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

by

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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

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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.

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May 2009
# TABLE OF CONTENTS

## Acknowledgments

1

## Preface

2

## CHAPTER 1

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

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 \( \textit{Saccharomyces cerevisiae} \) 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 \( \textit{Neurospora crassa} \) \( MAT \) 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 \( \textit{Ophiostoma} \) 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 \( \textit{Ophiostoma} \) 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 \( \textit{Ophiostoma} \) (Paoletti et al. 2006). These are the only data available on molecular aspects of the \( MAT \) genes in \( \textit{Ophiostoma} \) 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 homothally, 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

Yeast

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 MATa and MATα. The region is 642 base pairs (bp) in length for MATa 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 MATa 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 (α) to the other (α) (Strathern & Herskowitz 1979).

**Functioning of the MAT locus**

The dissimilar MATα 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 α1) involved in regulation of mating function (Astell et al. 1981). Another protein making up the MATα idiomorph, α2 inhibits the constitutive expression of MATα related genes, ensuring that the cell mating type represents MATα. Constitutive expression of MATα mating type genes results in a MATα mating specificity for any haploid cell not containing an expressed α cassette. In the diploid phase, the protein product of the MATα 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 MATα 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 \(a1\) polypeptide from *S. cerevisiae*. The highest level of conservation is found in a 15 aa region, the \(\alpha\)-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/\(\alpha\)-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 matA-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 \(\alpha\)-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* MATα1 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 mata-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 \textit{MAT} locus

From the above-mentioned and other studies on mating types, some general characteristics of the \textit{MAT} locus become clear. \textit{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 \textit{MAT-1} or \textit{MAT-2}. Although these idiomorphs are different, similarities exist between them (Fig. 4). Some of these are as follows:

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

- A single gene in each idiomorph is needed for a successful fertilization event. In \textit{MAT-1}, it is the \(\alpha\)-box containing gene and for \textit{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 \textit{C. heterostrophus} (Turgeon \textit{et al.} 1993).

- The conserved domains of the peptides encoded by these genes define the idiomorphs. An idiomorph that contains an \(\alpha\)-box motif is characterised as \textit{MAT-1}. Although genes at this idiomorph may also contain other functional domains (HMG-box or amphipathic helix), the presence of the \(\alpha\)-box is characteristic. For the \textit{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 \textit{MAT} genes containing the HMG DNA binding motif, share an intron of size 54-68 bp placed at a conserved serine position (Arie \textit{et al.} 1997, Coppin \textit{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 \(\alpha\)-box motif in all \textit{MAT-1} idiomorphs (Coppin \textit{et al.} 1997, Turgeon & Yoder 2000). This can also confirm the nature of a gene thought to be a \textit{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 \( \text{MAT} \) locus. This is in contrast to what is seen in \( S. \text{cerevisiae} \), 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 \textit{et al.} 2000).

These important points can guide the characterisation and study of \( \text{MAT} \) 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 \textit{et al.} 1997). Genome walking, using degenerate primers targeting these conserved regions, has also successfully been used (Arie \textit{et al.} 1997, Paoletti \textit{et al.} 2005) and could help with the isolation of the \( \text{MAT} \) idiomorphs.

**5. Ophiostoma ecology and taxonomy**

The genus \textit{Ophiostoma} forms part of an economically important group of fungi commonly referred to as the ophiostomatoid fungi (Wingfield \textit{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 \textit{Leptographium} and \textit{Pesotum}) with sticky drops of conidia (Wingfield \textit{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 \textit{Ophiostoma} and related genera (Aghayeva \textit{et al.} 2004, De Beer \textit{et al.} 2003,
Hausner \textit{et al.} 1993, Harrington \textit{et al.} 2001, Lim \textit{et al.} 2004, Spatafora & Blackwell 1994). One of the most comprehensive recent publications was by Zipfel \textit{et al.} (2006) in which a large sample of isolates was used in a multigenic phylogenetic study. Using data from the $\beta$-tubulin and nuclear large ribosomal subunit gene, 50 \textit{Ophiostoma} species were analysed. The description for \textit{Ophiostoma} was amended to accommodate species with \textit{Sporothrix}, \textit{Pesotum} and \textit{Hyalorhinocladiella} anamorphs. Furthermore, the teleomorph genera \textit{Grosmannia} and \textit{Ceratocystiopsis} were reinstated for two monophyletic lineages alongside \textit{Ophiostoma} within the Ophiostomatales.

\textit{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 \textit{Ophiostoma sensu stricto} (Fig. 5). Although the focus of the study was not on the subgroups within \textit{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, \textit{O. quercus}. This species has been isolated from \textit{Quercus} and \textit{Fagus} species in the Northern Hemisphere (Brasier & Kirk 1993) and on various hardwood species as well as on commercial \textit{Pinus} spp. in the Southern Hemisphere (De Beer \textit{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 \textit{et al.} 2003). Other species grouping closely in the DED - \textit{O. quercus} clade include \textit{O. auracariae}, as well as the conifer-infesting species \textit{O. canum, O. piceae, O. flexuosum} and \textit{O. distortum}. However, the relationships within and between these and other species groups in \textit{Ophiostoma} remain largely unresolved. Sequences of additional gene regions are necessary for further clarification.

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


Fig. 1. Life cycle of the fungus, *P. anserina* (from Coppin et al. 1997).
**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).
Fig. 3. The mating switch in yeast. A single mother cell, S (mating type $\alpha$) gives rise to two daughter cells, D1 ($\alpha$) and D2 ($a$). Daughter cell D1 gives rise to another daughter cell D1-1 ($\alpha$). In the fourth generation, eight cells were formed from the single mother cell S, four of these $a$ mating type and four of these $\alpha$ mating type (from Strathern & Herskowitz 1979).
**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.
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).
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 ☐.
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.

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 dH₂O). 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°C 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°C, followed by 35 cycles of 30 s at 95°C, 30 s at 50°C and 1 min at 72°C, followed by one cycle of 7 min at 72°C. 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 AmpliTag 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 x 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


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

<table>
<thead>
<tr>
<th>Isolate number</th>
<th>Host</th>
<th>Country</th>
<th>Collector</th>
<th>Mating group</th>
<th>Genbank acc. nr.</th>
<th>Original publication</th>
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<td>+</td>
<td>AF493241* FJ865416 FJ865421</td>
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</tr>
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<td>J Roux</td>
<td>-</td>
<td>FJ959044 FJ865415 FJ865425</td>
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</tr>
<tr>
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<td>27845</td>
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<td>Brasier &amp; Kirk 1993</td>
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<tr>
<td>27846</td>
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<td>UK</td>
<td>PT Scard</td>
<td>+</td>
<td>AF211838* FJ865410 FJ865427</td>
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</tr>
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<td>PT Scard</td>
<td>-</td>
<td>EF429089* FJ865412 FJ865428</td>
<td>Brasier &amp; Kirk 1993; De Beer et al. 2003</td>
</tr>
</tbody>
</table>

* 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.

<table>
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Table 3. Primers used in this study.

<table>
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<th>Primer name</th>
<th>Sequence (5’-3’)</th>
<th>Origin</th>
<th>MAT idiomorph targeted</th>
</tr>
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<td>OqMt1F</td>
<td>TGGCAAGAAAGGAAGACTGG</td>
<td>This study</td>
<td>MAT-1</td>
</tr>
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<td>OqMt1R</td>
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<td>This study</td>
<td>MAT-1</td>
</tr>
<tr>
<td>Seq9</td>
<td>GGGGATGTAAAAGGAAC</td>
<td>Paoletti et al. 2005</td>
<td>MAT-2</td>
</tr>
<tr>
<td>OqMt2</td>
<td>GCACACAACCTTTGCCAGGTA</td>
<td>This study</td>
<td>MAT-2</td>
</tr>
</tbody>
</table>
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*.

<table>
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<th>108</th>
<th>114</th>
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<tr>
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</tr>
<tr>
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<td>C</td>
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<td>A</td>
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<td>T</td>
<td>A</td>
<td>C</td>
<td>T</td>
</tr>
<tr>
<td><strong>O. h-u</strong></td>
<td>T</td>
<td>A</td>
<td>C</td>
<td>A</td>
<td>C</td>
<td>T</td>
<td>T</td>
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<td>C</td>
<td>G</td>
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<td>T</td>
</tr>
<tr>
<td><strong>O. quercus</strong></td>
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<td>G</td>
<td>C</td>
<td>A</td>
<td>A</td>
<td>T</td>
<td>C</td>
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</table>

|       |   | 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   | 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.
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).
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.
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 (e.g. 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 similar finding emerged when the HMG box of the MAT-2 gene was used as a phylogenetic character in the Ceratocystis coeruleascens 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 $\chi^2$ 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 (Grobbeelaar 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*. 67
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 dH₂O) for 4-6 weeks at 25° C. 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 MgCl₂, 0.2 mM of each dNTP and 0.2 mM of each primer. The reaction protocol was: one cycle of 5 min at 96° C, followed by 35 cycles of 30 s at 95° C, 30 s at 50° C and 1 min at 72° C, followed by one cycle of 7 min at 72° C. 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* (Withuhn 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, Withuhn 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


Table 1. Isolates used in this study.

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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.

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

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

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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 represent sequences acquired in the present study, while those in normal type were obtained from Genbank (accession numbers in Table 2).
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.
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* idiromorphs 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* idiromorph 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 idiromorph for phylogenetic inference. Overall, the results presented in this study represent a significant increase in the knowledge available on *MAT* genes in *Ophiostoma*.