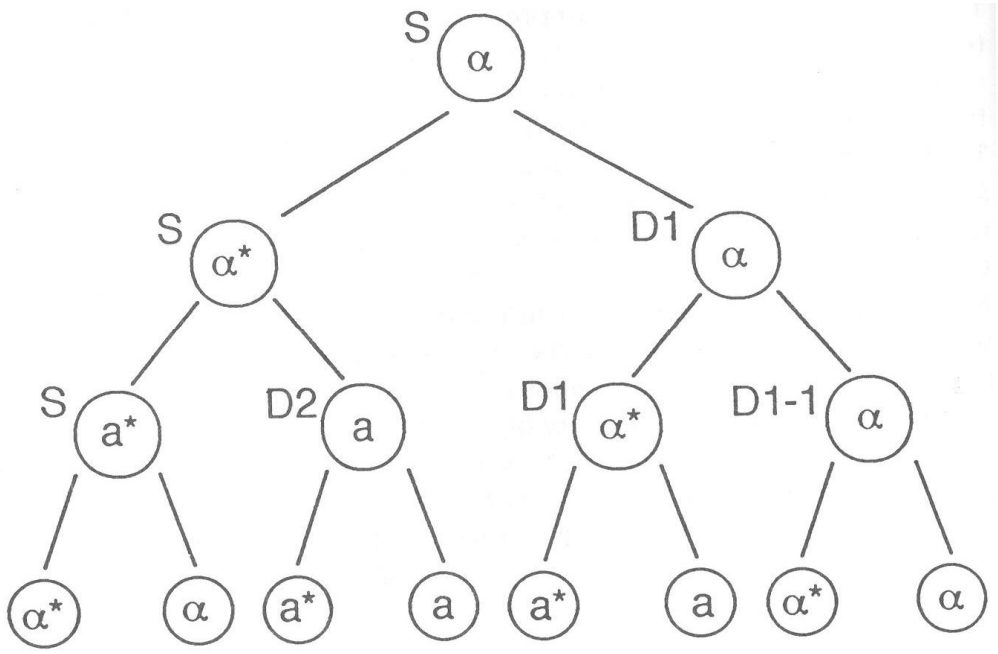

S
α
S
α*
D1
α
S
a*
D2
a
D1
α*
D1-1
α
α*
α
a*
a
a*
a
α*
α

Fig. 4. The organisation of the *MAT* locus of several heterothallic Ascomycetes. Note the conserved flanking regions, the genes found at each of the idiomorphs and the conserved introns in the genes (from Turgeon & Yoder 2000). Single lines: Flanking regions; Solid blocks: Idiomorphs; Arrows: Genes; Blocks on arrows: Conserved regions; Diamond shaped blocks: Introns.

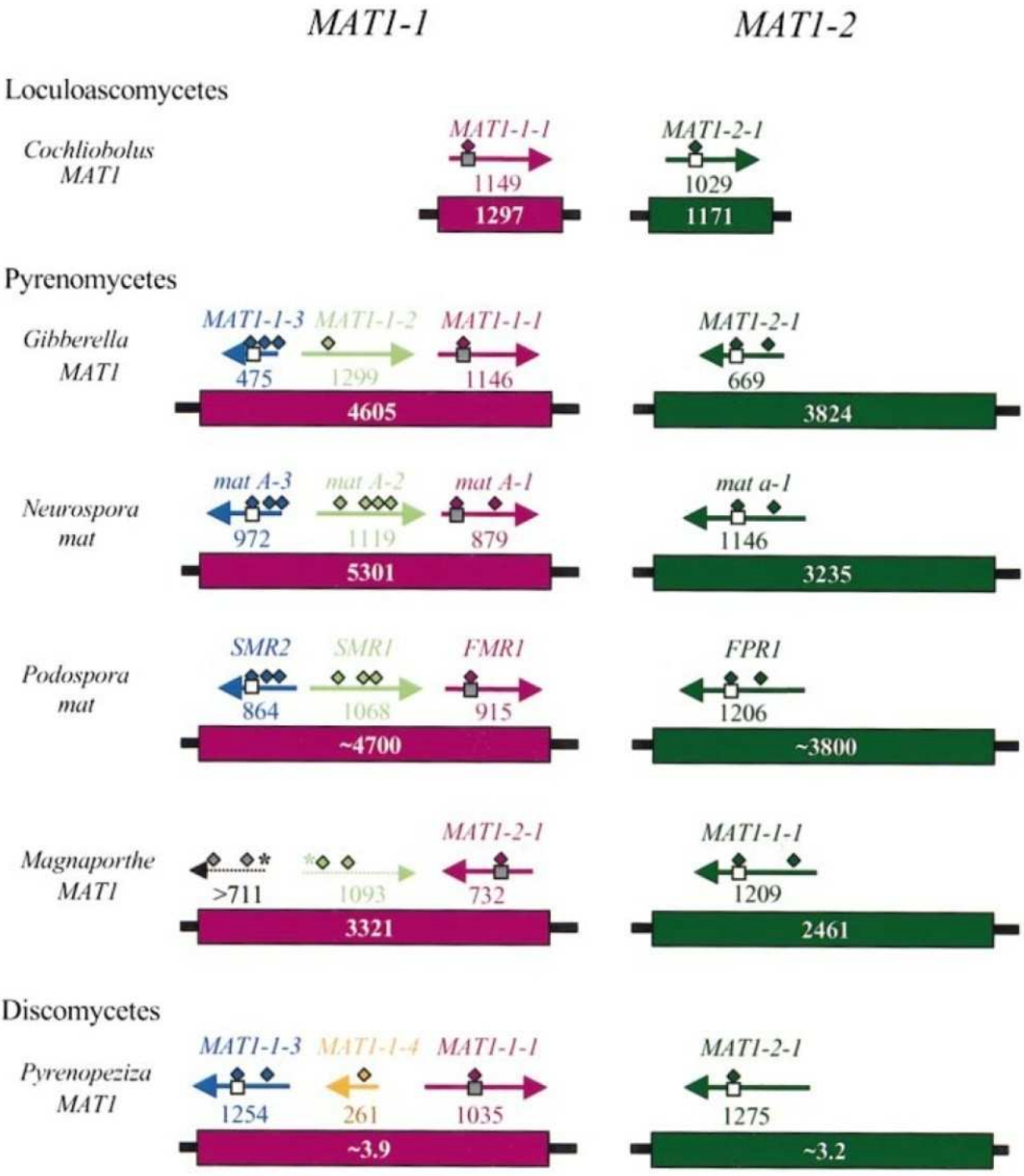

MAT1-1
MAT1-2
Loculoascomycetes
Cochliobolus MAT1
MAT1-1-1
1149
1297
MAT1-2-1
1029
1171
Pyrenomycetes
Gibberella MAT1
MAT1-1-3
475
MAT1-1-2
1299
MAT1-1-1
1146
4605
MAT1-2-1
669
3824
Neurospora mat
mat A-3
972
mat A-2
1119
mat A-1
879
5301
mat a-1
1146
3235
Podospora mat
SMR2
864
SMR1
1068
FMR1
915
~4700
FPR1
1206
~3800
Magnaporthe MAT1
>711
1093
MAT1-2-1
732
3321
MAT1-1-1
1209
2461
Discomycetes
Pyrenopeziza MAT1
MAT1-1-3
1254
MAT1-1-4
261
MAT1-1-1
1035
~3.9
MAT1-2-1
1275
~3.2

Fig. 5. The organisation of the genus *Ophiostoma* and related genera. Note the grouping of the DED pathogens with the fungus, *O. quercus* (from Zipfel *et al.* 2006).

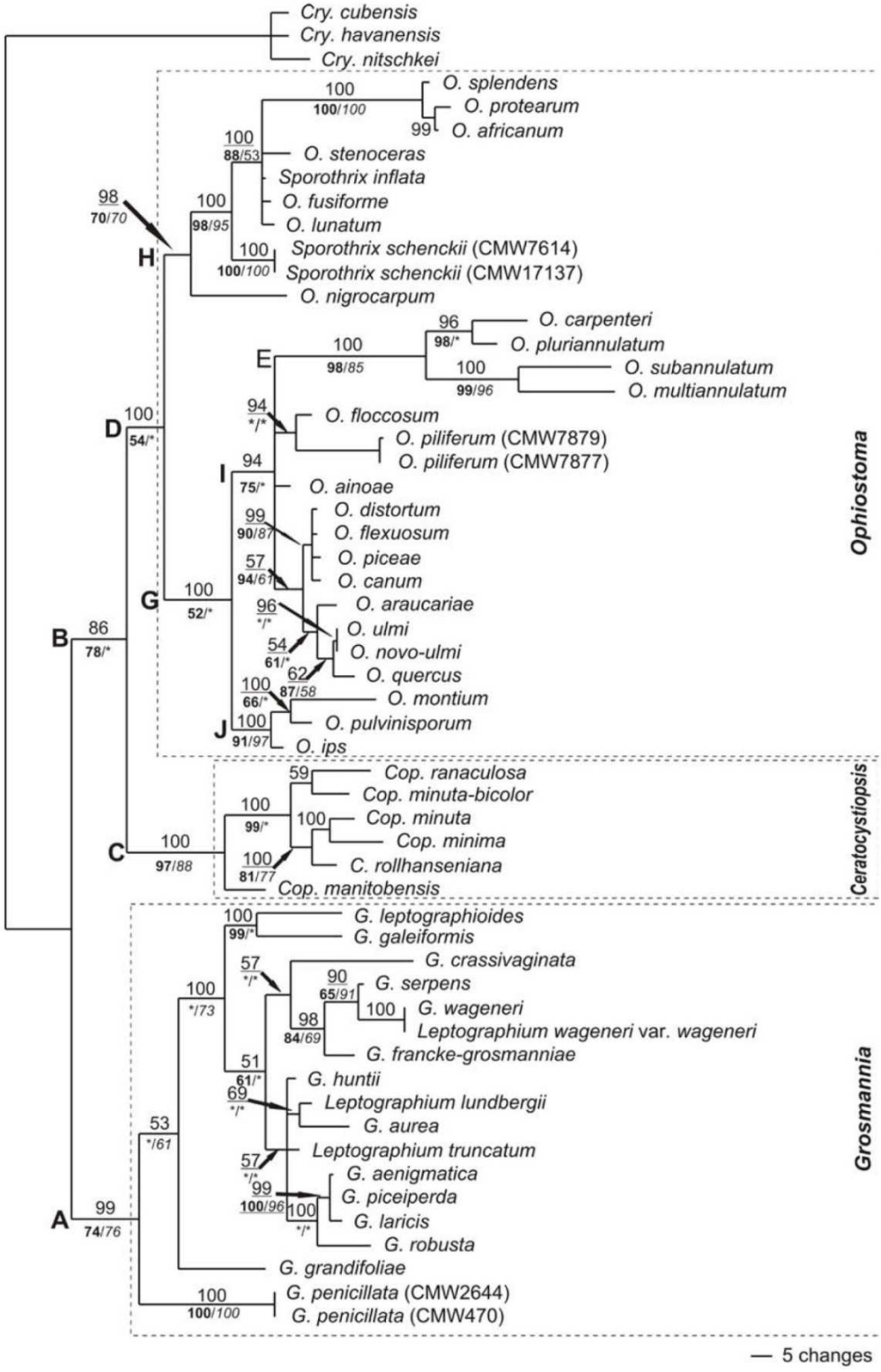
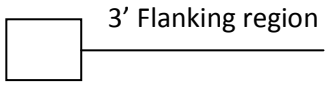

Cry. cubensis
Cry. havanensis
Cry. nitschkei
O. splendens
O. protearum
O. africanum
O. stenoceras
Sporothrix inflata
O. fusiforme
O. lunatum
Sporothrix schenckii (CMW7614)
Sporothrix schenckii (CMW17137)
O. nigrocarpum
O. carpenteri
O. pluriannulatum
O. subannulatum
O. multiannulatum
O. floccosum
O. piliferum (CMW7879)
O. piliferum (CMW7877)
O. ainoae
O. distortum
O. flexuosum
O. piceae
O. canum
O. araucariae
O. ulmi
O. novo-ulmi
O. quercus
O. montium
O. pulvinisporum
O. ips
Ophiostoma
Cop. ranaculosa
Cop. minuta-bicolor
Cop. minuta
Cop. minima
C. rollhanseniana
Cop. manitobensis
Ceratocystiopsis
G. leptographioides
G. galeiformis
G. crassivaginata
G. serpens
G. wageneri
Leptographium wageneri var. wageneri
G. francke-grosmanniae
G. huntii
Leptographium lundbergii
G. aurea
Leptographium truncatum
G. aenigmatica
G. piceiperda
G. laricis
G. robusta
G. grandifoliae
G. penicillata (CMW2644)
G. penicillata (CMW470)
Grosmannia
A
B
C
D
E
G
H
I
J
— 5 changes

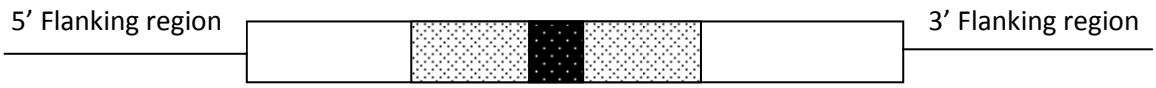
Fig. 6. The available information of the *MAT* idiomorphs from *O. novo-ulmi.* For *MAT-1,* the 3' flanking region, as well as 200 bp of sequence is available for the idiomorph. To which genes this sequence belongs, is currently not known. The full *MAT-2* idiomorph contains a single gene with one intron. Thin lines represent sequences flanking the idiomorphs. Thick blocks represent idiomorph-specific sequence. The *MAT-2* gene is indicated by ⊠ with the intron indicated by ■.

MAT-1



`

MAT-2



CHAPTER 2

Presence of both *MAT1-1* and *MAT1-2* mating idiomorph sequences in single isolates of the heterothallic fungus, *Ophiostoma quercus*

ABSTRACT

Ophiostoma quercus is a heterothallic Ascomycete with a global distribution. In heterothallic Ascomycetes, two opposite but distinct mating types control all sexual processes. Using mating crosses on agar plates, the heterothallic nature of *O. quercus* isolates was confirmed and mating types assigned. Primers were subsequently designed to target the *MAT-1* and *MAT-2* idiomorphs in all isolates. Interestingly, results showed that all ten isolates contained sequence fragments representing the *MAT-1* and *MAT-2* idiomorphs. This was unexpected as each isolate from a heterothallic species would typically contain only one of the two possible *MAT* idiomorphs. An atypical mating system of this nature is not without precedence in Ascomycetes, but has not been observed previously in *Ophiostoma* species.

INTRODUCTION

The organisation of mating type genes in Ascomycetes is highly conserved even between phylogenetically diverse species. Mating, the process central to sexual reproduction, is controlled by a single locus, *MAT-1* (Coppin *et al.* 1997, Turgeon & Yoder 2000). Two genetically distinct versions of the *MAT* locus are found, *MAT1-1* and *MAT1-2* usually referred to as *MAT-1* and *MAT-2*. Because these differ in gene content, function and nucleotide sequence, the term idiomorph is used to describe the two different forms (Metzenberg & Glass 1990).

The DNA sequences flanking the *MAT* idiomorphs share a high degree of similarity, but the idiomorphs themselves contain genes that are highly dissimilar. *MAT-1* typically contains one or more genes, while *MAT-2* usually contains a single gene, although multiple genes can be present (Coppin *et al.* 1997, Turgeon & Yoder 2000). Each idiomorph is characterised by a gene encoding a homeodomain protein. In the *MAT-1* idiomorph, this gene is always similar to the α-box motif of the *Saccharomyces cerevisiae MAT-a* protein (Coppin *et al.* 1997, Turgeon 1998). Likewise, the *MAT-2* idiomorph is always characterised by a High Mobility Group (HMG) motif (Arie *et al.* 1997, Coppin *et al.* 1997). Together the α-box and HMG-domain proteins regulate all processes involved in fertilization. Two fungal individuals, usually arising from single spores, each containing one of the two different idiomorphs, are referred to as belonging to opposite mating types. For a successful fertilization event to occur, the two opposite mating type isolates must make contact (Nelson 1996).

Three mating strategies are known to occur in the Ascomycetes (Nelson 1996). These include true homothallism where an individual arising from an uninucleate ascospore of a true homothallic species can complete the sexual cycle without an opposite mating partner, because both idiomorphs are present in its genome (Elliot 1994, Nelson 1996). Another form of sexual reproduction, known as pseudohomothallism, resembles homothallism in that a single ascospore can complete the sexual cycle independently. However, it differs from homothallism in that the ascospore harbours two nuclei, each containing one of the two idiomorphs (Coppin *et al*. 1997, Glass & Nelson 1994). The fusion of these two nuclei during meiosis results in sexual reproduction and ascospore formation. Heterothallism represents a third mating strategy and it occurs when one isolate must outcross with another of opposite mating type to reproduce sexually (Elliot 1994, Glass & Nelson 1994). Thus, when the two isolates come into contact and sexual reproduction is initiated, their hyphae fuse and the two

nuclei containing different *MAT* idiomorphs undergo karyogamy and meiosis resulting in haploid ascospores of opposite mating type.

Ophiostoma represents a large Ascomycete genus with a worldwide distribution (Wingfield *et al.* 1993). Most species have a close association with tree-infesting bark beetles and some cause serious tree diseases (Wingfield *et al*. 1993). One such group of species, including *O. ulmi* and *O. novo-ulmi,* are known as the Dutch Elm disease pathogens that have been responsible for the death of millions of Elm trees in the Northern Hemisphere (Brasier 1990). Little is known regarding the mating strategies of most *Ophiostoma* spp., but some species have been shown as strictly heterothallic (Brasier 1993, Gorton & Webber 2000, Harrington *et al.* 2001, Solla *et al.* 2008) while others are homothallic (Gorton & Webber 2000, Harrington *et al.* 2001, Zhou *et al*. 2004b).

Paoletti *et al.* (2005) considered the mating type genes of three heterothallic species, *O. ulmi, O. novo-ulmi* and *O. himal-ulmi* and provided the first genetic information for these genes in *Ophiostoma*. The authors were able to clone and sequence the full *MAT-2* idiomorph from the three species (Fig. 1). Subsequently they identified 820 bp (base pairs) of *MAT* sequence from *MAT-1* isolates (Paoletti *et al*. 2006). Of these, 620 bp were proposed to form part of the flanking region common to both idiomorphs and 200 bp were unique to the *MAT-1* idiomorph (Fig. 1). The availability of this sequence data establishes an opportunity to study these genes in other *Ophiostoma* species.

Ophiostoma quercus is closely related to *O. ulmi* and other hardwood-inhabiting species of *Ophiostoma* (Grobbelaar *et al*. 2009, Harrington *et al.* 2001, Kamgan Nkuekam *et al.* 2008b, Linnakoski *et al*. 2008, Zipfel *et al.* 2006). This fungus is not pathogenic, but it is a primary colonist of freshly made wounds on trees (Geldenhuis *et al.* 2004, Kamgan Nkuekam *et al.* 2008a, b) and typically imparts a stain to infected timber (Kim *et al.* 2005, 2007, Thwaites *et al.* 2005). Like most *Ophiostoma* spp., it is vectored by insects, but its relationship with bark beetles and other insects is non-specific (Linnakoski *et al*. 2008, Paciura *et al.* 2009, Zhou *et al*. 2004a, 2006). *Ophiostoma quercus* has a global distribution and has been reported from a wide variety of primarily hardwood hosts in both the Northern and Southern Hemispheres (Brasier & Kirk 1993, De Beer *et al.* 2003, Harrington *et al.* 2001). Several studies have shown that the fungus has a heterothallic mating system (Brasier & Kirk 1993, De Beer *et al.* 2003, Harrington *et al.* 2001, Kamgan Nkuekam *et al.* 2008b, Morelet 1992, Przybyl 1992).

The relatedness of *O. quercus* to the *O. ulmi* group and the availability of several opposite mating type isolates for *O. quercus* (Brasier & Kirk 1993, De Beer *et al*. 2003, Kamgan Nkuekam *et al.* 2008b) provided an opportunity to increase the base of knowledge regarding *MAT* genes in *Ophiostoma*. The aim of this study was thus to characterise the *MAT* genes in *O. quercus* by cloning and sequencing fragments of the *MAT-1* and *MAT-2* idiomorphs.

MATERIALS AND METHODS

Isolates and mating study

Ten *O. quercus* isolates representing opposite mating type strains, were selected from previous studies (Table 1) in which their mating specificity was determined (Brasier & Kirk 1993, De Beer *et al*. 2003, Kamgan Nkuekam *et al.* 2008b). The identity of the isolates was confirmed by sequencing the internal transcribed spacer regions ITS 1 and 2 of the ribosomal DNA operon, including the 5.8S gene (Table 1). For this verification step, the primer set ITS1F and ITS4 (Gardes & Bruns 1993, White *et al.* 1990) was used. The sequences were compared with those of authentic *O. quercus* isolates available from the NCBI (www.ncbi.nih.gov) database and recent studies such as those of Grobbelaar *et al.* (2009).

Heterothallic mating behaviour was confirmed for all the test isolates by conducting mating tests on sterilized *Quercus* twigs in agar plates as described by De Beer *et al.* (2003). All 10 *O. quercus* isolates were mated in all possible combinations, yielding 45 unique combinations, with an additional 10 control plates, where each isolate was mated against itself (Table 2, Fig. 2). A mating interaction was scored as negative if no perithecia were formed, if the perithecia contained no ascospores or if the ascospores were not viable when plated on 2% malt-extract agar media (MEA: 20 g malt extract [Biolab, Merck], 20 g agar [Biolab, Merck], 1L dH_2O). Positive mating responses were noted only when perithecia with viable ascospores were formed. The complete mating study was repeated once.

Amplification and sequencing of *MAT-1* and *MAT-2* gene regions

PCR, cloning and sequencing

Cultures were grown on 2% MEA at 25℃ for 4-6 weeks. Mycelium was scraped from the surface of the plates and genomic DNA was extracted using the method of Aljanabi & Martinez (1997). PCR reactions were done using 20-50 ng of DNA in a 25 ul PCR reaction

containing 1 U Roche Fast start *Taq* mixture (Roche Diagnostics, Mannheim, Germany), 1x PCR reaction buffer, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP and 0.2 mM of each primer. All reactions were performed on an Eppendorf thermocycler (Eppendorf AG, Mannheim, Germany) using the following conditions: one cycle of 5 min at 96℃, followed by 35 cycles of 30 s at 95℃, 30 s at 50℃ and 1 min at 72℃, follo wed by one cycle of 7 min at 72℃. PCR products were visualized by electrophoresis on a 2% (w/v) agarose gel and stained with ethidium bromide using UV illumination.

Two sets of primers were used in this study (Fig. 1, Table 3). Using the primer designing program Primer3 (frodo.wi.mit.edu/primer3/input.htm), primer set OqMt1F and OqMt1R were designed to target a 180 bp fragment specific to the *MAT-1* idiomorph (Fig. 1). To design the *MAT-1* specific primers, conserved regions of the *MAT-1* fragments from the following isolates were exploited: *O. ulmi* isolates GOLB4 (Genbank accession number: DQ013862) and H173 (DQ013859) and *O. novo-ulmi* isolates MAFg8 (DQ013868) and R67 (DQ013864) (Paoletti *et al.* 2006).

MAT-2 idiomorph sequences previously published (Paoletti *et al.* 2005) were used to exploit regions showing high similarity for design of a single new primer, OqMt2. This primer was then used in combination with Seq9 (Paoletti *et al.* 2005) to target a 1150 bp sequence that included the full *MAT*-2 open reading frame (ORF) and intron as well as 500 bp of non-coding sequence (Fig. 1). All PCR fragments were purified using the Roche high-pure PCR product purification kit (Roche Diagnostics, Mannheim, Germany) following the manufacturer's protocols.

All fragments were cloned using the pGem T-easy cloning kit (Promega, Madison, USA). Transformed colonies were identified with blue/white screening and the insert was amplified using colony PCR. Colony PCR was done using the universal plasmid primers (Sp6/T7) and the PCR protocol described above, but using 30 amplification cycles instead of 35 during step 2. Colonies representing *MAT-1* and *MAT-2* were sequenced in 10 ul volumes using the plasmid primers, a Big Dye cycle sequencing kit with Amplitaq DNA polymerase (Perkin-Elmer, Warrington, UK), following the manufacturer's protocols on an ABI PRISM 3300 Genetic Analyser (Applied Biosystems, Foster City, USA). Chromatograms obtained from sequencing were analysed using the Chromas Lite v. 2.01 (Technelysium Pty. Ltd.; http://www.technelysium.com.au) software package.

DNA sequence analysis

To confirm the identity of sequenced fragments, BLASTn comparisons were done using the NCBI database. Sequences of the *MAT-1* and *MAT-2* idiomorphs for the closely related species *O. ulmi*, *O. novo-ulmi* and *O. himal-ulmi* were downloaded from Genbank. These were aligned with sequences from the present study using the G-INS-i strategy in the online interface of the alignment program MAFFT v. 6 (Katoh *et al.* 2002).

The full *MAT-1* fragment was used in analysis of the DNA sequence. The fragment was aligned with sequences from the NCBI database (accession numbers shown in brackets) for *O. ulmi* isolates GOLB4 (DQ013862), H173 (DQ013859), PG389 (DQ013863), R21 (DQ013860) and *O. novo-ulmi* isolates ES1142 (DQ013875), H345 (DQ013871), H987 (DQ013870), M3 (DQ013869), MAFg8 (DQ013868), R67 (DQ013864), TOMIIb5 (DQ013861), US132 (DQ013865), US133 (DQ013866), US200 (DQ013867), V1 (DQ013872), YU16 (AJ972685) (Paoletti *et al.* 2006). For the *MAT-2* gene, only the 666 bp ORF and intron were used in the analysis. The region of interest was aligned to the following previously published sequences: *O. ulmi* isolate W9 (AY887023), *O. novo-ulmi* subsp. *americana* isolate US411 (AY887025), *O novo-ulmi* subsp. *novo-ulmi* isolate R66 (AY887028) and *O. himal-ulmi* isolate HP62 (AY887030) (Paoletti *et al*. 2005).

Phylogenetic analysis for the *MAT-2* gene was done using MEGA v. 4.0 (Tamura *et al.* 2007). A neighbour joining tree (using a Kimura 2-Parameter model) and maximum parsimony tree were constructed using the algorithms associated with MEGA. To generate confidence values for branching points, 1000 bootstrap repeats were used. For the analysis, the full dataset including all *O. quercus*, the single *O. ulmi*, the two *O. novo-ulmi* and the single *O. himal-ulmi* sequences were used.

RESULTS

Isolates and mating study

Comparisons of morphological characters and ITS sequences confirmed all isolates used in this study as *O. quercus*. All newly generated ITS sequences for these isolates were submitted to Genbank (Table 1).

Of the 55 mating reactions, none of the 10 self-fertilizations (e.g. 2521 vs. 2521) produced any perithecia or ascospores. Based on positive matings, the isolates were separated into

two groups of five isolates each representing the two mating types (*MAT-1* and *MAT-2*) typical for heterothallic mating species (Table 2).

Amplification and sequencing of the *MAT-1* and *MAT-2* gene regions

MAT-1 amplicons

Using the primer pair OqMt1F/OqMt1R specific for the *MAT-1* idiomorph, it was possible to amplify a single fragment of 180 bp from the genome of all 10 *O. quercus* isolates used in this study (Table 1). In two of the isolates (CMW1034 and CMW2521), an additional band of approximately 600 bp was co-amplified. This fragment was sequenced and it showed no similarity to any *MAT* gene or to any other sequence in the NCBI database. This fragment was thus ignored in further studies.

BLASTn analysis and comparison with previously published data confirmed that all 180 bp fragments amplified with primers OqMt1F and OqMt1R were from the *MAT* genes of *Ophiostoma* species. BLASTn results from the *MAT-1* specific fragment showed high similarity ($E=3 \times 10^{-86}$) to fragments previously amplified from *MAT-1* isolates of *O. novo-ulmi* and *O. ulmi* (Paoletti *et al.* 2006). None of the amplicons showed any sequence similarity to fragments previously amplified from the *MAT-2* idiomorph of the *O. ulmi* group. Comparisons of the *MAT-1* fragments with the *MAT-2* fragments generated in this study also showed no similarity in any of the sequences. The 10 *O. quercus MAT-1* fragments showed no sequence polymorphisms and were 100% identical on sequence level. Comparison of the 10 *MAT-1 O. quercus* sequences with the same region in *O. ulmi* and *O. novo-ulmi* showed no sequence differences between these three species.

MAT-2 amplicons

Using the idiomorph sequences from the *O. ulmi* group, the primer OqMt2 was designed to amplify the full ORF and intron in a single reaction. OqMt2/Seq9 (Fig. 1, Table 3) was used to amplify fragments of 1100 bp from all 10 isolates of *O. quercus* (Table 1). These fragments were sequenced and aligned to previously published *MAT-2* idiomorphs from *O. novo-ulmi*, *O. ulmi* and *O. himal-ulmi* (Paoletti *et al.* 2006). The final dataset consisted of 14 sequences. Of these, 10 sequences represented *O. quercus MAT-2*, a single sequence was of *O. ulmi MAT-2*, two represented *O. novo-ulmi MAT-2* and the remaining sequence represented *O. himal-ulmi MAT-2*.

A comparison between the 10 *O. quercus* sequences yielded no polymorphic sites. It was possible to identify 34 polymorphic sites between an isolate representing each of *O. quercus*, *O. novo-ulmi* subsp. *americana*, *O. novo-ulmi* subsp. *novo-ulmi*, *O. ulmi* and *O. himal-ulmi* (Table 4). Most of these polymorphisms were specific to *O. himal-ulmi*. A single polymorphism at position 64 was specific to *O. quercus*.
All the ORFs representing *O. quercus* contained a single intron at the conserved site seen for *O. novo-ulmi*. These introns spanned a conserved serine amino acid and was 57 bp in size for all sequences.

The predicted protein-coding region from the *MAT*-2 idiomorph yielded a protein 202 amino acids in length and closely resembled the *MAT*-2 protein from the *O. ulmi* group. Complete homology between *O. ulmi*, *O. novo-ulmi* and *O. himal-ulmi* was observed in the HMG box region. Only a single amino acid difference separated the *O. quercus MAT-2* protein from that of the *O. ulmi* group: amino acid 22 changes from a threonine to an alanine (Table 4).

The Neighbour Joining and Maximum Parsimony trees resulting from analysis of the full *MAT-2* gene and intron showed similar topologies with three distinct groups (Fig. 3). The first group contained all the sequences representing *O. quercus* and that of *O. ulmi*. This was expected as only a single polymorphism (at position 64) separated these sequences from each other. Group 2 contained the single *O. himal-ulmi* sequence. The third group contained all of the *O. novo-ulmi* sequences, but with a clear distinction between the two subspecies of *O. novo-ulmi*.

DISCUSSION

Isolates of *O. quercus* chosen for this study were typically heterothallic as has been shown previously for this fungus (Brasier & Kirk 1993, De Beer *et al.* 2003, Morelet 1992). In mating studies, all ten isolates behaved in a typical heterothallic manner, five as one mating type and the other five as the opposite mating type (Table 2). Therefore, it was unusual to find that both *MAT*-1 and *MAT*-2 gene sequences were present in all ten *O. quercus* isolates from both functional mating groups.

Using molecular techniques, fragments representing both the *MAT-1* and the *MAT-2* idiomorph were identified from all ten *O. quercus* isolates, irrespective of their mating behaviour. The *MAT-1* fragment was highly homologous to several fragments characterised as *MAT-1* in other *Ophiostoma* species in the study of Paoletti *et al.* (2006). Although these

fragments were very small (180 bp), they showed no homology to any region representing the *MAT-2* idiomorph.

A 666 bp fragment representing the full *MAT-2* ORF and intron was used to identify *MAT-2*. This was a large region and resembled the *MAT-2* idiomorph from the *O. ulmi* group in both nucleotide and protein sequence. A single intron spanned a conserved serine residue in all isolates of *O. quercus* used in this study. This was not surprising as this serine position is conserved in a number of species for which *MAT* information is available (Arie *et al.* 1997, Coppin *et al.* 1997, Paoletti *et al.* 2005). The gene contains an HMG-box motif seen as representative of the *MAT-2* idiomorph (Arie *et al.* 1997, Coppin *et al.* 1997).

The results of mating crosses underpinning this study dictate that each isolate should contain only one of the mating type idiomorphs as is expected for heterothallic species (Nelson 1996, Turgeon 1998). However, fragments of both idiomorphs were amplified in all ten selected isolates representing opposite mating types. This unusual result might be explained if an alternative *MAT* locus organisation is present, as is found for two heterothallic *Diaporthe* species designated as W and G types (Kanematsu *et al.* 2007). In that study, three genes were found at both the *MAT-1* and *MAT-2* idiomorphs. Interestingly, two of these genes were shared between the idiomorphs. The conserved domains present in these shared genes were an HMG box and a PPF domain (Proline-Proline-Phenylalanine). In addition to this, the *MAT-1* idiomorph contained a third gene with a characteristic α-box motif while the *MAT-2* idiomorph contained the characteristic HMG-box gene. A similar gene organisation could thus occur in *O. quercus*. The HMG-box *MAT-2* gene identified in the present study for the *MAT-2* idiomorph might be shared between both idiomorphs. To prove or disprove this, more sequence data for the *MAT-1* idiomorph of *O. quercus* will be required. If this proves to be the case, the sequence data for the *MAT-2* idiomorph will also need to be expanded.

An alternative explanation for the presence of both idiomorphs in all isolates is that the *MAT-2* gene might be non-functional in some of the isolates used in this study. This, however, seems highly unlikely as all 10 *MAT-2* ORFs encode a full predicted peptide. A non-functional control region or post-transcriptional modifications could provide the answer.

Sequence data for the *MAT* genes generated in one species (Paoletti *et al*. 2005, 2006) were used in the present study to extend our knowledge to a related species. A high level of sequence similarity allowed for primers designed on sequences of the *O. ulmi* group to be used in species of *O. quercus*. Three new primers were designed for use in conjunction with

previously designed primers. For both idiomorphs, fragments of the expected size were amplified and sequenced from all 10 isolates representing *O. quercus*. The amount of sequence conservation in both regions suggests that these primers might be useful in studies on other *Ophiostoma* species.

REFERENCES

Aljanabi, S.M. & Martinez, I. (1997) Universal and rapid salt-extraction of high quality genomic DNA for PCR-based techniques. *Nucleic Acids Research* **25**: 4692-4693

Arie, T., Christiansen, S.K., Yoder, O.C. & Turgeon, B.G. (1997) Efficient cloning of Ascomycete mating type genes by PCR amplification of the conserved *MAT* HMG box. *Fungal Genetics and Biology* **21**: 118-130

Brasier, C.M. (1990) China and the origins of Dutch Elm Disease: an appraisal. *Plant Pathology* **39**: 5-16

Brasier, C.M. (1993) The genetic system as a fungal taxonomic tool: gene flow, molecular variation and sibling species in the *'Ophiostoma piceae - O. ulmi'* complex and its taxonomic and ecological significance. In: *Ceratocystis and Ophiostoma: Taxonomy, Ecology and Pathogenicity* (M.J. Wingfield, K.A. Seifert & J. Webber, Eds), APS Press, St. Paul, Minnesota: 77-92

Brasier, C.M. & Kirk, S.A. (1993) Sibling species within *Ophiostoma piceae*. *Mycological Research* **97**: 811-816

Coppin, E., Debuchy, R., Arnaise, S. & Picard, M. (1997) Mating types and sexual development in filamentous Ascomycetes. *Microbiology and Molecular Biology Reviews* **61**: 411-428

De Beer, Z.W., Wingfield, B.D. & Wingfield, M.J. (2003) The *Ophiostoma piceae* complex in the Southern Hemisphere: a phylogenetic study. *Mycological Research* **107**: 469-476

Elliott, C.G. (1994) *Reproduction in fungi: Genetical and physiological aspects*. Chapman & Hall, London, New York

Gardes, M. & Bruns, T.D. (1993) ITS primers with enhanced specificity for Basidiomycetes - application to the identification of mycorrhizae and rusts. *Molecular Ecology* **2**: 113-118

Geldenhuis, M.M., Roux, J., Montenegro, F., De Beer, Z.W., Wingfield, M.J. & Wingfield, B.D. (2004) Identification and pathogenicity of *Graphium* and *Pesotum* species from machete wounds on *Schizolobium parahybum* in Ecuador. *Fungal Diversity* **15**: 135-149

Glass, N.L. & Nelson, M.A. (1994) Mating type genes in mycelial Ascomycetes. In *The Mycota I: Growth, Differentiation and Sexuality* (J.G.H. Wessels & F. Meinhardt, Eds), Springer-Verlag, Berlin, Germany: 295-306

Gorton, C. & Webber, J.F. (2000) Re-evaluation of the status of the bluestain fungus and bark beetle associate *Ophiostoma minus*. *Mycologia* **92**: 1071-1079

Grobbelaar, J., De Beer, Z.W., Aghayeva, D.N., Bloomer, P., Wingfield, M.J. & Wingfield, B.D. (2009) Delimitation of *Ophiostoma quercus* and its synonyms using multiple gene phylogenies. *Mycological Progress* In press. DOI: 10.1007/s11557-009-0594-4

Harrington, T.C., McNew, D., Steimel, J., Hofstra, D. & Farrell, R. (2001) Phylogeny and taxonomy of the *Ophiostoma piceae* complex and the Dutch Elm Disease fungi. *Mycologia* **93**: 111-136

Kamgan Nkuekam, G., Jacobs, K., De Beer, Z.W., Wingfield, M.J. & Roux, J. (2008a) *Ceratocystis* and *Ophiostoma* species including three new taxa, associated with wounds on native South African trees. *Fungal Diversity* **29**: 37-59

Kamgan Nkuekam, G., Jacobs, K., De Beer, Z.W., Wingfield, M.J. & Roux, J. (2008b) *Pesotum australi* sp. nov. and *Ophiostoma quercus* associated with *Acacia mearnsii* trees in Australia and Uganda, respectively. *Australasian Plant Pathology* **37**: 406-416

Kanematsu, S., Adachi, Y. & Ito, T. (2007) Mating-type loci of heterothallic *Diaporthe* spp.: homologous genes are present in opposite mating-types. *Current Genetics* **52**: 11-22

Katoh, K., Misawa, K., Kuma, K.I. & Miyata, T. (2002) MAFFT: a novel method for rapid sequence alignment based on fast Fourier transform. *Nucleic Acids Research* **30**: 3059-3066

Kim, G.-H., Kim, J.-J., Lim, Y.W. & Breuil, C. (2005) Ophiostomatoid fungi isolated from *Pinus radiata* logs imported from New Zealand to Korea. *Canadian Journal of Botany* **83**: 272-278

Kim, G.-H., Kim, J.-J. & Breuil, C. (2007) Sap-staining fungi from logs and boards of two commercially important pines in Korea. *Holzforschung* **61**: 333-336

Linnakoski, R., De Beer, Z.W., Rousi, M., Niemelä, P., Pappinen, A. & Wingfeld, M.J. (2008) Fungi including *Ophiostoma karelicum* sp. nov., associated with *Scolytus ratzeburgi* infesting birch in Finland and Russia. *Mycological Research* **112**: 1475-1488

Metzenberg, R.L. & Glass, N.L. (1990) Mating type and mating strategies in *Neurospora*. *BioEssays* **12**: 53-59

Morelet, M. (1992) *Ophiostoma querci* sur chêne en France. *Annales de la Societe des Sciences Naturelles et d'Archeologie de Toulon et du Var* **44**: 109-112

Nelson, M.A. (1996) Mating systems in Ascomycetes: a romp in the sac. *Trends in Genetics* **12**: 69-74

Paciura, D., Zhou, X.D., De Beer, Z.W., Jacobs, K. & Wingfield, M.J. (2009) Characterisation of synnematous bark beetle-associated fungi from China, including *Graphium carbonis* sp. nov. *Fungal Diversity* In press

Paoletti, M., Buck, K.W. & Brasier, C.M. (2005) Cloning and sequence analysis of the *MAT-B* (*MAT*-2) genes from the three Dutch Elm Disease pathogens, *Ophiostoma ulmi*, *O. novo-ulmi*, and *O. himal-ulmi*. *Mycological Research* **109**: 983-991

Paoletti, M., Buck, K.W. & Brasier, C.M. (2006) Selective acquisition of novel mating type and vegetative incompatibility genes via interspecies gene transfer in the globally invading eukaryote *Ophiostoma novo-ulmi*. *Molecular Ecology* **15**: 249-262

Przybyl, K. (1992) Some aspects on *Ophiostoma roboris* (syn. *O. querci*) studies. *Arboretum Kórnickie Rocznik* **37**: 61-73

Solla, A., Dacasa, M.C., Nasmith, C., Hubbes, M. & Gil, L. (2008) Analysis of Spanish populations of *Ophiostoma ulmi* and *O. novo-ulmi* using phenotypic characteristics and RAPD markers. *Plant Pathology* **57**: 33-44

Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* **24**: 1596-1599

Thwaites, J.M., Farrell, R.L., Duncan, S.M., Reay, S.D., Blanchette, R.A., Hadar, E., Hadar, Y., Harrington, T.C. & McNew, D. (2005) Survey of potential sapstain fungi on *Pinus radiata* in New Zealand. *Journal of Botany* **43**: 653-663

Turgeon, B.G. (1998) Application of mating type gene technology to problems in fungal biology. *Annual Review of Phytopathology* **36**: 115-137

Turgeon, B.G. & Yoder, O.C. (2000) Proposed nomenclature for mating type genes of filamentous Ascomycetes. *Fungal Genetics and Biology* **31**: 1-5

White, T.J., Bruns, T., Lee, S., & Taylor, J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR protocols: A guide to methods and applications* (M.A. Innis, D.H. Gelfand, J.J. Snindky & T.J. White, Eds), Academic Press Inc., San Diego, California: 315-322

Wingfield, M.J., Seifert, K.A. & Webber, J.F. (1993) *Ceratocystis and Ophiostoma: Taxonomy, ecology, and pathogenicity.* APS Press, St. Paul, Minnesota

Zhou, X.D., De Beer, Z.W., Ahumada, R., Wingfield, B.D. & Wingfield, M.J. (2004a) *Ophiostoma* and *Ceratocystiopsis* spp. associated with two pine-infesting bark beetles in Chile. *Fungal Diversity* **15**: 261-274

Zhou, X., De Beer, Z.W., Cibrian, D., Wingfield, B.D. & Wingfield, M.J. (2004b) Characterisation of *Ophiostoma* species associated with pine bark beetles from Mexico, including *O. pulvinisporum* sp. nov. *Mycological Research* **108**: 690-698

Zhou, X., De Beer, Z.W. & Wingfield, M.J. (2006) DNA sequence comparisons of *Ophiostoma* spp., including *Ophiostoma aurorae* sp. nov., associated with pine bark beetles in South Africa. *Studies in Mycology* **55**: 269-277

Zipfel, R.D., De Beer, Z.W., Jacobs, K., Wingfield, B.D. & Wingfield, M.J. (2006) Multi-gene phylogenies define *Ceratocystiopsis* and *Grosmannia* distinct from *Ophiostoma*. *Studies in Mycology* **55**: 75-97

Table 1. *Ophiostoma quercus* isolates used in this study. Mating groups were arbitrarily assigned to indicate the mating specificity of the 10 isolates.

Isolate number[a]		Host	Country	Collector	Mating group	Genbank acc. nr.			Original publication
CMW	**other**					**ITS**	**MAT1**	**MAT2**	
2520	CBS 116321	*Eucalyptus* chips	South Africa	ZW de Beer	+	AF493241*	FJ865416	FJ865421	De Beer *et al.* 2003
2521		*Eucalyptus* chips	South Africa	ZW de Beer	-	FJ441283*	FJ865417	FJ865420	De Beer *et al.* 2003
14307		*Acacia mearnsii*	Uganda	J Roux	-	FJ959044	FJ865415	FJ865425	Kamgan Nkuekam *et al.* 2008b
17256[b]		*A. mearnsii*	Uganda	J Roux	+	FJ959042	FJ865411	FJ865422	Kamgan Nkuekam *et al.* 2008b
17257[b]		*A. mearnsii*	Uganda	J Roux	+	FJ959045	FJ865414	FJ865424	Kamgan Nkuekam *et al.* 2008b
17258[b]		*A. mearnsii*	Uganda	J Roux	-	FJ959043	FJ865418	FJ865423	Kamgan Nkuekam *et al.* 2008b
27845	H 2190	*Quercus* sp.	Canada	K Seifert	+	AF211840*	FJ865419	FJ865426	Brasier & Kirk 1993
27846	H 1039	*Quercus* sp.	UK	PT Scard	+	AF211838*	FJ865410	FJ865427	Brasier & Kirk 1993; De Beer *et al.* 2003
27847	H 920	*Quercus* sp.	UK	JN Gibbs	-	AF081134*	FJ865413	FJ865429	Brasier & Kirk 1993
27848	H 1042	*Quercus* sp.	UK	PT Scard	-	EF429089*	FJ865412	FJ865428	Brasier & Kirk 1993; De Beer *et al.* 2003

[a] CMW = Culture collection of the Forestry and Biotechnology Institute (FABI), University of Pretoria, South Africa; CBS = Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; H = From the collection of Brasier & Kirk (1993).

[b] All three these single spore isolates were obtained from one isolate, CMW 5826, as tester strains for *O. quercus* (Kamgan Nkuekam *et al.* 2008b).

* Sequences already in NCBI database from previous studies.

Table 2. Results of the mating type study. 1-10 indicates the ten isolates used in this study. + perithecia with viable ascospores, – no perithecia. The compatible mating reactions between the two opposite mating types are highlighted.

Isolate	1	2	3	4	5	6	7	8	9	10
1	-									
2	-	-								
3	-	-	-							
4	-	-	-	-						
5	-	-	-	-	-					
6	+	+	+	+	+	-				
7	+	+	+	+	+	-	-			
8	+	+	+	+	+	-	-	-		
9	+	+	+	+	+	-	-	-	-	
10	+	+	+	+	+	-	-	-	-	-

Table 3. Primers used in this study.

Primer name	Sequence (5'-3')	Origin	*MAT* idiomorph targeted
OqMt1F	TGGCAAGAAAGGAAGACTGG	This study	*MAT-1*
OqMt1R	GCGTTATTGGGAGACAGGAA	This study	*MAT-1*
Seq9	GGGGATGTAAAAGGAAC	Paoletti *et al.* 2005	*MAT-2*
OqMt2	GCACACAACTTTGCCAGGTA	This study	*MAT-2*

Table 4. Polymorphisms between the four species of *Ophiostoma* discussed in this study. Shaded is position 64, the only polymorphism unique to *O. quercus* and the polymorphism responsible for the amino acid change seen in *O. quercus*. O. n-u s. a = *O. novo-ulmi* subsp. *americana*, O. n-u s. n-u = *O. novo-ulmi* subsp. *novo-ulmi*, O. h-u = *O. himal-ulmi*.

	12	**64**	**108**	**114**	**129**	**150**	**155**	**156**	**158**	**165**	**169**	**174**	**175**	**178**	**180**	**258**	**321**
O. n-u s. a	C	A	A	G	C	C	C	C	T	C	G	C	G	C	G	G	C
***O. n-u s.* n-u**	C	A	A	G	C	C	C	C	T	C	G	C	G	C	G	C	C
O. ulmi	C	A	C	A	A	T	C	C	C	T	A	C	A	T	A	C	T
O. h-u	T	A	C	A	C	T	T	T	C	C	A	T	G	C	G	C	T
O. quercus	C	G	C	A	A	T	C	C	C	T	A	C	A	T	A	C	T

	335	**341**	**345**	**359**	**405**	**471**	**483**	**508**	**529**	**558**	**574**	**588**	**592**	**598**	**609**	**630**	**654**
O. n-u s. a	G	T	A	A	C	G	G	C	A	A	G	C	A	T	G	C	A
***O. n-u s.* n-u**	G	T	A	A	C	G	G	C	G	A	G	C	A	C	G	C	A
O. ulmi	A	T	G	A	C	G	G	C	G	A	A	T	A	C	G	T	G
O. h-u	A	C	G	G	T	A	C	T	G	T	A	T	G	C	T	C	A
O. quercus	A	T	G	A	C	G	G	C	G	A	A	T	A	C	G	C	G

Fig. 1. Figure representing the available information (Paoletti *et al*. 2005, 2006) on the two fungal *MAT* idiomorphs for the genus *Ophiostoma*. Indicated by arrows are the binding position of primers used in this study.

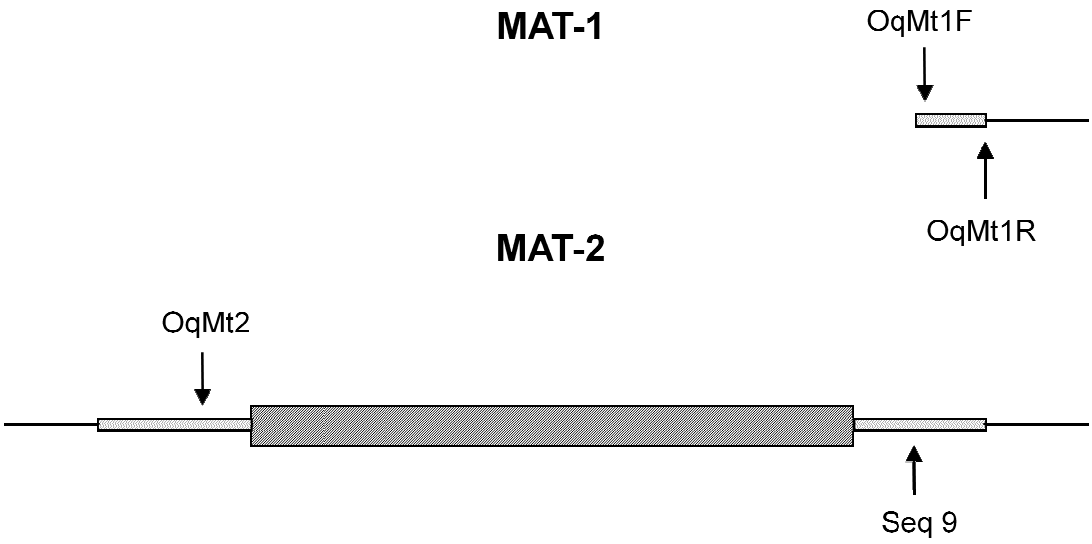

MAT-1
OqMt1F
OqMt1R
MAT-2
OqMt2
Seq 9

Fig. 2. Positive mating reaction between two isolates of opposite mating type on an agar plate. Perithecia (small black structures) were formed in the interaction zone (box) between the two pieces of inoculum (indicated by isolate numbers).

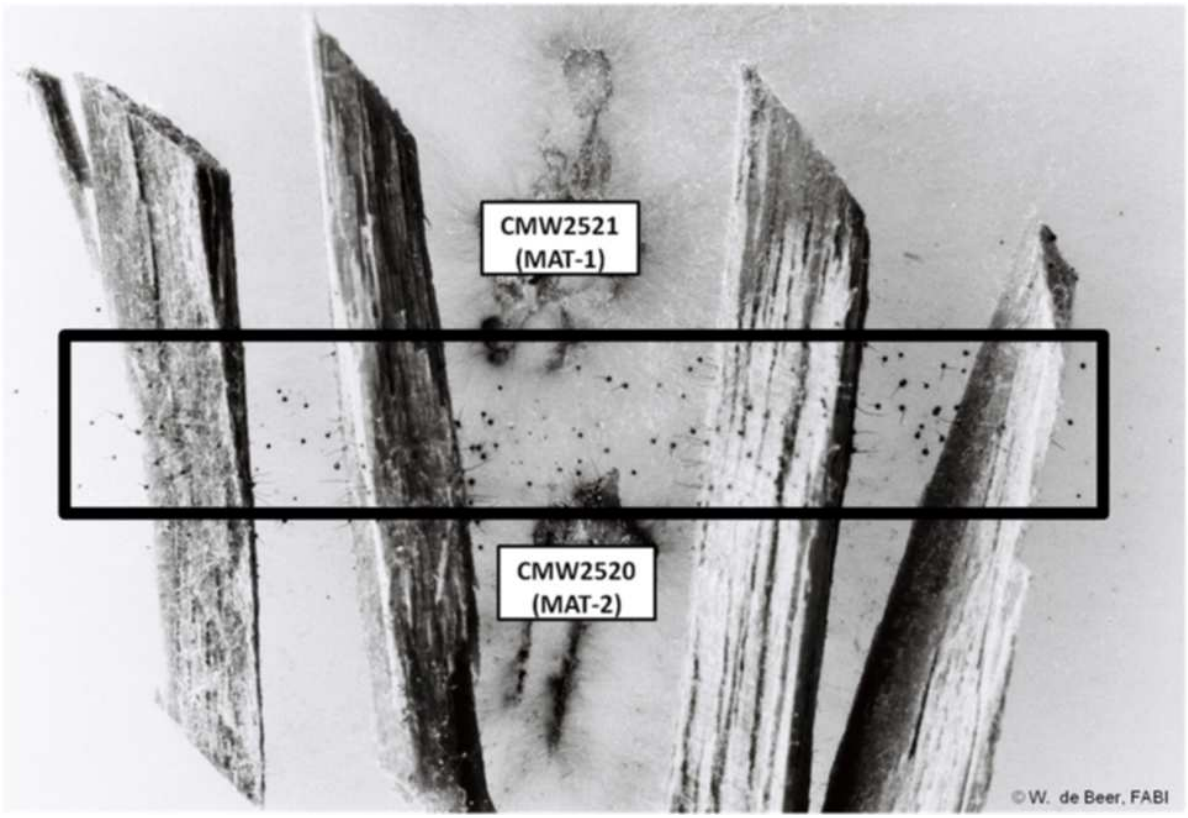
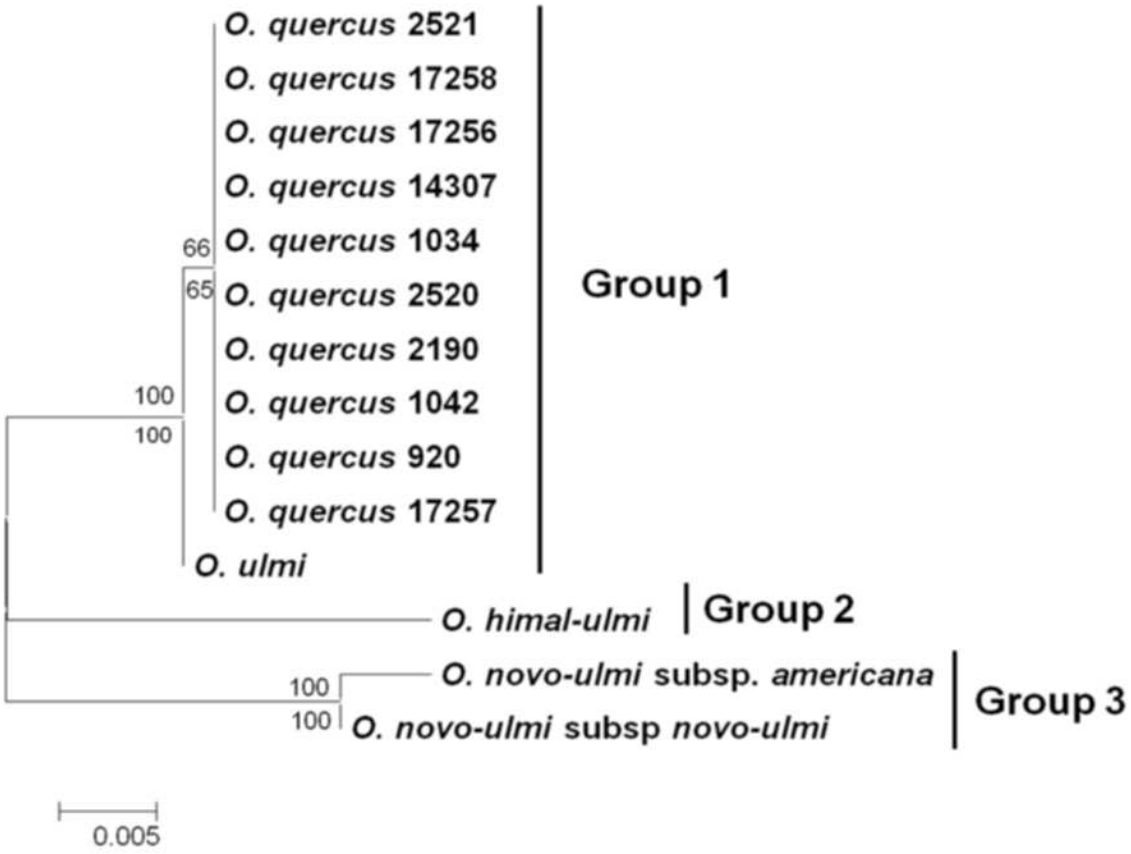

CMW2521
(MAT-1)
CMW2520
(MAT-2)
©W. de Beer, FABI

Fig. 3. NJ tree constructed from *MAT-2* gene and intron. Shown above branching points are confidence levels from the NJ tree bootstrap, with those from MP analysis below.


O. quercus 2521
O. quercus 17258
O. quercus 17256
O. quercus 14307
O. quercus 1034
O. quercus 2520
O. quercus 2190
O. quercus 1042
O. quercus 920
O. quercus 17257
O. ulmi
Group 1
66
65
100
100
O. himal-ulmi
Group 2
O. novo-ulmi subsp. americana
O. novo-ulmi subsp novo-ulmi
Group 3
100
100
0.005

CHAPTER 3

A *MAT1-2-1* phylogeny for *Ophiostoma* spp. related to *O. quercus*

ABSTRACT

Multi-gene analysis are increasingly being used to improve phylogenetic resolution of fungi. This study considers the value of using the *MAT1-2-1* gene for phylogenetic studies on species of *Ophiostoma*. The *MAT1-2-1* gene was amplified and sequenced from 17 species of *Ophiostoma*. Sequence variation and phylogenetic usefulness of the region was assessed through several assays. A comparison to the commonly used phylogenetic region, the ITS region, was used to determine the applicability of the *MAT1-2-1* gene region for phylogenetics in *Ophiostoma*. *MAT1-2-1* was found to show very low levels of sequence variation. This highly conserved region proved to be no more informative than commonly used phylogenetic gene regions such as the rRNA genes.

INTRODUCTION

Conservation of the mating type genes (*MAT*) across related species has been of interest since the earliest molecular studies on filamentous Ascomycetes. Glass *et al*. (1988) first cloned the *MAT* genes for a filamentous Ascomycete, *Neurospora crassa*. In the same study genomes of various other *Neurospora* species were probed using the *N. crassa MAT* sequence. Although a variety of reproductive strategies (heterothallism, homothallism and pseudohomothallism) are represented by these *Neurospora* species, the authors were able to identify fragments homologous to the *N. crassa MAT* genes in all isolates (Glass *et al.* 1988). This provided the first indication that sequences were conserved for the *MAT* region across related species of fungi. Subsequently, *MAT* gene data have become available for a large number of fungal species (eg. McGuire *et al*. 2001, Ramirez-Prado *et al.* 2008, Stergiopoulos *et al*. 2007).

The availability of large amounts of *MAT* sequence has led to questions regarding the means in which this data might be used (Turgeon 1998). It has for example been suggested that *MAT* genes might provide better phylogenetic resolution with less sequencing effort than the more commonly used gene regions (Turgeon 1998). A phylogenetic analysis of *Cochliobolus* spp. using 234 base pair (bp) aligned *MAT1-2-1* HMG box sites provided equal or even better resolution than using a 1343 bp aligned ribosomal internal transcribed spacer regions (ITS) and glyceraldehyde-3-phosphate dehydrogenase (GPD) data set (Turgeon 1998). A similiar finding emerged when the HMG box of the *MAT-2* gene was used as a phylogenetic character in the *Ceratocystis coerulescens* complex (Witthuhn *et al*. 2000). However, *MAT* gene regions do not always yield effective phylogenetic resolution, as for example in studies on *Fusarium* (Steenkamp *et al*. 2000) and *Ascochyta* (Barve *et al*. 2003). Likewise, a study comparing the *MAT* regions to other common regions found no support for the *MAT* genes providing a superior phylogenetic signal in *Fusarium* (O'Donnell *et al*. 2004). The authors speculated that different regions of the *MAT* idiomorph might be better suited to addressing such questions, depending on the aim of the study. They thus suggested that a pilot study examining different regions of the full *MAT-2* idiomorph for applicability in phylogenetics might be useful (O'Donnell *et al*. 2004).

Mating genes provide useful data that allow for the statistical testing of random mating. The inherent lack of heterozygosity disqualifies statistical tests based on Hardy-Weinberg assumptions (Milgroom 1996). However, *MAT* genes provide a solution to this problem for heterothallic species. Under randomly mating conditions, a 1:1 ratio of MAT-1 to MAT-2

isolates is expected (Barve *et al*. 2003, Milgroom 1996). The availability of *MAT* primers makes it possible to test for random mating using a χ^2 analysis. This provides a statistical indication for the presence or absence of random mating in a population (Barve *et al*. 2003).

Ophiostoma ulmi and *O. novo-ulmi* are responsible for the Dutch Elm disease pandemics that resulted in the death of millions of Elm trees throughout the Northern Hemisphere (Brasier 1990, Brasier & Kirk 1993). In an effort to locate the origin of these pathogens, a third, related species *O. himal-ulmi,* was identified from the Himalayan Mountains (Brasier & Mehrotra 1995). Paoletti *et al*. (2005) succeeded in cloning the full *MAT-2* idiomorph for these three closely related species. The authors identified a single gene, *MAT-1-2-1* (abbreviated as *MAT-2*) encoding an HMG-box domain characteristic of the *MAT-2* protein (Arie *et al*. 1997, Coppin *et al*. 1997). The coding region consists of two exons interrupted by a single intron.

Using the *MAT-2* DNA sequences of the *O. ulmi*-group, the corresponding region from the closely related heterothallic species, *O. quercus,* was cloned and sequenced (Chapter 2 of this thesis). A comparison of the nucleotide differences revealed 34 variable positions (5% of the full gene and intron) between the *MAT-2* genes of *O. ulmi*, the two *O. novo-ulmi* subspecies, *O. himal-ulmi* and *O. quercus*. This high level of sequence conservation was expected as the *O. ulmi*-group is very closely related to *O. quercus* (Harrington *et al*. 2001, Zipfel *et al*. 2006). Many *Ophiostoma* species related to the above-mentioned species are morphologically similar and difficult to distinguish from each other, even when phylogenetic data from gene regions such as ITS, which is most commonly used for species delineation in this group, are available (Harrington *et al*. 2001, Zhou *et al*. 2004, Villarreal *et al*. 2005). For this reason, other gene regions have been considered for these fungi including the β-tubulin (Aghayeva *et al*. 2004, Chung *et al.* 2006, Gorton *et al*. 2004, Linnakoski *et al*. 2008) and the Elongation factor 1-α (EF 1-α) region (Jacobs *et al*. 2004). In a recent study on *O. quercus* and its relatives using four gene regions, EF 1-α showed the highest diversity, followed by β-tubulin, ITS and Histone (Grobbelaar *et al.* 2009). With multi-gene phylogenies becoming standard procedure for phylogenetic studies, the *MAT* genes should be considered for their possible value and this would have the added benefit of revealing information about the mating behaviour of a particular species.

The aim of this study was to test primers developed for *O. quercus* (Chapter 2 of this thesis) on related *Ophiostoma* species. In addition, this study assessed the usefulness of the *MAT-2* gene sequence for phylogenetic analysis in the genus *Ophiostoma*.

MATERIALS AND METHODS

Isolates

Twenty three isolates representing 17 species of *Ophiostoma* were used (Table 1). All cultures are stored in the culture collection (CMW) of the Forestry and Biotechnology Institute (FABI) in Pretoria, South Africa. The identity of the isolates was confirmed based on morphology and, where necessary, sequencing of the ITS regions. For this, primer set ITS1F (Gardes & Bruns 1993) and ITS4 (White *et al.* 1990) were used. Confirmation of the ITS sequences was done by BLASTn analysis to the Genbank database (www.ncbi.nih.gov) and comparisons to the FABI database that includes unpublished sequences.

Amplification of genomic regions

The cultures used in the study were grown on 2% malt-extract agar media (MEA: 20 g malt extract [Biolab, Merck], 20 g agar [Biolab, Merck], 1L dH_2O) for 4-6 weeks at 25℃. Genomic DNA was extracted from mycelium scraped off agar in Petri dishes by using a salt-extraction method (Aljanabi & Martinez 1997). PCR reactions were done with an Eppendorf cycler machine (Eppendorf, Mannheim, Germany). The PCR mixture contained: 20-50 ng of DNA, 1 U Roche Fast start *Taq* mixture, 1X PCR reaction buffer, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP and 0.2 mM of each primer. The reaction protocol was: one cycle of 5 min at 96℃, followed by 35 cycles of 30 s at 95℃, 30 s at 50℃ and 1 min at 72℃, followed by one cycle of 7 min at 72℃. All PCR products were visualized by electrophoresis on a 2% (w/v) agarose gel stained with ethidium bromide and illuminated by UV light.

Amplification of the *MAT-2* gene region was done using primer pair seq9 (5' GGGGATGTAAAAGGAAC 3') (Paoletti *et al*. 2005) and OqMt2 (5' GCACACAACTTTGCCAGGTA 3') (Chapter 2 of this thesis). This PCR targeted an 1150 bp sequence that included the full *MAT*-2 open reading frame (ORF) and intron, as well as 484 bp of non-coding sequence. All PCR fragments were purified using the Roche high-pure PCR product purification kit (Roche Diagnostics, Mannheim, Germany) following the manufacturer's protocol.

Amplified fragments were cloned using the pGem T-easy cloning kit (Promega, Madison, USA). Using blue/white screening, transformed colonies were identified and the insert was re-amplified using colony PCR. Colony PCR was done using the PCR method described

above with the only change being 30 amplification cycles instead of 35 cycles during step 2. The universal plasmid primers Sp6 and T7 were used and the insert was sequenced in 10 ul volumes using the plasmid primers, a Big Dye cycle sequencing kit with Amplitaq DNA polymerase (Perkin-Elmer, Warrington, UK), following the manufacturer's protocols on an ABI PRISM 3300 Genetic Analyser (Applied Biosystems, Foster City, USA). Chromatograms obtained from sequencing were analysed using the Chromas Lite v. 2.01 (Technelysium Pty Ltd; http://www.technelysium.com.au) software package. A total of three inserts were sequenced for each isolate and the consensus sequence was identified. If this was not possible, a further seven inserts were sequenced and the consensus sequence was determined.

DNA analysis

To confirm that sequences were those of the *MAT-2* idiomorph, a BLASTn search was done in the NCBI database. Sequenced fragments were aligned and visually compared to those for the closely related species *O. ulmi*, *O. novo-ulmi*, *O. himal-ulmi* and *O. quercus* from previous studies (Table 2) (Paoletti *et al.* 2005, Chapter 2 of this thesis).

For sequence analysis, representative *MAT-2* sequences from the Genbank database were imported into the dataset. These sequences were representative of *O. ulmi*, *O. novo-ulmi* (both subspecies *novo-ulmi* and *americana*), *O. himal-ulmi* and *O. quercus* (Table 2) (Paoletti *et al*. 2005, Chapter 2 of this thesis). All sequence alignments were done using the E-INS-i strategy in the online alignment program MAFFT v. 6 (Katoh *et al.* 2002). For phylogenetic analysis only the *MAT-2* gene of 666 bp were used. This included the full 609 bp ORF and a 57 bp intron. After alignment of the sequences, two measures of similarity as well as a phylogenetic analysis were produced.

Similarity statistics were produced by the sequence identity matrix and sequence difference count matrix commands of the BioEdit software package v. 7.0.9.0 (Hall 1999). The number of differences and percentage sequence similarity between sequences were then determined.

For phylogenetic analysis, Modeltest v. 3.7 (Posada 1998) was used to determine the appropriate substitution model to use in analysis. A GTR+I+G model were selected using the Akaike Information Criterion (AIC). Using PhyML v. 3.0 (Guindon *et al*. 2005) a Maximum Likelihood (ML) phylogenetic tree was produced (Fig. 1). The same dataset was subjected to

a Neighbor joining (NJ) analysis in the program MEGA v. 4.0 (Tamura *et al.* 2007), using the Kimura 2-Parameter model.

For a comparison to the *MAT-2* phylogenetic analysis, a second dataset was analysed. ITS sequences from a large group of *Ophiostoma* species were used from both this study (Table 1) and the NCBI database. ML analysis was done as described for the *MAT-2* data set. A GTR+I+G substitution model was selected based on the AIC used in Modeltest v. 3.7 (Posada 1998).

RESULTS

Isolates

The identity of all isolates was confirmed base on morphology and ITS sequence comparisons for selected isolates. All sequences generated in this study were submitted to Genbank (Table 1).

Amplification of genomic regions

Primer set OqMt2/Seq9 yielded a single 1150 bp band in all 27 isolates used. After cloning and sequencing, alignment to sequences retrieved from NCBI as well as BLASTn analysis confirmed that all fragments were similar to those previously published for the *MAT-2* genes (Paoletti *et al*. 2005). The final dataset consisted of 36 sequences representing 18 species of *Ophiostoma* and included all fragments generated in this study and those imported from Genbank (Table 2).

DNA analysis

For further analysis of the *MAT-2* data, only the 666 bp consisting of the full ORF and intron were considered. A total of 43 sites were polymorphic in this dataset. Based on similarity statistics, isolates were placed in nine groups containing sequences with no polymorphisms within a group (Table 3). The similarity between groups ranged from 99.8% to 95.4% (Table 3). Although there were 40 polymorphic bases between Groups 1 and 4, the majority of the groups differed by only a small number of polymorphisms.

Group 1 contained three *O. novo-ulmi* subsp. *americana* isolates from NCBI (Table 4). Group 2 included the two *O. novo-ulmi* subsp. *novo-ulmi* sequences from NCBI and one from this study. Groups 3 and 4 consisted of a *O. himal-ulmi* sequence from NCBI and one from this study, respectively. These two sequences exhibited eight polymorphisms between them. Group 5 contained two *O. ulmi* isolates from NCBI together with a sequence for *O. araucariae* produced in this study. The sequences of two *O. novo-ulmi* isolates from Austria constituted Groups 6 and 7 respectively, while Group 8 included only one isolate of *O. flexuosum*. The last group (Group 9) contained 14 species with no polymorphisms between the isolates (Table 4). These species all contained *MAT-2* genes identical to *O. quercus* and included the single isolate of *O. ulmi* used in this study. The difference between the *O. ulmi* isolates from NCBI (Group 5) and the isolate from the current study (in Group 9) was a single polymorphism at position 65.

The ML and NJ trees constructed from the *MAT-2* data presented similar topologies. Only the tree obtained from the ML analysis is presented (Fig. 1) and this revealed three major groups with good statistical support (A-C). Group A had a sub-clade that represented all the species with *MAT-2* sequences identical to *O. quercus* (Group 9 based on similarity statistics). This group included the single *O. ulmi* sequence from the present study, as well as the *O. flexuosum* isolate (Group 8). A second sub-group of Group A (Fig. 1) included all of the *O. ulmi* isolates from NCBI and the *O. araucariae* isolate from the present study and thus representing Group 5 based on the similarity statistics. Group B included all sequences representing *O. novo-ulmi* from Genbank and those from this study (sequence Groups 1, 2, 6 and 7). Group C contained the sequences of the two *O. himal-ulmi* isolates (sequence Groups 3 and 4).

The ITS tree (Fig. 2) had a similar topology to that presented for *Ophiostoma* species in earlier studies based on different gene regions (Harrington et al. 2001, Zipfel et al. 2006, Linnakoski et al. 2008). Several well defined species complexes were evident and this provided a good measure of the relatedness of the species included in the present study.

DISCUSSION

The present study evaluated the applicability of the *MAT-2* gene as a phylogenetic tool for the genus *Ophiostoma*. The justification to do so emerged from recent evidence suggesting that this gene region might be useful in phylogenetic studies on the fungi (O'Donnell *et al.* 2004, Turgeon 1998). Such additional gene regions could thus add to the multi-gene

phylogenies that have been favoured in phylogenetic analysis across several kingdoms including the fungi (Zhang *et al*. 2008), plants (Bouchenak-Khelladi *et al.* 2008), animals (Light & Reed 2009) and protista (Shalchian-Tabrizi *et al*. 2008). This is also consistent with the fact that a multi-gene approach provides more accurate trees (Gadagkar *et al*., 2005), a strongly supported estimate of phylogenies (Driskell *et al.* 2004) and a multitude of other benefits (Barrett *et al*. 1991, Fitzpatrick *et al*. 2006).

MAT sequences have not been commonly used for phylogenetics in fungi. The earliest example demonstrated successfully the resolution provided by the *MAT-2* HMG box region for *Cochliobolus* spp. (Turgeon 1998) and this view was supported in a later study on *Ceratocystis* species (Witthuhn *et al*. 2000). However, only a small number of other studies have evaluated this region for phylogenetic analysis. Currently there is no consensus as to the usefulness of this region for phylogenetics with opinions varying from their being useful to relatively uninformative (Barve *et al*. 2003, O'Donnell *et al.* 2004, Steenkamp *et al.* 2000, Turgeon 1998). One of the limitations of *MAT* regions for phylogenetics is that a universal primer set are not available as is the case for other gene regions.

This study evaluated a previously described primer set (Chapter 2 of this thesis) designed for the *MAT-2* idiomorph of *Ophiostoma*. One of these primers had previously been used for the *O. ulmi*-group (Paoletti *et al*. 2005) and the other was specifically designed for *O. quercus* (Chapter 2 of this thesis). These primers have not previously been tested across a wide range of *Ophiostoma* species, but they were useful in this study, increasing the number of available *MAT-2* gene sequences for *Ophiostoma* species. In this study a fragment of 1100 bp from the *MAT-2* idiomorph was amplified from a large number of different *Ophiostoma* species. In each case the expected size fragment was produced using the same PCR conditions. We are confident that this primer set will be useful across additional species of *Ophiostoma*. The combination of these primers with a suitable primer set for the *MAT-1* idiomorph should also provide a rapid and reliable PCR screening method for mating type assignment in the genus *Ophiostoma*.

The availability of sequence data for the *MAT-2* gene and the inability of ITS to separate certain species (Harrington *et al*. 2001, Roets *et al*. 2007, Kamgan Nkuekam *et al*. 2008) prompted this pilot study to assess the applicability of this region for *Ophiostoma*. Of the 1100 bp fragment amplified from all isolates, the current study focussed on only 666 bp. These included the coding region (with the HMG box region) as well as a single intron (Paoletti *et al.* 2005). This type of analysis has previously been used for other genera such

as *Fusarium* (O'Donnell *et al*. 2004) and *Ceratocystis* (Witthuhn *et al*. 2000). In this pilot study, a small fragment of the full *MAT-2* idiomorph was evaluated for use in phylogenetic analysis.

From the ML tree produced using *MAT-2* gene data, four major phylogenetic groups with very little diversity within them, were identified (Fig. 1). This is as opposed to the ITS tree showing various well-defined species and groups of species (Fig. 2). Some interesting groupings of species in the *MAT-2* tree was observed. The first subgroup of Group A contained the only isolate of *O. ulmi* used in this study. This isolate was placed in this group based on a single polymorphism in position 65 previously shown to be unique to *O. quercus* (Chapter 2 of this thesis). This is in contrast to a previous study where no polymorphisms between any *O. ulmi* isolates were observed (Paoletti *et al*. 2005). The second subgroup within Group 1 demonstrated the 100% homology in the *MAT-2* genes between *O. ulmi* isolates from NCBI and *O. araucariae*. It was shown that there is a close relationship between the *O. ulmi*-group and *O. quercus* (Harrington *et al*. 2001), but in the current analysis, *O. ulmi* was separated from all other isolates except *O. araucariae*. Usually, *O. araucariae* is associated with the *O. quercus*/*O. ips* clade (Zipfel *et al.* 2006). The presence of *O. araucariae* with *O. ulmi* might point to a unique association in the *MAT-2* genes between these species and is worthy of further study.

Group B contained all isolates representing *O. novo-ulmi* from the current and a previous study (Paoletti *et al*. 2005). Good bootstrap support for the clade indicates a clear distinction between these species and other species of *Ophiostoma*. A sub-group with strong bootstrap support contained all isolates identified as *O. novo-ulmi* subsp *americana*. One isolate of *O. novo-ulmi* used in this study was identical to *O. novo-ulmi* subsp *novo-ulmi* isolates from Genbank. The remaining two *O. novo-ulmi* isolates from this study grouped separately from both *O. novo-ulmi* subspecies obtained from NCBI. ITS analysis groups all *O. novo-ulmi* sequences together distinct from other species but related to *O. karelicum* (Fig. 2). The last group identified was the *O. himal-ulmi* group. As expected, this group contained both isolates of *O. himal-ulmi* found in the dataset. *Ophiostoma himal-ulmi* is a well defined species that can be distinguished using ITS data as well.

Results of this study made it possible to established that there is very little variation in the *MAT-2* gene for the genus *Ophiostoma*. This was unexpected as several previous studies identified only the smaller HMG coding region and intron as a suitable phylogenetic marker (O'Donnell *et al*. 2004, Witthuhn *et al*. 2000). Also, 34 variable positions were previously

found in the *MAT-2* gene of only four *Ophiostoma* species (*O. ulmi, O. novo-ulmi* (2 subspecies), *O. himal-ulmi* and *O. quercus*) (Paoletti *et al*. 2005, Chapter 2 of this thesis). In this study, the number of variable positions was increased to only 43 when considering 18 species of *Ophiostoma*. The lack of sequence variation means that the *MAT-2* coding region is not a suitable candidate for phylogenetic analysis.

The use of the *MAT-2* gene in *Ophiostoma* does not appear to provide a superior alternative to the rRNA region or β-tubulin region currently used for phylogenetic analysis (De Beer *et al*. 2003, Grobbelaar *et al*. 2009, Harrington *et al.* 2001, Zipfel *et al.* 2006). The full *MAT-2* idiomorph for *Ophiostoma* contains several other regions including non-coding regions under lower evolutionary pressure. Unfortunately these regions are currently only available for *O. ulmi*, *O. novo-ulmi* and *O. himal-ulmi* (Paoletti *et al*. 2005). Using the starting point of *MAT-2* information provided here, the isolation and characterisation of the full *MAT-2* idiomorph from other *Ophiostoma* species should be possible. This will provide sequence information to allow testing of different regions within this idiomorph for phylogenetics. Ultimately the *MAT-2* idiomorph might provide a necessary basis to better understand the diverse species in the genus *Ophiostoma*.

REFERENCES

Aghayeva, D.N., Wingfield, M.J., de Beer, Z.W. & Kirisits, T. (2004) Two new *Ophiostoma* species with *Sporothrix* anamorphs from Austria and Azerbaijan. *Mycologia* **96**: 866-878

Aljanabi, S.M. & Martinez, I. (1997) Universal and rapid salt-extraction of high quality genomic DNA for PCR-based techniques. *Nucleic Acids Research* **25**: 4692-4693

Arie, T., Christiansen, S.K., Yoder, O.C. & Turgeon, B.G. (1997) Efficient cloning of Ascomycete mating type genes by PCR amplification of the conserved *MAT* HMG box. *Fungal Genetics and Biology* **21**: 118-130

Barrett, M., Donoghue, M.J. & Sober, E. (1991) Against consensus**.** *Systematic Zoology* **40**: 486-493

Barve, M.P., Arie, T., Salimath, S.S., Muehlbauer, F.J. & Peever, T.L. (2003) Cloning and characterization of the mating type (*MAT*) locus from *Ascochyta rabiei* (teleomorph: *Didymella rabiei*) and a *MAT* phylogeny of legume-associated *Ascochyta* spp. *Fungal Genetics and Biology* **39**: 151-167

Bouchenak-Khelladi, Y., Salamin, N., Savolainen, V., Forest, F., van der Bank, M., Chase, M.W. & Hodkinson, T.R. (2008) Large multi-gene phylogenetic trees of the grasses (Poaceae): Progress towards complete tribal and generic level sampling. *Molecular Phylogenetics and Evolution* **47**: 488-505

Brasier, C.M. (1990) China and the origins of Dutch Elm disease: An appraisal. *Plant Pathology* **39**: 5-16

Brasier, C.M. & Kirk, S.A. (1993) Sibling species within *Ophiostoma piceae*. *Mycological Research* **97**: 811-816

Brasier, C.M. & Mehrotra, M.D. (1995) *Ophiostoma himal-ulmi* sp. nov., a new species of Dutch Elm disease fungus endemic to the Himalayas. *Mycological Research* **99**: 205-215

Chung, W.-H., Kim, J.-J., Yamaoka, Y., Uzunovic, A., Masuya, H. & Breuil, C. (2006) *Ophiostoma breviusculum* sp. nov. (Ophiostomatales, Ascomycota) is a new species in the *Ophiostoma piceae* complex associated with bark beetles infesting larch in Japan. *Mycologia* **98**: 801-814

Coppin, E., Debuchy, R., Arnaise, S. & Picard, M. (1997) Mating types and sexual development in filamentous Ascomycetes. *Microbiology and Molecular Biology Reviews* **61**: 411-428

De Beer, Z.W., Wingfield, B.D. & Wingfield, M.J. (2003) The *Ophiostoma piceae* complex in the Southern Hemisphere: A phylogenetic study. *Mycological Research* **107**: 469-476

Driskell, A.C., Ané, C., Burleigh, J.G., McMahon, M.M., O'Meara, B.C. & Sanderson, M.J. (2004) Prospects for building the tree of life from large sequence databases. *Science* **306**: 1172-1174

Fitzpatrick, D.A., Logue, M.E., Stajich, J.E. & Butler, G. (2006) A fungal phylogeny based on 42 complete genomes derived from supertree and combined gene analysis. *BMC Evolutionary Biology* **6**: 99-113

Gadagkar, S.R., Rosenberg, M.S. & Kumar, S. (2005) Inferring species phylogenies from multiple genes: Concatenated sequence tree versus consensus gene tree. *Journal of Experimental Zoology* **304**: 64-74

Gardes, M. & Bruns, T.D. (1993) ITS primers with enhanced specificity for Basidiomycetes - application to the identification of mycorrhizae and rusts. *Molecular Ecology* **2**: 113-118

Glass, N.L., Vollmer, S.J., Staben, C., Grotelueschen, J., Metzenberg, R.L. & Yanofsky, C. (1988) DNAs of the two mating-type alleles of *Neurospora crassa* are highly dissimilar. *Science* **241**: 570-573

Gorton, C., Kim, S.H., Henricot, B., Webber, J. & Breuil, C. (2004) Phylogenetic analysis of the bluestain fungus *Ophiostoma minus* based on partial ITS rDNA and β-tubulin gene sequences. *Mycological Research* **108**: 759-765

Grobbelaar, J., De Beer, Z.W., Aghayeva, D.N., Bloomer, P., Wingfield, M.J. & Wingfield, B.D. (2009) Delimitation of *Ophiostoma quercus* and its synonyms using multiple gene phylogenies. *Mycological Progress* In press. DOI: 10.1007/s11557-009-0594-4

Guindon, S., Lethiec, F., Duroux, P. & Gascuel, O. (2005) PHYML Online - a web server for fast maximum likelihood-based phylogenetic inference. *Nucleic Acids Research (Web Server Issue)* **33**: W557-W559

Hall, T.A. (1999) BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**: 95-98

Harrington, T.C., McNew, D., Steimel, J., Hofstra, D. & Farrell, R. (2001) Phylogeny and taxonomy of the *Ophiostoma piceae* complex and the Dutch Elm disease fungi. *Mycologia* **93**: 111-136

Jacobs, K., Bergdahl, D.R., Wingfield, M.J., Halik, S., Seifert, K.A., Bright, D.E. & Wingfield, B.D. (2004) *Leptographium wingfieldii* introduced into North America and found associated with exotic *Tomicus piniperda* and native bark beetles. *Mycological Research* **108**: 411-418

Kamgan Nkuekam, G., Jacobs, K., De Beer, Z.W., Wingfield, M.J. & Roux, J. (2008) *Pesotum australi* sp. nov. and *Ophiostoma quercus* associated with *Acacia mearnsii* trees in Australia and Uganda, respectively. *Australasian Plant Pathology* **37**: 406-416

Katoh, K., Misawa, K., Kuma, K.I. & Miyata, T. (2002) MAFFT: A novel method for rapid sequence alignment based on fast fourier transform. *Nucleic Acids Research* **30**: 3059-3066

Light, J.E. & Reed, D.L. (2009) Multigene analysis of phylogenetic relationships and divergence times of primate sucking lice (Phthiraptera: Anoplura). *Molecular Phylogenetics and Evolution* **50**: 376-390

Linnakoski, R., De Beer, Z.W., Rousi, M., Niemelä, P., Pappinen, A. & Wingfeld, M.J. (2008) Fungi including *Ophiostoma karelicum* sp. nov., associated with *Scolytus ratzeburgi* infesting birch in Finland and Russia. *Mycological Research* **112**: 1475-1488

McGuire, I.C., Marra, R.E., Turgeon, B.G. & Milgroom, M.G. (2001) Analysis of mating-type genes in the chestnut blight fungus, *Cryphonectria parasitica. Fungal Genetics and Biology* **34**: 131-144

Milgroom, M.G. (1996) Recombination and the multilocus structure of fungal populations. *Annual review of Phytopathology* **34**: 457-77

O'Donnell, K., Ward, T.J., Geiser, D.M., Kistler, H.C. & Aoki, T. (2004) Genealogical concordance between the mating type locus and seven other nuclear genes supports formal recognition of nine phylogenetically distinct species within the *Fusarium graminearum* clade. *Fungal Genetics and Biology* **41**: 600-623

Paoletti, M., Buck, K.W. & Brasier, C.M. (2005) Cloning and sequence analysis of the *MAT-B* (*MAT-2*) genes from the three Dutch Elm disease pathogens, *Ophiostoma ulmi*, *O. novo-ulmi*, and *O. himal-ulmi*. *Mycological Research* **109**: 983-991

Posada, D. (1998) Modeltest: Testing the model of DNA substitution. *Bioinformatics* **14**: 817-818

Ramirez-Prado, J.H., Moore, G.G., Horn, B.W. & Carbone, I. (2008) Characterization and population analysis of the mating-type genes in *Aspergillus flavus* and *Aspergillus parasiticus. Fungal Genetics and Biology* **45**: 1292-1299

Roets, F., De Beer, Z.W., Wingfeld, M.J., Crous, P.W. & Dreyer, L.L. (2008) *Ophiostoma gemellus* and *Sporothrix variecibatus* from mites infesting *Protea* infructescences in South Africa. *Mycologia* **100**: 496-510

Shalchian-Tabrizi, K., Minge, M.A., Espelund, M., Orr, R., Ruden, T., Jakobsen, K.S., Cavalier-Smith, T. (2008) Multigene phylogeny of Choanozoa and the origin of animals. *PLoS ONE* **3**: e2098.

Steenkamp, E.T., Wingfield, B.D., Coutinho, T.A., Zeller, K.A., Wingfield, M.J., Marasas, W.F. & Leslie, J.F. (2000) PCR-based identification of *MAT-1* and *MAT-2* in the *Gibberella fujikuroi* species complex. *Applied and Environmental Microbiology* **66**: 4378-4382

Stergiopoulos, I., Groenewald, M., Staats, M., Lindhout, P., Crous, P.W. & De Wit, P.J.G.M. (2007) Mating-type genes and the genetic structure of a world-wide collection of the tomato pathogen *Cladosporium fulvum*. *Fungal Genetics and Biology* **44**: 415-429

Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* **24**: 1596-1599

Turgeon, B.G. (1998) Application of mating type gene technology to problems in fungal biology. *Annual Review of Phytopathology* **36**: 115-137

Villarreal, M., Rubio, V., De Troya, M.T. & Arenall, F. (2005) A new *Ophiostoma* species isolated from *Pinus pinaster* in the Iberian Peninsula. *Mycotaxon* **92**: 259-268

White, T.J., Bruns, T., Lee, S., & Taylor, J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR protocols: A guide to methods and applications* (M.A. Innis, D.H. Gelfand, J.J. Snindky & T.J. White, Eds), Academic Press Inc., San Diego, California: 315-322

Witthuhn, R.C., Harrington, T.C., Steimel, J.P., Wingfield, B.D. & Wingfield, M.J. (2000) Comparison of isozymes, rDNA spacer regions and *MAT-2* DNA sequences as phylogenetic characters in the analysis of the *Ceratocystis coerulescens* complex. *Mycologia* **92**: 447-452

Zhang, Y., Jeewon, R., Fournier, J. & Hyde, K.D. (2008) Multi-gene phylogeny and morphotaxonomy of *Amniculicola lignicola*: a novel freshwater fungus from France and its relationships to the *Pleosporales*. *Mycological Research* **112**: 1186-1194

Zhou, X., De Beer, Z.W., Cibrian, D., Wingfield, B.D. & Wingfield, M.J. (2004) Characterisation of *Ophiostoma* species associated with pine bark beetles from Mexico, including *O. pulvinisporum* sp. nov. *Mycological Research* **108**: 690-698

Zipfel, R.D., De Beer, Z.W., Jacobs, K., Wingfield, B.D. & Wingfield, M.J. (2006) Multi-gene phylogenies define *Ceratocystiopsis* and *Grosmannia* distinct from *Ophiostoma*. *Studies in Mycology* **55**: 75-97

Table 1. Isolates used in this study.

						Genbank acc. nr.	
Species	**CMW**[a]	**Other**	**Host**	**Country**	**Collector**	**ITS**	***MAT-2***
O. ainoae	1903	CBS 118672	*Picea abies*	Norway	O Olsen	FJ59048	FJ959054
O. araucariae	671	CBS 114.68	*Araucariae* sp.	Chile	H Butin	-	FJ959053
O. borealis	18953	GNT 677.2	*Betula* logs		NG Kamgan	Pending	FJ959055
	18956	CBS 123220	*Betula* logs		NG Kamgan	Pending	FJ959056
O. canum	5023	CBS 118668	*Tomicus minor*	Austria	T Kirisits	-	FJ959057
O. catonianum	11535	CBS 263.35	*Pyrus communis*	Italy	Unknown	AF198243	FJ959058
O. distortum	467	CBS 397.77	*Picea engelmannii*	Unknown	RW Davidson	AY924386	FJ959059
O. flexuosum	907	CBS 208.83	*Picea abies*	Norway	H Solheim	AY924387	FJ959071
O. floccosum	12622	CBS 123601	*Pinus sylvestris*	Austria	T Kirisits	FJ430472	FJ959061
	12623	CTK 123	*P. sylvestris*	Austria	T Kirisits	FJ430473	FJ959060
O. himal-ulmi	22729	CBS 374.67	*Ulmus wallichiana*	India	H Rebel	FJ430474	FJ959072
O. ips	5089		*Pinus radiata*	Chile	MJ Wingfield	AY546701	FJ959062
	6445	A9812J41.1	*Pinus patula*	South Africa	X Zhou	-	FJ959063
O. karelicum	23109	CBS 123218	*Betula pendula*	Finland	M Rousi	-	FJ959064
O. multi-annulatum	2567	CBS 357.77	*Pinus* sp.	USA	RW Davidson	FJ959049	FJ959065
O. novo-ulmi	16178	AT21	*U. glabra*	Austria	S Krumbock, T Kirisits	FJ959046	FJ959050
	16179	AT73	*U. glabra*	Austria	S Krumbock, T Kirisits	FJ959047	FJ959052
	16184	AT149	*U. glabra*	Austria	H Konrad	-	FJ959051
O. piliferum	7879	CBS 129.32	Unknown	Unknown	H Diddens	AF221070	FJ959066
O. pulvinisporum	9024	SIPS6	*Pinus maximinoi*	Mexico	X Zhou	AY546714	FJ959068
	9028	SIPS10	*P. maximinoi*	Mexico	X Zhou	-	FJ959067
O. tsotsi	5943	CBS 116323	*Acacia mearnsii*	Uganda	J Roux	EF408599	FJ959069
O. ulmi	25028	CBS 102.63	*U. hollandica*	Netherlands	FW Holmes	DQ198232	FJ959070

[a] CMW = Culture collection of the Forestry and Biotechnology Institute (FABI), University of Pretoria, South Africa; CBS = Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

Table 2. *MAT-2* sequences from Genbank used in this study.

Species	Isolate number	Genbank number	Reference
O. ulmi	W9	AY887023	Paoletti *et al*. 2005
	PG450	AY887022	Paoletti *et al*. 2005
O. novo-ulmi subsp. *novo-ulmi*	R66	AY887028	Paoletti *et al*. 2005
	V19	AY887029	Paoletti *et al*. 2005
O. novo-ulmi subsp. *americana*	W2	AY887026	Paoletti *et al*. 2005
	PG402	AY887024	Paoletti *et al*. 2005
	US411	AY887025	Paoletti *et al*. 2005
O. himal-ulmi	HP62	AY887030	Paoletti *et al*. 2005
O. quercus	2520	FJ865421	Chapter 2 of this thesis
	2521	FJ865420	Chapter 2 of this thesis

Table 3. Number of differences (top) and percentage similarity (bottom) between the nine similarity groups based on *MAT-2* sequences.

Group	1	2	3	4	5	6	7	8	9
1	0 (100)								
2	3 (99.5)	0 (100)							
3	28 (95.7)	25 (96.2)	0 (100)						
4	30 (95.4)	27 (95.9)	8 (98.7)	0 (100)					
5	20 (96.9)	17 (97.4)	20 (96.9)	24 (96.3)	0 (100)				
6	4 (99.3)	1 (99.8)	26 (96.0)	28 (95.7)	18 (97.2)	0 (100)			
7	4 (99.3)	1 (99.8)	26 (96.0)	28 (95.7)	18 (97.2)	2 (99.6)	0 (100)		
8	22 (96.6)	19 (97.1)	22 (96.6)	26 (96.0)	2 (99.6)	20 (96.9)	20 (96.9)	0 (100)	
9	21 (96.8)	18 (97.2)	21 (96.8)	25 (96.2)	1 (99.8)	19 (97.1)	19 (97.1)	1 (99.8)	0 (100)

Table 4. Nine similarity groups defined by sequence conservation between the *MAT-2* sequences.

Group	Species	Isolate number	Reference
1	*O. novo-ulmi* subsp. *Americana*	W2	Paoletti *et al.* 2005
		US411	Paoletti *et al.* 2005
		PG402	Paoletti *et al.* 2005
2	*O. novo-ulmi* subsp. *novo-ulmi*	V19	Paoletti *et al.* 2005
		R66	Paoletti *et al.* 2005
		CMW 16184	This study
3	*O. himal-ulmi*	HP62	Paoletti *et al.* 2005
4	*O. himal-ulmi*	22729	This study
5	*O. ulmi*	PG450	Paoletti *et al.* 2005
		W9	Paoletti *et al.* 2005
	O. araucariae	671	This study
6	*O. novo-ulmi*	CMW 16178	This study
7	*O. novo-ulmi*	CMW 16179	This study
8	*O. flexuosum*	CMW 907	This study
9	*O. quercus*	CMW 2520	Chapter 2 of this thesis
		CMW 2521	Chapter 2 of this thesis
	O. ainoae	CMW 1903	This study
	O. borealis	CMW 18953	This study
	O. borealis	CMW 18956	This study
	O. canum	CMW 5023	This study
	O. catonianum	CMW 11535	This study
	O. distortum	CMW 467	This study
	O. floccosum	CMW 12622	This study
		CMW 12623	This study
	O. ips	CMW 5089	This study
		CMW 6445	This study
	O. karelicum	CMW 23109	This study
	O. multiannulatum	CMW 2567	This study
	O. piliferum	CMW 7879	This study
	O. pulvinisporum	CMW 9024	This study
		CMW 9028	This study
	O. tsotsi	CMW 5943	This study
	O. ulmi	CMW 25028	This study

Fig. 1. Phylogram resulting from a Maximum Likelihood analysis of the *MAT-2* sequence data. Bootstrap support values above nodes were obtained with ML analysis, while values below nodes originated from the NJ analysis. Taxon labels in bold type represents sequences acquired in the present study, while those in normal type were obtained from Genbank (accession numbers in Table 2).

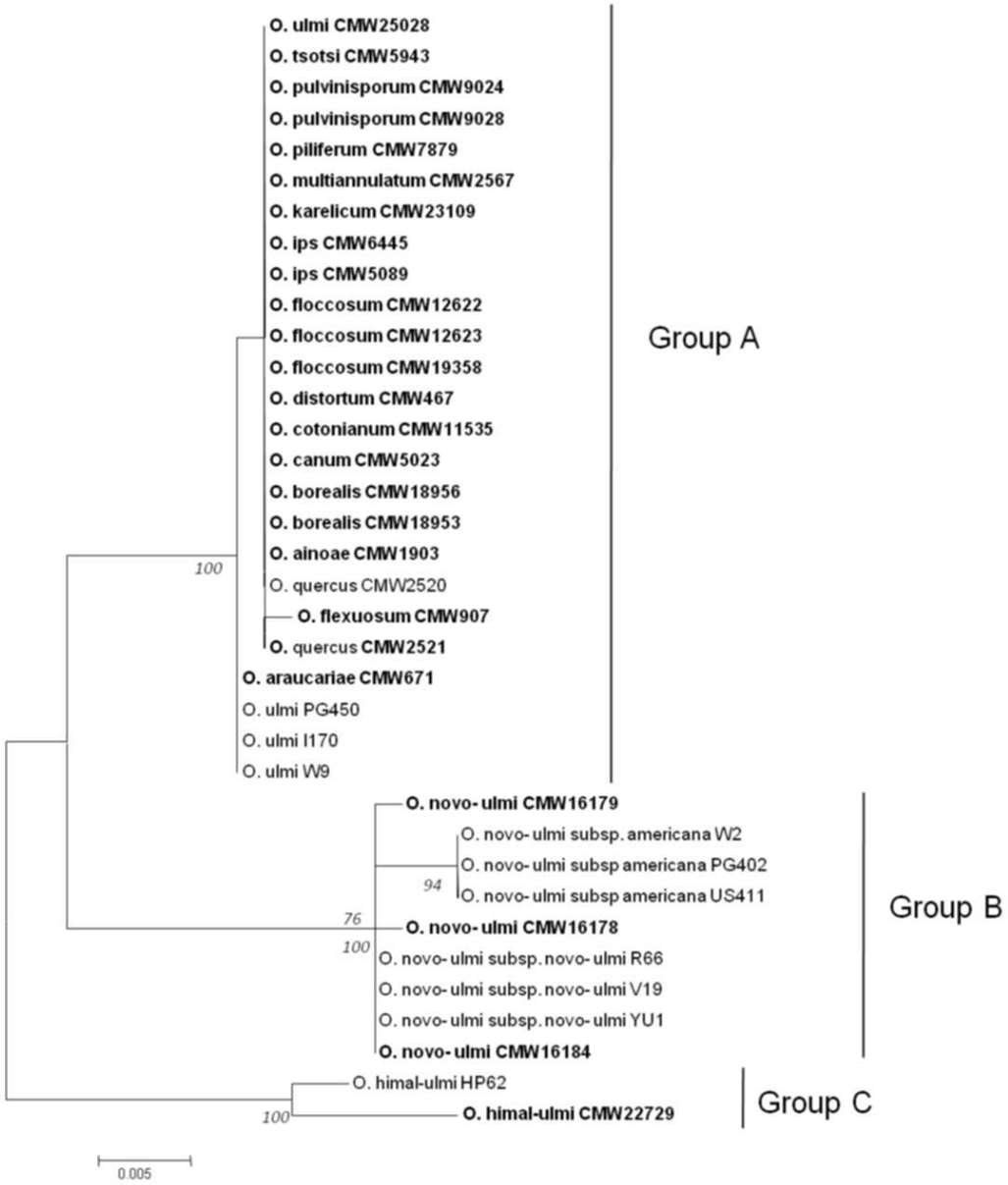
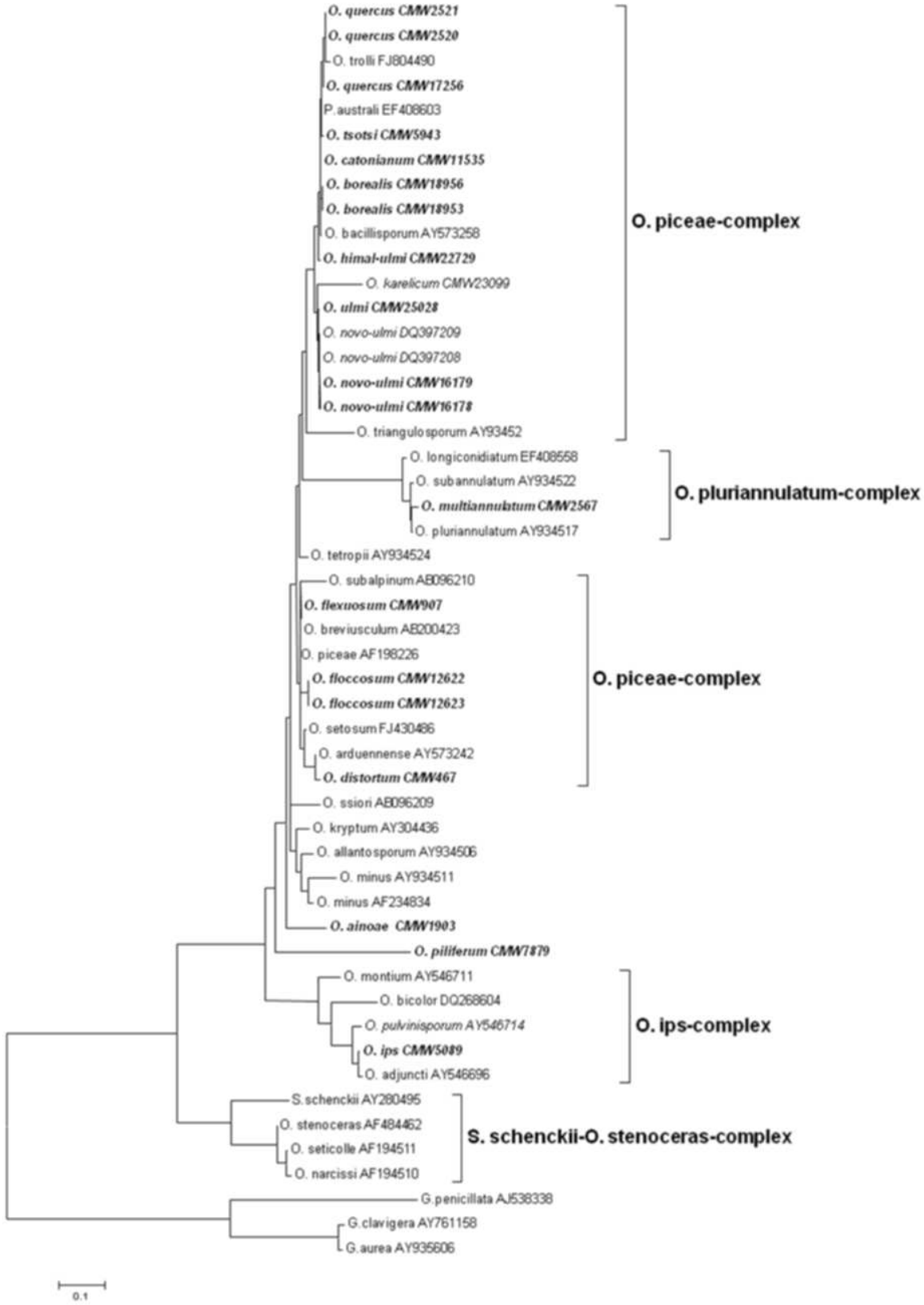

O. ulmi CMW25028
O. tsotsi CMW5943
O. pulvinisporum CMW9024
O. pulvinisporum CMW9028
O. piliferum CMW7879
O. multiannulatum CMW2567
O. karelicum CMW23109
O. ips CMW6445
O. ips CMW5089
O. floccosum CMW12622
O. floccosum CMW12623
O. floccosum CMW19358
O. distortum CMW467
O. cotonianum CMW11535
O. canum CMW5023
O. borealis CMW18956
O. borealis CMW18953
O. ainoae CMW1903
O. quercus CMW2520
O. flexuosum CMW907
O. quercus CMW2521
O. araucariae CMW671
O. ulmi PG450
O. ulmi I170
O. ulmi W9
Group A
O. novo-ulmi CMW16179
O. novo-ulmi subsp. americana W2
O. novo-ulmi subsp americana PG402
O. novo-ulmi subsp americana US411
O. novo-ulmi CMW16178
O. novo-ulmi subsp. novo-ulmi R66
O. novo-ulmi subsp. novo-ulmi V19
O. novo-ulmi subsp. novo-ulmi YU1
O. novo-ulmi CMW16184
Group B
O. himal-ulmi HP62
O. himal-ulmi CMW22729
Group C
100
94
76
100
100
0.005

Fig. 2. Phylogram obtained with a Maximum Likelihood analysis of the ITS region for selected species of *Ophiostoma*. Bootstrap values are indicated on branching points. The tree is rooted against three *Grosmannia* species. ITS sequences of isolates used in this study are indicated in bold type and italics. Species names included in this study but represented by isolates from other studies are shown in italics. All accession numbers are for the ITS sequence in the Genbank database.


O. quercus CMW2521
O. quercus CMW2520
O. trolli FJ804490
O. quercus CMW17256
P. australi EF408603
O. tsotsi CMW5943
O. catonianum CMW11535
O. borealis CMW18956
O. borealis CMW18953
O. bacillisporum AY573258
O. himal-ulmi CMW22729
O. karelicum CMW23099
O. ulmi CMW25028
O. novo-ulmi DQ397209
O. novo-ulmi DQ397208
O. novo-ulmi CMW16179
O. novo-ulmi CMW16178
O. triangulosporum AY93452
O. piceae-complex
O. longiconidiatum EF408558
O. subannulatum AY934522
O. multiannulatum CMW2567
O. pluriannulatum AY934517
O. pluriannulatum-complex
O. tetropii AY934524
O. subalpinum AB096210
O. flexuosum CMW907
O. breviusculum AB200423
O. piceae AF198226
O. floccosum CMW12622
O. floccosum CMW12623
O. setosum FJ430486
O. arduennense AY573242
O. distortum CMW467
O. piceae-complex
O. ssiori AB096209
O. kryptum AY304436
O. allantosporum AY934506
O. minus AY934511
O. minus AF234834
O. ainoae CMW1903
O. piliferum CMW7879
O. montium AY546711
O. bicolor DQ268604
O. pulvinisporum AY546714
O. ips CMW5089
O. adjuncti AY546696
O. ips-complex
S. schenckii AY280495
O. stenoceras AF484462
O. seticolle AF194511
O. narcissi AF194510
S. schenckii-O. stenoceras-complex
G. penicillata AJ538338
G. clavigera AY761158
G. aurea AY935606
0.1

SUMMARY

Although the genetic aspects of mating are a rapidly expanding field of study, little information is available for the genus *Ophiostoma*. The first *MAT* information for the genus focussed on only three species and this was as such, hardly representative of the genus. In this study, existing DNA sequence data were used as a starting point to expand the available knowledge on mating genes to other species of *Ophiostoma*.

Ophiostoma quercus, one of the better-studied species of *Ophiostoma* was the focus of the initial investigation. The heterothallic mating strategy of *O. quercus* was confirmed and isolates of both mating-types were used for the molecular analysis of the *MAT* genes. Regions of both *MAT* idiomorphs were observed in both mating-type isolates. This discovery was unexpected and suggests an unconventional mating organisation for *O. quercus* as compared to other heterothallic fungal species. Such a system is not unprecedented for fungi, but is unique for the genus *Ophiostoma*.

The primers developed for *O. quercus* were tested in isolates representing 17 species of *Ophiostoma*. These primers were used successfully to amplify a large segment of the *MAT-2* idiomorph in all isolates tested. This significantly expanded on the amount of data available for the *MAT* genes of *Ophiostoma*. Analysis showed that these isolates share a high amount of conservation in the *MAT-2* open reading frame. This region of the genome is, therefore, not useful for phylogenetic analyses. However, the availability of primers for the region might facilitate testing of other areas of the full idiomorph for phylogenetic inference. Overall, the results presented in this study represent a significant increase in the knowledge available on *MAT* genes in *Ophiostoma*.