Taxonomy, phylogeny and species diversity in the *Ophiostoma quercus* complex

Submitted by

Joha Grobbelaar
B.Sc. Hons

A thesis submitted in partial fulfilment of the requirements for the degree

MAGISTER SCIENTIAE

In the faculty of Natural and Agricultural Sciences, Department of Genetics, Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria, South Africa

January 2009

Study leaders:
Prof. Paulette Bloomer
Prof. Michael J. Wingfield
Prof. Brenda D. Wingfield

DECLARATION

I, the undersigned, hereby declare that the thesis submitted herewith for the degree Magister Scientiae to the University of Pretoria, contains my own independent work and has hitherto not been submitted by me for a degree at this or any other tertiary institution.

Joha W. Grobbelaar

January 2009

THIS THESIS IS DEDICATED TO MY LATE PARENTS

CHRISTO AND WILNA GROBBELAAR

THANK YOU FOR THE OPPORTUNITY TO STUDY

I SHOULD ALLOW ONLY MY HEART TO HAVE IMAGINATION, AND FOR THE REST RELY ON MEMORY, THAT LONG-DRAWN SUNSET OF ONE'S PERSONAL TRUTH

VLADIMIR NABOKOV

TABLE OF CONTENTS

CHAPTER 6

INTRASPECIFIC VARIATION IN A GENERALIST ASCOMYCETE FUNGUS, *OPHIOSTOMA QUERCUS*, REVEALS GENETIC STRUCTURE AT DIFFERENT GEOGRAPHIC SCALES

ACKNOWLEDGEMENTS

I wish to convey my sincere appreciation and admiration to the following people:

Professors *Mike and Brenda Wingfield*, for always believing in me, their passion and positive attitude towards science, for the world class facility I had the privilege of working in and their understanding and guidance.

Prof. *Paulette Bloomer*, for the grace and determination with which you taught me to persevere, your enthusiasm throughout this project and for always having an open door and good advice when I needed it. I'm truly indebted.

Wilhelm de Beer, who unselfishly dedicated hours of work to this thesis, who taught me about many of the finer details in science and in writing good manuscripts and for listening and sharing your thoughtful views on science and life.

My constant mentor, friend and colleague, *Irene Barnes* for your knowledge and understanding, your involvement in every area over the last five years, and the skill and precision you taught me to do good science.

My laboratory mates, *Dina Paciura, Maria-Noel Cortinas, Grace Nakabonge, Ryan Nadel, Alvaro* and *Blanca Duran* and *Gudrun Dittrich Schroeder* for your magnificent friendship and interest in my research and for supplying many laughs in lab 2-21.

Renate Zipfel, Dr Jane Wright and *Dr Bernard Slippers* for your advice and assistance with analysing the many results, and for your guidance in preparing manuscripts.

To fellow companions, past and present, in the FABI family who supported, guided and shared their experience and expertise.

My sister, *Annemarie*, who supported me throughout my entire degree, lovingly helped and encouraged me. I'm so proud of you.

Prof. *Eefke Beyers*, whose unconditional friendship and deep understanding I could not have relied on more. Your discerning views and many shared experiences have helped me make sense of this one life I have to live and to give it everything. Thank you.

My friends and family for your enduring love and words of encouragement when times were hard.

I would also like to thank the Tree Protection Co-operative Programme, National Research Foundation (NRF) and University of Pretoria the Department of Science and Technology (DST)/ NRF Centre of Excellence in Tree Health Biotechnology, South Africa for providing financial support.

PREFACE

The genus *Ophiostoma* is one of the ascomycete genera most frequently found associated with blue-stain of sapwood. The discolouration of wood reduces its value and therefore these fungi are considered to be economically important to timber industries globally. Although morphological characteristics are valuable for the differentiation of species, they are not particularly helpful in identifying closely related taxa within *Ophiostoma*. The *O. piceae* complex forms a monophyletic cluster within *Ophiostoma* and accommodates several different species that infect hardwood and coniferous hosts. Some of these are causal agents of sapstain, while others are serious tree pathogens in their own right.

The taxonomy of fungi in the *O. quercus* - *O. piceae* complex has been confused since the description of *O. quercus* from dying oak in 1926 from Yugoslavia. Morphologically, *O. quercus* resembles *O. piceae,* which was described earlier from conifers. These two species were considered as synonyms for more than 30 years, however, contemporary studies have shown that the two species are phylogenetically distinct. In **Chapter 1** of this thesis, the current knowledge on the taxonomy, biology and evolution of *O. quercus* and some of its close relatives in the *O. piceae* complex is reviewed.

In central and eastern Europe, a number of species morphologically similar to *O. quercus* have been implicated as part of a larger complex of factors contributing to oak decline, also known as tracheomycosis of oak. These include *O. fagi* from *Fagus* in Germany, *O. catonianum* from *Pyrus* in Italy, *O. roboris* and *O. valachicum* from *Quercus* in Romania, and *O. kubanicum* from *Quercus* in Russia. Overlapping morphological characters and a lack of authentic specimens and cultures have confused the taxonomy of *O. quercus* and these morphologically similar species, which have consequently been regarded as possible synonyms of *O. quercus*. The objective in **Chapter 2** was to redefine *O. quercus sensu stricto* by using a multigene phylogeny based on four independent nuclear gene regions to confirm the identity of all the isolates and clarify which species are its

synonyms. This work was co-authored with DN Aghayeva, ZW de Beer, P Bloomer, MJ Wingfield and BD Wingfield and submitted to Mycological Progress for publication.

The diversity of *Ophiostoma quercus* remains poorly understood and the high degree of morphological variability in isolates of *O. quercus* is misleading, possibly as a result of ongoing incipient speciation. One of the objectives of this research was to clarify what is being observed in *O. quercus* by developing species specific polymorphic microsatellite markers. **Chapter 3** describes the development and characterization of polymorphic markers for *O. quercus*. This work was published in Molecular Ecology Resources (2008) and co-authored with I Barnes, M-N Cortinas, P Bloomer, MJ Wingfield and BD Wingfield.

Sequence data generated using the polymorphic markers developed in Chapter 3 gave rise to many different haplotypes and a high number of polymorphisms which led to further investigation of the nuclear DNA. The result was the discovery of a new species. **Chapter 4** presents the description of *Ophiostoma tsotsi* sp.nov. from hardwoods found in Africa. This work was co-written with ZW de Beer, P Bloomer, M J Wingfield and BD Wingfield and has been prepared for submission to FEMS Microbiology letters.

A co-operative project between the South African and Chinese governments provided an opportunity for isolates of *O. quercus* to be collected in China. DNA sequence comparison revealed that the species was in fact the newly described *O. tsotsi*. **Chapter 5** presents a new report of *O. tsotsi* from *Eucalyptus* pulpwood chips in China. The work is co-authored with ZW de Beer, P Bloomer, MJ Wingfield, XD Zhou and BD Wingfield, which has been written and prepared for MycoScience, a journal published by the Mycological Society of Japan.

Studies and collections that formed part of this thesis led to a larger question relating to the intraspecific variation in *O. quercus* and this was considered in **Chapter 6**. Here the diversity of *O. quercus* is evaluated using four species-specific polymorphic markers on a global collection of isolates to reveal information about the genetic variation. We were

thus able to consider this variation at three levels of geographic partitioning; regional, continental and global.

This thesis has laid the foundation for further investigation in *O. quercus* regarding its ecology, epidemiology and molecular biology. The opportunity to study these aspects as well as the biology, pathogenicity, and ecological role of *O. tsotsi* has also transpired. In view of the relatedness of both *O. quercus* and *O. tsotsi* to the Dutch elm disease fungi, these aspects deserve serious consideration as numerous questions still remain regarding the role of these economically important fungi to the global forestry industry. This thesis is presented as a series of chapters in which chapters 2, 3, 4, 5 and 6 are in manuscript format. Consequently each chapter represents an independent article and repetition between these chapters has been unavoidable.

LIST OF TABLES

LIST OF FIGURES

Fig 1 Phylogram resulting from a Bayesian Monte Carlo Markov chain (MCMC) analyses of the ITS sequences. The posterior probability (pp) support values are given first followed by the ML bootstrap (bs) values (1000 replicates) for each node respectively (scores of 0.90 pp or 70 bs and less have not been included and are indicated with '-'. Bootstrap support values (1000 replicates) above 75% for Maximum parsimony are indicated with bold lines at the relevant branching points. Isolate numbers of unique sequences obtained in this study are printed in bold type.

Fig 2 Phylogram resulting from a Bayesian Monte Carlo Markov chain (MCMC) analyses of the β-tubulin sequences. The posterior probability (pp) support values are given first followed by the ML bootstrap (bs) values (1000 replicates) for each node respectively (scores of 0.90 pp or 70 bs and less have not been included and are indicated with '-'. Bootstrap support values (1000 replicates) above 75% for Maximum parsimony are indicated with bold lines at the relevant branching points. Isolate numbers of unique sequences obtained in this study are printed in bold type.

Fig 3 Phylogram resulting from a Bayesian Monte Carlo Markov chain (MCMC) analyses of the TEF1-α sequences. The posterior probability (pp) support values are given first followed by the ML bootstrap (bs) values (1000 replicates) for each node respectively (scores of 0.90 pp or 70 bs and less have not been included and are indicated with '-'. Bootstrap support values (1000 replicates) above 75% for Maximum parsimony are indicated with bold lines at the relevant branching points. Isolate numbers of unique sequences obtained in this study are printed in bold type.

Fig 4 Comparison of mean growth on MEA (three isolates per tested species, (± standard deviation) of *O.quercus* (purple) and *O. tsotsi* (grey) held at different temperatures (grown 10 d in the dark).

Fig 5 Morphological characteristics of *Ophiostoma tsotsi* (from holotype) (CMW 3117 x CMW15239) 1. Perithecium (bar = 100 µm), 2. Scanning electron micrograph (SEM) of ostiolar hyphae at the end of a perithecial neck (bar = 10 µm), 3. Ascospores, side view above and face view below (bar = 10 µm), 4. Synnematous *Pesotum* (bar = 100 µm) 5. SEM of a young conidiophore (bar = 10 µm), 6. SEM of conidia (bar = 5 µm), 7.

Sporothrix conidiogenous cell (bar = 100 µm), 8. SEM of a *Sporothrix* anamorph (bar = 10 µm), 9. SEM of a *Sporothrix* conidiogenous cell with emerging conidia (bar = 10 µm).

CHAPTER 5

Fig 1 Phylogram resulting from a Bayesian Monte Carlo Markov chain (MCMC) analysis of the β-tubulin sequences. The posterior probability (pp) support values are given first followed by the ML bootstrap (bs) values (1000 replicates) in bold type for each node respectively (scores of 0.95 pp or 70 bs and less have not been included). T indicates ex-type isolates of species and NT points out the ex-neotype.

CHAPTER 6

Figs. 1a, b, c and d Haplotype networks for *O. quercus* isolates for each of the independent SSR markers. Oqrc2 **(a)**, Oqrc12 **(b)**, Oqrc18 **(c)** and Oqrc19 **(d)**. Each circle represents a unique sequence-based haplotype. One mutational step (one base pair difference) between haplotypes is represented by a line. The size of the haplotype circle represents the frequencies of the haplotypes and is colour-coded by continent. The largest haplotype has the highest frequency in each network and is presumably the ancestral haplotype. The smallest coloured circle represents one occurrence of a haplotype. Nodes on the lines indicate unsampled or extinct haplotypes. Sample areas for each haplotype were grouped according to continents: Europe (green) [AU, Austria; FR, France; NO, Norway], Asia (red) [AZ, Azerbaijan; BH, Bhutan; JAP, Japan], Africa (blue) [MA, Malawi; SA, South Africa; TZ, Tanzania; UG, Uganda], South America (pink) [CH, Chile; UR, Uruguay], Australasia (yellow) [AUS, Australia; NZ, New Zealand]

Figs. 2a, b, c and d Haplotype networks for the 46 Norwegian *O. quercus* isolates for each of the independent SSR markers. Oqrc2**(a)**, Oqrc12**(b)**, Oqrc18**(c)** and Oqrc19**(d)**. Each circle represents a unique sequence-based haplotype. One mutational step (one base pair difference) between haplotypes is represented by a line. The size of the haplotype circle represents the frequencies of the haplotypes and is colour-coded by continent. The largest haplotype has the highest frequency in each network and is presumably the ancestral haplotype. The smallest coloured circle represents one occurrence of a haplotype. Nodes on the lines indicate unsampled or extinct haplotypes. Sample areas for

each haplotype were grouped according to Northern Norway (red) and Southern Norway (yellow).

Figs. 3a and b Absolute values of the rate of change of the likelihood distribution (mean ± SD) (a) and the rate of change in the log probability of data between successive *K* values (b).

Figs. 3c, d and e Bar graphs showing the average population assignments when *K*=2 (c), 3 (d) and 4 (e) respectively. Population 1 (green), population 2 (blue), population 3 (yellow) and population 4 (pink). Each country is indicated by a number, 1-Australia, 2-Austria, 3-Azerbaijan, 4-Bhutan, 5-Chile, 6-France, 7-Japan, 8-Malawi, 9-New Zealand, 10-Norway, 11-South Africa, 12-Tanzania, 13-Uganda, 14-Uruguay.

Figs 4 World map showing the samples of *O. quercus* used in this study with an average assignment of 80% or higher assignment to a cluster at *K*=4 for each country. Correlated allele frequencies amongst the isolates are assumed as a result of shared ancestry.

CHAPTER 1

LITERATURE REVIEW: *OPHIOSTOMA QUERCUS* WITH SPECIAL REFERENCE TO THE *O. PICEAE* COMPLEX

1.0 Introduction

Ophiostoma spp. are amongst the most common fungi resulting in blue-stain in logs and lumber. This staining occurs on both the surface of wood as dark spots and streaks, as well as within wood. The blue-stain fungi colonise through wounds on trees and logs, and sapwood of lumber (Seifert 1993). Factors influencing *Ophiostoma* colonisation of timber can be either biotic or abiotic. Although most of these fungi exhibit only low levels of pathogenicity, trees affected by drought and environmental pressures or mass attack by aggressive beetles result in easy infection, and a rapid decline in tree health (Frisullo *et al*. 1989; Minkevich 1962; Oleksyn & Przybyl 1987; Simonin *et al*. 1994).

Species in the genus *Ophiostoma* are characterized by their dark, flask-shaped ascomata giving rise to sticky droplets at the tips of extended ascomatal necks (Upadhyay 1981). These characteristics correspond to adaptations for insect-vectored dispersal of their reproductive spores (Francke-Grosmann 1967; Münch 1907). The vectors include diverse arthropod taxa resulting in close fungal-insect associations that are well recognized (Francke-Grosmann 1967; Jacobs & Wingfield 2001; Klepzig *et al.* 2001).

Most *Ophiostoma* spp. are vectored by insects, either specifically by bark beetles or by casual insects, for example flies and nitidulids, that visit freshly made wounds on trees. The genus *Ophiostoma* also includes species that are highly pathogenic and responsible for significant economic losses of forest crops worldwide. Well-documented examples of tree pathogens are the Dutch elm disease fungi causing pandemics in Europe and North America (Brasier 1991).

The *Ophiostoma piceae* species complex forms a distinct monophyletic lineage within *Ophiostoma* and incorporates a number of economically important species. These include saprophytic agents of blue-stain isolated from a variety of coniferous and angiosperm hosts as well as the more virulent Dutch elm diseases pathogens isolated from *Ulmus* spp. As stricter quarantine measures are applied and consumer demand for clean wood increases, a worldwide, renewed interest in the fungi and other organisms responsible for blue-stain (Seifert 1993). This also comes at a time when there is an immense pressure to

reduce chemical use (Gorton *et al*. 2004) and to find eco-friendly solutions to problems associated with blue-stain. This review focuses on the causal agents of blue-stain specifically in the *O. piceae* complex with an emphasis on *O. quercus*.

2.0 What is *Ophiostoma*?

Species of *Ophiostoma* are Ascomycete fungi in the order Ophiostomatales and family Ophiostomataceae. This is one of the largest ascomycete genera with over 160 species described (Upadhyay 1981; Wingfield *et al*. 1993). *Ophiostoma* has also been plagued by a complex taxonomic history. This is largely due to the morphological convergence in unrelated taxa that benefit from a largely symbiotic life history associated with insects (Hausner *et al.* 1993b; Wingfield *et al.* 1991).

During the previous century, correctly naming species in *Ophiostoma* based on morphological characteristics was challenging. In 1919, H. & P. Sydow described the genus *Ophiostoma* based on the type species *O. piliferum* (Fr.) H. & P. Sydow. However, a great deal of controversy existed since most American taxonomists considered species that are now known to be unrelated (Griffin 1968; Hunt 1956) in the genus *Ceratocystis* Ell. & Halst. *sensu lato.* This incorporated the diverse genera *Ceratocystis* Ellis & Halst. *sensu stricto, Ophiostoma* H. Syd. & P. Syd., and *Ceratocystiopsis* Upadh. & Kendr.

The advent of cell wall biochemistry in the 1970's brought new methods to delimit and diagnose fungal groups. This lead to the discovery that *Ophiostoma* species have cellulose and rhamnose in their cell walls, while these compounds are absent in *Ceratocystis* (De Hoog & Scheffer 1984). Another factor distinguishing *Ophiostoma* spp. from the rest of the ascomycetes, is their ability to tolerate the antibiotic cycloheximide, a protein synthesis inhibitor, at high concentrations (Harrington 1981). Later, molecular techniques based on DNA data re-emphasized and supported the separation of *Ceratocystis* and *Ophiostoma* (Hausner 1993b; Spatafora &Blackwell 1994) into separate genera.

Phylogenetic reconstruction presents the most robust (Taylor *et al*. 2000) route to defining *Ophiostoma* and *Ceratocystis*. The first study in which DNA sequence data were

applied for this purpose was that of Spatafora and Blackwell (1994). This was followed by a number of studies that included *Ceratocystis* and *Ophiostoma*, the most recent of which is a product from the AFTOL (www.aftol.org) consortium (Zhang *et al.* 2006). More specifically within *Ophiostoma*, monophyletic lineages are emerging (Zipfel *et al*. 2006) and these are being provided with names. As a result, true *Ophiostoma* spp. have been shown to form an independent monophyletic lineage that groups distinctly from the genus *Grosmannia* Goidanich (with their *Leptographium* Lagerberg & Melin anamorphs) and from the genus *Ceratocystiopsis* Upadhyay & Kendrick (with their *Hyalorhinocladiella* Upadhyay & Kendrick anamorphs), which were previously regarded as representing a single genus (Upadhyay 1981; Zipfel *et al*. 2006).

As knowledge and methods for species delineation becomes more sophisticated and sampling and surveys move into previously unexplored areas, the number of recorded *Ophiostoma* species will also increase. Thus, in just four years, the ISI Web of Knowledge has recorded over 120 reports or publications on *Ophiostoma* species, many of these being new species descriptions (http://.isiknowledge.com). This is a trend that is likely to continue into the future with many new species and groupings emerging in *Ophiostoma sensu lato*.

3.0 The *Ophiostoma piceae* complex

A significant assemblage of fungi in the genus *Ophiostoma* and one that represents a monophyletic group (Harrington *et al*. 2001) is the so-called *Ophiostoma piceae* complex. Species in this complex include numerous species found on hardwoods and coniferous trees. Also included in this complex are the well-known Dutch elm disease (DED) fungi, *O. ulmi, O. novo-ulmi* and *O. himal-ulmi* (Brasier, 1990; Brasier & Mehrotra 1995).

Species in the *Ophiostoma piceae* complex were initially characterized as those fungi that have synnematous *Pesotum* anamorphs as well as micronematous *Sporothrix* synanamorphs (Brasier 1993; Harrington *et al*. 2001). The presence of these two anamorph states is what sets the complex apart from the rest of the species of

Ophiostoma. Harrington (2001) provided a review of species belonging to the *O. piceae* complex and phylogenetic analyses revealed two clades. Species in the one clade cause blue-stain in mostly coniferous trees and those in the other are predominantly found on hardwoods (Brasier 1993; De Beer *et al*. 2003b; Harrington *et al*. 2001).

The *O. piceae* complex *sensu* Harrington *et al.* (2001) listed the hardwood host species as *O. quercus* (Georgev.) Nannf, *O. catonianum* (Goid) Goid., *O. ulmi* (Buisman) Nannf, *O. novo-ulmi* and *O. himal-ulmi*. Those associated with coniferous hosts were *O. piceae* (Münch) H. & P. Sydow, *O. canum*, (Münch) H. & P. Sydow, *O. setosum* Uzunovic, Seifert, Kim & Breuil and *O. floccosum* Mathiesen. In recent years, several additional species in the *O. piceae* complex have been described. These include *O. distortum* and *O. triangulosporium* (Hausner & Reid 2003), *O. kryptum* (Jacobs & Kirisits 2003), *O. tetropii* (Jacobs *et al*. 2003), *O. subalpinum, O. ssiori* (Masuya *et al*. 2003), *O. flexuosum, O. bacillisporum* (Villarreal *et al*. 2005), *O. arduennense* (Carlier *et al*. 2006), *O. breviusculum* (Chung *et al*. 2006), *O. ainoae, O. araucariae* (Zipfel *et al*. 2006), *O. karelicum* (Linnakoski *et al*. 2008) and *P. australi* (Kamgan *et al*. 2008a). Interestingly, some of these species were found to be genetically related to the complex, however, not necessarily having both a *Pesotum* and *Sporothrix* anamorph. Therefore, species in the complex are no longer classified as those with both *Pesotum* and *Sporothrix* states alone, and the value of using DNA sequence comparisons to analyse relationships between species in the complex is increasingly important.

While DNA sequence comparisons have become essential to define phylogenetic relationships in fungi such as those in the *O. piceae* complex, this is a topic which is still relatively underdeveloped. It is thus emerging that phylogenetic inference in *Ophiostoma* based on small numbers of gene regions can be misleading (Kim & Breuil 2001; Schroeder *et al*. 2001) and that protein coding genes produce better resolution at the species level (Lim *et al*. 2004d; Roets *et al*. 2006; De Meyer *et al*. 2008). For example ITS sequence data do not distinguish *O. piceae* from *O. canum,* however morphologically and biologically these two species are distinct (Harrington *et al*. 2001). Consequently, phylogenetic reconstruction with β-tubulin and translation elongation factor 1-α gene sequences has been shown to improve the resolution between closely related *Ophiostoma*

species (Grobbelaar *et al.* 2009; Jacobs & Kirisits 2003; Kim *et al*. 2004; Lim *et al*. 2004). It also highlights the importance of utilising a multi gene approach to classify relationships amongst species as has been strongly debated in reviews of this topic (Dettman *et al.* 2003; Taylor *et al.* 2000).

The known taxa in the *O. piceae* complex now include 23 distinct species collected in many different countries (Chung *et al.* 2006; De Beer *et al*. 2003b; Harrington *et al*. 2001). The species are predominantly opportunistic saprophytes but also include tree pathogens, well known for causing devastating diseases (Brasier 1991; Brasier & Mehotra 1995). These species remain an interesting group of taxa with a diverse symptomalogy, host range and geographic distribution, leaving many unanswered questions regarding their population structure and genetic diversity.

3.1.1 *O. ulmi, O. novo-ulmi* and *O. himal-ulmi*

The DED fungi, *Ophiostoma ulmi* and *O. novo-ulmi* are closely related to *O. quercus* and also cluster within the hardwood group of the *O. piceae* complex (Harrington *et al*. 2001). These pathogens are amongst the most damaging known, have the ability fo kill trees and decimated elm tree populations. In the Northern Hemisphere devastating pandemics started in the early 1900's and destroyed numerous elms (Brasier 1991). The origin of the DED fungi remains uncertain and was complicated by the diagnosis of the two genetically divergent subgroups of the pathogen, which are now treated as distinct species *O. ulmi* and *O. novo-ulmi* (Brasier 1991; 1993). A third species in the complex, *O. himal-ulmi* is believed to be native to the Himalayas where it was associated with bark beetles in elm branches (Brasier & Mehrotra 1995). The native range of *O. ulmi* and *O. novo-oulmi* has never been determined with certainty although it is thought to have its origins in the East (Brasier 1990). Thus, the discovery of *O. himal-ulmi* emerged from efforts to find the former two fungi in their native environment (Brasier & Mehrotra 1995).

DED infection results in the wilt of trees during their growth period or where the vulnerability of the host is high (Brasier 1991). The pathogen grows as mycelium within the sap-wood and secretes the wilt toxin, cerato-ulmin (Bowden *et al*. 1994; Temple & Horgen 2000), which disrupts the water supply of the tree leading to tylosis formation (a

defence response of the host to interfere with microbial invasion) in the vessels. When the cambium has been killed, the fungus begins to grow deeper into the viable bark tissues (phloem). As a result, the disease leads to rapid cell death destroying mature elm trees rapidly (Gagnon 1967; Stipes & Campana 1981).

Ophiostoma novo-ulmi is the more aggressive than *O. ulmi* and was responsible for the second DED pandemic in the early 1970's (Brasier 1991). This pandemic destroyed the majority of elms across most of North America, Europe and a large part of Asia. As *O. novo-ulmi* spread across the Northern Hemisphere countries, it replaced the less aggressive *O. ulmi* that caused the first pandemic fifty years earlier (Brasier 2000). Mating type studies revealed that a succession of clonal populations with a single mating type was responsible for the original pandemic of *O. novo-ulmi* (Paoletti *et al.* 2006).

Two forms of *O. novo-ulmi* are recognized and described as subspecies (Brasier 1979; 1990). These are the European EAN race and the North American NAN races of the pathogen. These differ biologically and a partial fertility barrier exists between them, however, EAN x NAN hybrids have been found in nature (Brasier 1991). Studies of natural hybridization have revealed further evidence of recombination between the two subspecies of *O. novo-ulmi* (Konrad *et al*. 2002). Brasier *et al* (1998) also discovered rare inter-specific hybrids between *O. ulmi* and *O. novo-ulmi*. These recombinant genotypes were likely as a result of *O. novo-ulmi* invading territory previously occupied by *O. ulmi* and served as a genetic bridge between the two species to acquire certain pathogenicity genes (Brasier *et al*. 1998). This ability to transfer genes resulted in *O. novo-ulmi* rapidly adapting to new environments as an invasive pathogen, and may explain how the second pandemic was even more destructive than the first (Paoletti *et al.* 2006).

The close relationship of the DED fungi in the *O. piceae* complex and other hardwood colonising *Ophiostoma* species (Brasier 1993) should invite serious consideration of the potential pathogenicity of the other species. Relatively little is known about the ecological role of many of the remaining species and their ability to establish and invade novel forest ecosystems and hybridise with other species.

3.1.2 *O. quercus* and *O. piceae*

Ophiostoma piceae was first described from sap-stained pine and spruce in Germany (Münch 1907), and most frequently affects coniferous timber. In contrast, *O. quercus* was initially isolated and described as *Ceratostomella querci* from oak in the former Yugoslavia in 1926 by Petor Georgevitch (1874-1947) (Georgevitch 1926). The taxonomy of *O. piceae* and *O. quercus* based solely on morphology resulted in the two species being treated as a single taxon for more than half a century (Griffin 1968; Hunt 1956; Przybyl 1992; Przybyl & De Hoog 1989; Upadhyay 1981). Many of these reports also incorrectly referred to *O. piceae* when *O. quercus* was isolated and vice versa.

Brasier and Kirk (1989) suggested that the hardwood and coniferous forms of *O. piceae* may be a discrete species. Based on temperature growth studies, Brasier and Stephens (1993) were able to distinguish the hardwood form of *O. piceae* from the coniferous form of the fungus based on temperature-growth response while mating behaviour studies between the two forms showed them to be intersterile (Brasier & Kirk 1993). Additional studies including RFLP data and phylogenetic analyses have since confirmed this observation (Halmschlager *et al*. 1994; Harrington *et al*. 2001; Kim *et al*. 1999; Morelet 1992; Pipe *et al*. 1995a) and *O. piceae* and *O. quercus* are now widely recognised as two very distinct species within the *O. piceae* complex.

Around the same time as the description of *O. quercus,* a number of other *Ophiostoma* species morphologically similar to *O. quercus,* were described from oak and other hardwood trees. These are:

Ophiostoma fagi (Loos) Nannf., in Melin & Nannf., Sven. Skogsvardsforen Tidskr. 32: 408. 1934;

Ophiostoma catonianum (Goid.) Goid., Boll Staz. Patol. Veg. Roma, n.s. 15: 125. 1935;

Ophiostoma roboris Georgescu & Teodoru, Anal. Inst. Cerc. Exp. For., Ser. 1, 11: 207. 1948;

Ophiostoma valachicum Georgescu & Teodoru, Anal. Inst. Cerc. Exp. For., Ser. 1, 11: 198. 1948;

Ophiostoma kubanicum Sherbin-Parfenenko, Rak. Sos. Bol. List. Porod (Moscow) p. 49. 1953;

Ophiostoma fagi was isolated from *Fagus* in Germany by Loos (1932) who demonstrated that it causes blue-stain on pinewood and has been suggested as a synonym of *O. quercus* (Harrington *et al*. 2001). An ex-type isolate of *O. fagi* was deposited by Goidánich and is held at CBS. *O. roboris* (Georgescu *et al*. 1948) and *O. valachicum* (Georgescu *et al*. 1948) isolated from *Quercus* in Romania as well as *O. kubanicum* (Sczerbin-Parfenenko 1953) from *Quercus* in Russia and *O. catonianum* from *Pyrus* in Italy (Goidánich 1935) have too been considered likely synonyms of *O. quercus* for many years because of their morphological similarity (Brasier & Kirk 1993; Przybyl & De Hoog 1989) and lack of authentic herbarium material in certain cases. In vitro, an isolate of *O. fagi* (CBS 236.32) mated with *O. quercus* and a so called isolate of *O. roboris* from Azerbaijan also paired with *O. quercus* isolates producing perithecia and ascospores (Guseinov 1984). Consequently these two species were reduced to synonymy with *O. quercus* (Brasier & Kirk 1993; Harrington *et al*. 2001).

Additional ITS sequence data and mating compatibility studies have also supported the view that *O. fagi* and *O. roboris* are synonyms of *O. quercus* but *O. catonianum* was proposed to be a separate species (Harrington *et al*. 2001). Unfortunately Georgevitch (1927) did not preserve type material or culture of the original isolate, described as *Ceratostomella querci*, isolated from dying oak in 1926 (Georgevitch 1926). Therefore, Morelet (1992) designated neotype material derived from two French isolates for *O. quercus* (Morelet 1992). The name *O. quercus* is favored rather than the name *O. querci,* as referred to by some authors considering the grammatical rules of Latin. The correct form is *O. quercus*, as is the case in classical Latin in the genitive form of *Quercus* ('U'-declension) (De Beer *et al*. 2003a).

O. quercus is ubiquitous on wood, has a worldwide distribution on hardwood and less commonly on coniferous tree species and is thought to be native to the Northern

Hemisphere (Harrington *et al*. 2001; Przybyl & Morelet 1993). However, a number of recent studies have showed that *O. quercus* is much more widely distributed on woody substrates than previously recognized, with reports of the fungus coming from countries such as Australia (Harrington *et al.* 2001), Brazil and Japan (De Beer *et al*. 2003b), Ecuador (Geldenhuis *et al.* 2004), Chile (Zhou *et al*. 2004), Canada, (Brasier & Kirk 1993; Kim *et al.* 1999), Korea (Harrington *et al.* 2001), New Zealand (Thwaites *et al.* 2005), Uruguay (Harrington *et al.* 2001), the USA (Kim *et al.* 1999), South Africa (De Beer *et al.* 2003b; Zhou *et al*. 2006, Kamgan Nkuekam *et al.* 2008a) and Uganda (Kamgan Nkuekam *et al.* 2008b). These reports of the fungus have been from a large variety of non-native and native hardwood trees and less commonly non-native plantation pines (De Beer *et al.* 2003b, Zhou *et al.* 2004, 2006, Thwaites *et al.* 2005). Some of these could have been accidentally introduced, but many are also likely to be native to the Southern Hemisphere, which is far from completely sampled.

3.2 Morphology

The genus *Ophiostoma* is characterized by a life cycle that is separated into a teleomorph (sexual) and anamorph (asexual) reproductive state. In the teleomorph, the ascospores (sexual spores) are produced in asci (sacs) found inside ascomata (fruiting bodies). Although features of the ascocarps (pigmentation, length of perithecial neck, absence or presence of an ostiole, or of ostiolar hyphae) and ascospores are valuable for the differentiation of species, they may not be particularly useful in identifying closely related species within *Ophiostoma* since ascomatal morphology is known to be very variable among its species (Hausner *et al*. 1993b; Upadhyay 1981).

Species in the *O. piceae* complex have either one or two anamorph states that are accommodated in the genus names *Pesotum* Crane & Schoknecht and *Sporothrix* Hektoen & Perkins Nicot & Mariat (Okada *et al*. 2000). In the *Pesotum* state, conidiophores are present as a collection of spore bearing stalks known as synnemata. Conidia are produced at the apices of these structures in slimy masses from conidiogenous cells that proliferate recurrently. In the *Sporothrix* state, micronematous

conidiophores (synanamorph) have prominent denticles on which conidia are produced (De Hoog 1993).

The morphology and conidial ontogeny, while defining the anamorph genera of *Ophiostoma* spp., remain challenging (Wingfield *et al*. 1991). *Hyalorhinocladiella* H.P. Upadhyay & W.B. Kendr., and *Leptographium* Lagerb. & Melin, are two more generic names for anamorphic states of species in *Ophiostoma.* Recently, the genera *Ceratocystiopsis* H.P. Upadhyay & W.B. Kendr. and *Grosmannia* Goid. were re-instated to accommodate two strongly supported lineages of *Ophiostoma* spp. with known teleomorph states, further subdividing the genus (Zipfel *et al*. 2006).

Observations of culture morphology in the *O. piceae* complex have revealed that the DED fungi, *O. ulmi, O. novo-ulmi* and *O. himal-ulmi,* as well as *O. quercus* and *O. setosum* produce characteristic concentric rings of aerial mycelium when cultured in the laboratory (Harrington *et al*. 2001). *Ophiostoma roboris* and *O. fagi* were compared to *O. quercus* based on the size of synnemata and found to be in the same range (Halmschlager *et al*. 1994). When comparing *O. quercus* and *O. piceae* anamorphs, *O. quercus* produces markedly light-brown protoperithecia while those of *O. piceae* are black (Harrington *et al*. 2001). However, the anamorph morphology remains unconvincingly reliable for taxonomic purposes.

In the coniferous clade of the *O. piceae* complex (Harrington *et al*. 2001), *O. piceae* conidia are cylindrical to obovoid whereas the conidia of *O. canum* are globose. In *O. piceae* the synnemata are always black, however the synnemata of *O. canum* have brown stipes and in *O. floccosum* the synnemata are red-brown with lateral knobs (Harrington *et al*. 2001). *O. setosum* is reported as having darkly pigmented synnemata with dark seta-like hyphae extending from the stipe into apices (Uzunovic *et al*. 2000).

In general, species in the *O. piceae* complex have dark coloured ascomata that are well adapted for anthropod-mediated dispersal with long perithecial necks, surrounded by prominent ostiolar hyphae (Münch 1907). Also in most cases, species in this complex have reniform ascospores without thickened outer walls (Hedgcock 1906; Upadhyay 1981).

3.3 Reproduction

The majority of *Ophiostoma* species rely on a heterothallic mating system that enforces outbreeding (Wingfield *et al*. 1993). A single bi-allelic mating type locus (MAT) has been identified with the MAT-1 and the MAT-2 genes referred to as idiomorphs (Brasier 1993; Paoletti *et al*. 2005). As a result of heterothallism, individuals carry only one idiomorph. *Ophiostoma catonianum* is the only species in the *O. piceae* complex that Goidanich (1935) reported as being homothallic. Paring isolates of opposite mating type in one species readily produces ascomata and ascospores. Studies have shown that different species in the *O. piceae* complex frequently do not cross hybridise with closely related species (Brasier 2000; Harrington *et al*. 2001).

The species barriers in fungi are apparently robust (Kohn 2005) and therefore the inability to cross (the biological species concept) could be a useful characteristic in delimiting species. However, a system of mostly reproductive isolation, known as partial interfertility, exists between certain species of *Ophiostoma* (Brasier 1993) and it is not uncommon to find such partially fertile crosses in fungi. They have a lower ascospore germination percentage with fewer ascospores produced than seen in intraspecific crosses (Harrington & McNew 1998). Barriers involved in these crosses are likely to be postzygotic resulting in hybrids of reduced fertility and highlights the complicated interactions between these species and the intricate factors involved in reproductive isolation. Sterility or the observation of aborted asci serves as strong evidence that species are biologically distinct (Brasier 1990; Brasier & Kirk 1993). However, Brasier (1993) observed the formation of sterile perithecia in interspecific pairings between *O. novo-ulmi* and *O. piceae,* as well as between *O. ulmi* and *O. quercus*. Therefore, genetic exchange may occur during interspecific crosses when both species colonise the same host (coniferous or hardwood species) (Pipe *et al*. 1995a).

Heterothallism may be beneficial when studying mating populations to identify distinct biological species and mating types. However, in certain cases the possibility remains for closely related species to mate when their mating and intersterility genes are compatible.

There are well known examples in fungi where speciation occurs without full reproductive isolation (Dettman *et al*. 2003; Kohn 2005; Taylor *et al*. 2000) and as a result, drives research into the precise and complex mechanisms of reproduction in fungal systems. Sustained anthropogenic introductions and habitat modification impact on interbreeding and the mixing of taxa and may be a major contributor to driving the degree of biodiversity and genetic variability (Brasier, 2000; Desprez-Loustau *et al.*, 2007).

3.4 Ecology

Ophiostoma piceae and *O. quercus* are two of the blue-stain fungi most frequently isolated and have revealed a broad geographic range. Trees that are physiologically weakened, freshly sawn lumber or recently felled stumps and timber that has a high moisture content are likely substrates for infection (Gorton *et al*. 2004). *Ophiostoma piceae* is most commonly found on Pinaceae species throughout Europe, North America and Japan (Brasier & Kirk 1993; Halmschlager *et al*. 1994; Kim *et al*. 1999; Morelet 1992; Pipe *et al*. 1995a). The fungus has also been isolated from exotic *Pinus radiata* in New Zealand and Chile and from *Pinus* species in South Africa (De Beer *et al*. 2003b; Thwaites *et al*. 2005).

Ophiostoma quercus is widespread throughout Europe and North America, apparently occurring more frequently on hardwoods, especially oak, than on conifers (Brasier & Kirk 1993; Halmschlager *et al*. 1994; Kim *et al*. 1999; Pipe *et al*. 1995a). Harrington *et al.* (2001) identified isolates of *O. quercus* from *P. radiata* in Australia, *Eucalyptus* sp. in Uruguay and *Pinus* sp. in Korea. *O. quercus* has also been found on *Quercus* and *Fagus* species in the Northern Hemisphere and has been associated with oak decline in central and Eastern Europe and in Russia (Cech *et al*. 1990; Ivanchenko 1957; Minkevich 1962; Uroševic 1987).

In Ecuador, the *Pesotum* anamorph of *O. quercus* has been isolated from machete wounds on *Schizolobium parahybum* (Geldenhuis *et al*. 2004). In South Africa, *O. quercus* was reported to be distributed extensively on both native and exotic hardwood hosts (De Beer

et al. 1995). The occurrence of *O. quercus* on *Pinus* sp. in North America as well as in the Southern Hemisphere may be as a result of it not being native to these regions and having been introduced from Europe (Brasier & Kirk 1993; Harrington *et al*. 2001).

There are many questions that remain to be answered regarding the possible origins of *O. quercus.* In order to resolve this question, a large, world-wide collection of isolates would be required and studies aimed at a population biology level would reveal knowledge of *O. quercus* in particular regarding the diversity of the species and gene flow between isolates.

3.5 Insect association and dissemination

Vectors play a key role in dispersing species in the genus *Ophiostoma,* which is one of the largest groups of insect dispersed pyrenomycetes. In terms of biology, the fungus appears to be a casual associate of many different bark beetles and other tree-wound visiting insect species (Kirisits 2004, Zhou *et al*. 2004, 2006, Romón *et al*. 2007, Kamgan Nkuekam *et al*. 2008a, b, Linnakoski *et al*. 2008). These arthropod taxa include mainly bark beetles, longhorn beetles (*Cerambycidae*) and mites (*Acari*) (Barras & Perry 1975; Bridges & Moser 1983; 1986; Upadhyay 1981). Dying stems and branches of trees killed by fungal pathogens attract egg-laying female bark beetles that create larval galleries in these vulnerable sites. The sexual and asexual spores of the fungus stick to the bodies of the young beetles, which at maturation fly to undamaged branches and thereby transfer the spores. These fungal–insect associations have in some instances been shown to be mutualistic (Francke-Grosmann 1967; Jacobs & Wingfield 2001; Klepzig *et al.* 2001).

Water potential and abiotic factors may alter the result of competitive interactions among bark beetle associated fungi such that bark beetle success is favoured in conditions of decreasing water potential (Klepzig *et al*. 2004). Certain insects have the potential to carry a mixture of both saprophytic and pathogenic species. Sometimes saprophytic species have been shown to out compete the pathogenic species (Gibbs 1980). This important mutualistic relationship has been displayed by the members of the *O. piceae*

complex and certain sapwood inhabiting beetles (Juzwik *et al*. 1998; Juzwik & French 1983).

The majority of research on the vectors and dissemination of members of the *O. piceae* complex relates to Dutch elm disease, identifying the scolytid beetles *(Coleoptera: Scolytidae)* with the spread of this highly pathogenic disease (Brasier 1990). Other than the classic studies regarding dissemination and vectors that has been done on the DED fungi, there have been various studies on this topic in other parts of the world. For example in Japan, a new species of *Ophiostoma* possessing a *Pesotum* anamorph has recently been isolated from bark beetles and their galleries found in *Abies* species (Yamaoka *et al*. 2004). In Canada, *Tetropium fuscum* (Fabr.) (Coleoptera: Cerambycidae), an exotic species of longhorn beetle, was identified along with two of the most frequently isolated fungi associated with the beetle, namely *O. piceae* and *Pesotum fragrans* (Jacobs *et al*. 2003). Field experiments in the north central states of the USA detected sap-feeding beetles (*Coleoptera: Nitidulidae*) to be associated with *O. quercus* and *Ceratocystis fagacearum* (Juzwik *et al*. 1998).

Although the majority of members of the *O. piceae* complex is dispersed with the facilitation of sticky spore drops (Malloch & Blackwell 1993), species in the *O. piceae* complex can also produce dry asexual spores. These dry propagules (*Sporothrix*) can be wind-dispersed (Crane & Schoknecht 1973; Malloch & Blackwell 1993). Understanding the methods of dispersal of the sexual and asexual spores has implications for managing introductions of *O. quercus*.

3.6 Molecular taxonomy

Molecular tools have been used widely to refine the classification and to better understand the ecology of the ophiostomatoid fungi during the course of the last two decades. The pathogens *O. ulmi* and *O. novo-ulmi* were distinguished using randomly amplified polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP) markers (Pipe *et al*. 1995b). Kim *et al.* (1999) developed a rapid, uncomplicated method to detect *O. piceae* and *O. quercus* within four hours using the polymerase chain

reaction (PCR). Likewise, phylogenetic analyses have strongly supported the separation of the complex into the hardwood and coniferous host groupings (Harrington *et al*. 2001).

In cases where limited morphological characters hinder identification decisions such as in *Ophiostoma*, DNA sequence comparison for independent loci can present accurate, improved assessment (Hausner *et al*. 1993b). Most new species descriptions in the *O. piceae* complex published in the last five years contains an aspect of DNA sequence data of one or more loci to support the evidence to delineate and characterise a species (Chung *et al.* 2006; Jacobs & Kirisits 2003; Masuya *et al.* 2003).

An advancement in *Ophiostoma* genomics was the establishment of the Canadian *Ophiostoma* genome project, a collaboration between research teams from four different universities, who have identified more than 750 expressed sequence tags (EST's) in *Ophiostoma novo-ulmi* showing homology with other fungal genes of known function (Bernier *et al*. 2004). The *Ophiostoma* nuclear genome has approximately 8000-10,000 genes (Kupfer *et al*. 1997) and extends over 30 to 40 million nucleotides (Dewar *et al*. 1997). Pulsed-field gel electrophoresis (PFGE) has been used to separate chromosomes in 71 isolates representing seven species of *Ophiostoma*, six of which are included in the *O. piceae* complex, however, no consistent association could be recognized between karyotype and geographic origin or host (Bernier *et al*. 2004).

In the past, molecular systematics of *Ophiostoma* species was predominantly based on the nuclear rDNA gene (the ITS region and LSU region) (Hausner *et al*. 1993a; Jacobs *et al*. 2001, Schroeder *et al.* 2001). Since the ITS region has wide interspecific but less intraspecific variability, this demonstrated to be useful in identifying fungi at species level (Gardes & Bruns 1993). However, Kim and Breuil (2001) demonstrated that *O. quercus* and *O. piceae* commonly share at least two types of ITS sequences. Therefore, in closely related species of *Ophiostoma*, additional sequence data from single copy protein coding genes may be necessary to accurately identify and assess genetic variation in distinct species as several recent studies have shown (Lim *et al*. 2004; De Meyer *et al*. 2008; Roets *et al*. 2008).

To date, very little is known regarding the intraspecific variation of most species in the *O. piceae* complex. This is with the exception of the well documented DED fungi that have been studied intensively for variability (Brasier *et al*. 1998; Konrad *et al*. 2002). Furthermore, *O. piceae* populations in Canada were assessed for their genetic variability (Gagne *et al*. 2001). However, no studies have yet been done on the intraspecific variation of *O. quercus*.

4.0 Conclusions and objectives

The genus *Ophiostoma* is a diverse and interesting group of typically insect associated fungi, harbouring several well-known pathogen species, commonly found on angiosperm and coniferous wood. The capacity to colonize wounds on a wide range of hosts, their association with many different insect vectors and world-wide distribution encourages many questions regarding the origin and ecology of these pathogens and saprophytes.

Ophiostoma quercus is a morphologically variable species that appears to be a casual associate of many different bark beetles and other tree-wound visiting insect species. *O. quercus* isolates consistently display considerable variation in their DNA sequences revealing a high level of genetic diversity. Using molecular sequence data has aided efforts to map boundaries for species delineation and to recognize a new closely related species, which are treated in this thesis.

Intraspecific studies on *O. quercus* will contribute to a deeper understanding of the worldwide distribution and origin of this fungus. In this thesis, I have made an effort to answer some of the compelling questions using molecular markers specifically developed for *O. quercus*. In future, the biology, pathogenicity, and ecological role in invading forest ecosystems may be better appreciated for this fungus.

5.0 References

Barras SJ, Perry TJ, 1975. Interrelationships among microorganisms, bark or ambrosia beetles, and woody host tissue: an annotated bibliography. *U.S. Department of*

Agricultural Forest Service General Technical Report SO-10. Southern Forest Experiment Station, New Orleans, U.S.A, pp. 1956-1974.

Bernier L, Breuil C, Hintz WE, Horgen PA, Jacobi V, Dufour J, Aoun M, Bouvet G, Kim SH, Diguistini S, Tanguay P, Eades J, Burgess S, de la Bastide P, Pinchback M, Tadesse Y, 2004. The Canadian *Ophiostoma* genome project. *Invest. Agrar: Sist Recur For* **13**: 105-117.

Bowden CG, Hintz WE, Jeng RS, Hubbes M, Horgen PA, 1994. Isolation and characterization of the cerato-ulmin gene of the Dutch elm disease pathogen, *Ophiostoma ulmi*. *Current Genetics* **25:** 323-329.

Brasier CM, 1979. Dual origin of recent Dutch elm disease outbreaks in Europe. *Nature* **281**: 78-80.

Brasier CM, 1990. China and the origins of Dutch elm disease: an appraisal. *Plant pathology* **39**: 5-16.

Brasier CM, 1991. *Ophiostoma novo-ulmi* sp. nov., causative agent of current Dutch elm disease pandemics. *Mycopathologia* **115**: 151-161.

Brasier CM, 1993. The genetic system as a fungal taxonomic tool: gene flow, molecular variation and sibling species in the '*Ophiostoma piceae* - *Ophiostoma ulmi*' complex and it's taxonomic and ecological significance, in: Wingfield MJ, Seifert KA, Webber JF (Eds), *Ceratocystis and Ophiostoma: Taxonomy, Ecology and Pathogenicity*. The American Phytopathological Society, St. Paul, Minnesota, pp. 77-92.

Brasier CM, 2000. The rise of the hybrid fungi. *Nature* **55**: 134-135.

Brasier CM, Kirk SA, 1989. European oak decline. Status of *O. piceae* on hardwoods and conifers. *Forest Research*, London, UK, pp. 47-48.

Brasier CM, Kirk SA, 1993. Sibling species within *Ophiostoma piceae*. *Mycological Research* **97**: 811-816.

Brasier CM, Mehrotra MD, 1995. *Ophiostoma himal-ulmi* sp. nov., a new species of Dutch elm disease fungus endemic to the Himalayas. *Mycological Research* **99**: 205-215.

Brasier CM, Stephens TM, 1993. Temperature-growth responses distinguish the OPC and OPH sibling species within *'Ophiostoma piceae'*. *Mycological Research* **97**: 1416-1418.

Brasier CM, Kirk SA, Pipe ND, Buck KW, 1998. Rare interspecific hybrids in natural populations of the Dutch elm disease pathogens *Ophiostoma ulmi* and *O. novo-ulmi*. *Mycological Research* **102:** 45-57.

Bridges JR, Moser JC, 1983. Role of two phoretic mites in transmission of the blue-stain fungus, *Ceratocystis minor*. *Ecological Entomology* **8**: 9-12.

Bridges JR, Moser JC, 1986. Relationship of phoretic mites (*Acari: Tarsonemidae*) to the blue-staining fungus, *Ceratocystis minor*, in trees infested by southern pine beetle (*Coleoptera: Scolytidae*). *Environmental Entomology* **15**: 951-953.

Carlier F-X, Decock C, Jacobs K, Maraite H, 2006. *Ophiostoma arduennense* sp. nov. (Ophiostomatales, Ascomycota) from *Fagus sylvatica* in southern Belgium. *Mycological Research* **110:** 801-810.

Cech T, Donaubauer E, Tomiczek C, Leontovyc R, Yde-Anderson A, Delatour C, Wulf A, Toth J, Vajna L, Vammini A, Luisi N, Oosterbaan A, Kowalski T, Gibbs JN, Jurc D, 1990. Oak Decline and the Status of *Ophiostoma* spp. on oak in Europe, *EPPO Bulletin,* pp. 405-422.

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

Crane JL, Schoknecht JD, 1973. Conidiogenesis in *Ceratocystis ulmi, Ceratocystis piceae* and *Graphium penicillioides*. *American Journal of Botany* **60:** 346-354.

De Beer ZW, Wingfield MJ, Kemp GHJ, 1995. First report of *Ophiostoma querci* in South Africa. *South African Journal of Science* **91** (Abstract).

De Beer ZW, Glen HF, Wingfield BD, Wingfield MJ, 2003a. *Ophiostoma quercus* or *Ophiostoma querci*? *Mycotaxon* **LXXXVI**: 211-214.

De Beer ZW, Wingfield BD, Wingfield MJ, 2003b. The *Ophiostoma piceae* complex in the Southern Hemisphere: a phylogenetic study. *Mycological Research* **107**: 469-476.

De Hoog GS, Scheffer RJ, 1984. *Ceratocystis* versus *Ophiostoma:* a reappraisal. *Mycologia* **76**: 292-299.

De Hoog GS, 1993. *Sporothrix*-like anamorphs of *Ophiostoma* species and other fungi, in: Wingfield MJ, Seifert KA, Webber JF (Eds), *Ceratocystis and Ophiostoma: Taxonomy, Ecology, and Pathogenicity*. The American Phytopathological Society, St. Paul, Minnesota, pp. 53-60.

De Meyer EM, De Beer ZW, Summerbell RC, Moharram AM, De Hoog GS, Vismer HF, Wingfield MJ, 2008. Taxonomy and phylogeny of new wood- and soil-inhabiting *Sporothrix* species in the *Ophiostoma stenoceras-Sporothrix schenckii* complex. *Mycologia* **100**: 647-661.

Desprez-Loustau ML, Robin C, Buee M, Courtecuisse R, Garbaye J, Suffert F, Sache I, Rizzo DM, 2007. The fungal dimension of biological invasions. *Trends in Ecology & Evolution* **22**: 472-480.

Dettman JR, Jacobson DJ, Taylor JW, 2003. A multilocus genealogical approach to phylogenetic species recognition in the model eukaryote *Neurospora*. *Evolution* **57**: 2703-2720.

Dewar K, Bousquet J, Dufour J, Bernier L, 1997. A meiotically reproducible chromosome length polymorphism in the ascomycete fungus *Ophiostoma ulmi (sensu lato)*. *Molecular and General Genetics* **255**: 38-44.

Francke-Grosmann H, 1967. *Ectosymbiosis in wood-inhabiting insects*. In: Henry SM ed. Symbiosis, Vol II. New York: Academic Press. pp. 171-180.

Frisullo S, Mannerucci F, Luisi N, 1989. Funghi cromogeni associati al deperimento delle querce. *Micologia Italiana* **18**: 77-86.

Gagne P, Yang D-Q, Hamelin RC, Bernier L, 2001. Genetic variability of Canadian populations of the sapstain fungus *Ophiostoma piceae*. *Phytopathology* **91**: 369-376.

Gagnon C, 1967. Histochemical studies on the alteration of lignin and pectic substances in white elm infected by *Ceratocystis ulmi. Canadian Journal of Botany* **45:** 1619-1623.

Gardes M, Bruns TD, 1993. ITS primers with enhanced specificity for basidiomycetes - application to the identification of mycorrhizae and rust. *Molecular Ecology* **2**: 113-118.

Geldenhuis MM, Roux J, Montenegro F, De Beer ZW, Wingfield MJ, Wingfield BD, 2004. Identification and pathogenicity of *Graphium* and *Pesotum* species from machete wounds on *Schizolobium parahybum* in Ecuador. *Fungal Diversity* **15**: 135-149.

Georgescu C, Teodoru I, Badea M, 1948. Uscarea in Massa a Stejarului. *Análele Institutului de Cercetãri Forestiere al Romãniei* **XI** : 185-217.

Georgevitch Y, 1926. *Ceratostomella querci* n.sp. *Comptes Rendus Hebdomadaires des Séances de l'Académie des Sciences* **183** : 759-761.

Georgevitch Y, 1927. *Ceratostomella quercus* n. sp. Ein Parasit der slawonischen Eichen. *Biologia Generalis* **3:** 245-252.

Gibbs JN, 1980. Role of *Ceratocystis piceae* in preventing infection by *Ceratocystis fagacearum* in Minnesota. *Transactions of the British Mycological Society* **74**: 171-174.

Goidánich G, 1935. Uno nuova specie di *Ophiostoma* vivente sul pero ed alcune osservazioni sull'esatta posizione sistematica della forma ascofora e delle forme

metagenetiche del genere. *Bolletino della Stazione di Patologia Vegetale di Roma* **15**: 122-168.

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

Griffin HD, 1968. The genus *Ceratocystis* in Ontario. *Canadian Journal of Botany* **46**: 689-718.

Grobbelaar JW, Aghayeva DN, De Beer ZW, Bloomer P, Wingfield MJ, Wingfield BD, 2009. Delimitation of *Ophiostoma quercus* and its synonyms using multiple gene phylogenies. Chapter Two.

Guseinov ES, 1984. Vascular dieback of oak in Azerbaijan. *Mikologiya/ Fitopatologiya* **18**: 144-149.

Halmschlager E, Messner R, Kowalski T, Prillinger H, 1994. Differentiation of *Ophiostoma piceae* and *Ophiostoma quercus* by Morphology and RAPD Analysis. *Systematic and applied Microbiology* **17**: 554-562.

Harrington TC, 1981. Cycloheximide sensitivity as a taxonomic character in *Ceratocystis*. *Mycologia* **73**: 1123-1129.

Harrington TC, McNew DL, 1998. Partial Interfertility Among the *Ceratocystis* Species on Conifers. *Fungal Genetics and Biology* **25:** 44-53.

Harrington TC, McNew D, Steimel J, Hofstra D, Farrell R, 2001. Phylogeny and taxonomy of the *Ophiostoma piceae* complex and the Dutch elm disease fungi. *Mycologia* **93**: 111-136.

Hausner G, Reid J, Klassen GR, 1993a. *Ceratocystiopsis*: a reappraisal based on molecular criteria. *Mycological Research* **97**: 625-633.

Hausner G, Reid J, Klassen GR, 1993b. Grouping of isolates and species of *Ceratocystis sensu lato* on the basis of molecular and morphological characters, in: Wingfield MJ, Seifert KA, Webber JF (Eds), *Ceratocystis and Ophiostoma: Taxonomy, Ecology and Pathogenicity*. The American Phytopathological Society, St. Paul, Minnesota, pp. 93-104.

Hausner G, Reid J, 2003. Notes on *Ceratocystis brunnea* and some other *Ophiostoma* species based on partial ribosomal DNA sequence analysis. *Canadian Journal of Botany* **81**: 865-876.

Hedgcock GG, 1906. Studies upon some chromogenic fungi which discolor wood. *Missouri Botanical Garden Annual Report* **17**: 59–114.

Hunt J, 1956. Taxonomy of the Genus *Ceratocystis*. *Lloydia* **19**: 1-58.

Ivanchenko YN, 1957. [The causes of Oak wilt in the Lipetsky garden in the Saval'sky Forest (in Russian)]. *Trud. vsesoyuz. Inst. Zashch. Rast.* **8**: 221-225.

Jacobs K, Kirisits T, 2003. *Ophiostoma kryptum* sp.nov. from *Larix decidua* and *Picea abies* in Europe, similar to *O. minus*. *Mycological Research* **107**: 1231-1242.

Jacobs K, Wingfield MJ, 2001. *Leptographium species: Tree pathogens, insect associates, and agents of blue-stain.* The American Phytopathological Soceity, St. Paul, Minnesota.

Jacobs K, Wingfield MJ, Wingfield BD, 2001. Phylogenetic relationships in *Leptographium* based on morphological and molecular characters. *Canadian Journal of Botany* **79**: 719-732.

Jacobs K, Seifert KA, Harrison KJ, Kirisits T, 2003. Identity and phylogenetic relationships of ophiostomatoid fungi associated with invasive and native *Tetropium* species (Coleoptera: Cerambycidae) in Atlantic Canada. *Canadian Journal of Botany* **81**: 316-329.

Juzwik J, French DW, 1983. *Ceratocystis fagacearum* and *C. piceae* on the surfaces of free-flying and fungus-mat-inhabiting nitidulids. *Phytopathology* **73**: 1164-1168.

Juzwik J, Cease KR, Meyer JM, 1998. Acquisition of *Ophiostoma quercus* and *Ceratocystis fagacearum* by Nitidulids from *O. quercus* - Colonized Oak Wilt Mats. *Plant Disease* **82**: 239-243.

Kamgan Nkuekam G, Jacobs K, De Beer ZW, Wingfield MJ, Roux J, 2008a. *Ceratocystis* and *Ophistoma* species, including three new taxa, associated with wounds on native South African trees. *Fungal Diversity* **29**: 37-59.

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

Kim SH, Breuil C, 2001. Common nuclear ribosomal internal transcribed spacer sequences occur in the sibling species *Ophiostoma piceae* and *O. quercus*. *Mycological Research* **105**: 331-337.

Kim SH, Uzunovic A, Breuil C, 1999. Rapid Detection of *Ophiostoma piceae* and *O. quercus* in Stained Wood by PCR. *Applied and Environmental Microbiology* **65**: 287-290.

Kim J, Lim YW, Wingfield MJ, Breuil C, G.H. K, 2004. *Leptographium bistatum* sp. nov., a new species with a *Sporothrix* synanamorph from *Pinus radiata* in Korea. *Mycological Research* **108**: 699-706.

Klepzig KD, Flores-Otero J, Hofstetter RW, Ayres MP, 2004. Effects of available water on growth and competition of southern pine beetle associated fungi. *Mycological Research* **108**: 183-188.

Klepzig KD, Moser JC, Lombardero MJ, Ayres MP, 2001. Symbiosis and competition: Complex interactions among beetles, fungi and mites. *Symbiosis* **30**: 83-96.

Kirisits T, 2004. Fungal associates of European bark beetles with special emphasis on the Ophiostomatoid fungi. In: Lieutier F, Day KR, Battisti A, Grégoire J-C, Evans H (eds), Bark and Wood Boring Insects in Living Trees in Europe, a Synthesis. Kluwer Academic Publishers, Dordrecht, pp 181-235.

Kohn LM, 2005. Mechanisms of Fungal Speciation. *Annual Review of Phytopathology* **43**: 279-308.

Konrad H, Kirisits T, Riegler M, Halmschlager E, Stauffer C, 2002. Genetic evidence for natural hybridization between the Dutch elm disease pathogens *Ophiostoma novo-ulmi* ssp. *novo-ulmi* and *O. novo-ulmi* ssp. *americana*. *Plant pathology* **51**: 78-84.

Kupfer DM, Reece CA, Clifton SW, Roe BA, Prade RA, 1997. Multicellular ascomycetous fungal genomes contain more than 8000 genes. *Fungal Genetics and Biology* **21**: 364-372.

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

Linnakoski R, De Beer ZW, Rousi M, Niemela P, Pappinen A, Wingfield MJ, 2008. Fungi including *Ophiostoma karelicum* sp. nov., associated with *Scolytus ratzeburgi* infesting birch in Finland and Russia. *Mycological Research* **112**: 1475-1488.

Loos W, 1932. Über eine buchenholzbewohnende *Ceratostomella*, *Ceratostomella fagi* nov. sp. *Archiv für Microbiologie* **3**: 370-383.

Malloch D, Blackwell M, 1993. Dispersal biology of the ophiostomatoid fungi. In *Ceratocystis and Ophiostoma*: *Taxonomy, Ecology and Pathogenicity*. The American Phytopathological Society, St. Paul, Minnesota.

Masuya H, Kubono T, Ichihara Y, 2003. *Ophiostoma ssiori* sp. nov. (Ophiostomatales, Ascomycetes) isolated from a bark beetle in *Prunus* species. *Bulletin of the National Science Museum,, Tokyo* **29**: 35-43.

Minkevich II, 1962. A vascular disease of Oak. *Review of Applied Mycology* **42**: 416 (Abstract).

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

Münch E, 1907. Die Blaufäle des Nadelholzes. I. *Naturwissenschaftliche Zeitschrift für Forst- und Landwirtschaft* **5**: 531-573.

Okada G, Jacobs K, Kirisits T, G.W. L-S, Seifert KA, Sugita T, Takematsu A, Wingfield MJ, 2000. Epitypification of *Graphium penicillioides* Corda, with comments on the phylogeny and taxonomy of graphium-like synnematous fungi. *Studies in Mycology* **45**: 169-188.

Oleksyn J, Przybyl K, 1987. Oak decline in the Soviet Union - Scale and hypotheses. *European Journal of Forest Pathology* **17**: 321-336.

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

Pipe ND, Buck KW, Brasier CM, 1995a. Genomic fingerprinting supports the separation of *Ophiostoma piceae* into two species. *Mycological Research* **99**: 1182-1186.

Pipe ND, Buck KW, Brasier CM, 1995b. Molecular relationships between *Ophiostoma ulmi* and the NAN and EAN races of *O. novo-ulmi* determined by RAPD markers. *Mycological Research* **99**: 653-658.

Przybyl K, Morelet M, 1993. Morphological differences between *Ophiostoma piceae* and *O. querci*, and among *O. querci* isolates. *Cryptogamie, Mycologie* **14**: 219-228.

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

Przybyl K, De Hoog GS, 1989. On the variability of *Ophiostoma piceae*. *Antonie van Leeuwenhoek* **55**: 177-188.

Roets F, De Beer ZW, Dreyer LL, Zipfel RD, Crous PW, Wingfield MJ, 2006. Multigene phylogeny for *Ophiostoma* spp. reveals two new species from *Protea* infructescences. *Studies in Mycology* **55:** 199-212.

Roets F, De Beer ZW, Wingfield MJ, Crous PW, Dreyer LL, 2008. *Ophiostoma gemellus* and *Sporothrix variecibatus* from mites infesting *Protea* infructescences in South Africa. *Mycologia* **100**: 496-510.

Romón P, Zhou X, Iturrondobeitia JC, Wingfield MJ, Goldarazena A, 2007. *Ophiostoma* species (Ascomycetes: Ophiostomatales) associated with bark beetles (Coleoptera: Scolytinae) colonizing *Pinus radiata* in northern Spain. *Canadian Journal of Microbiology* **53**: 756-767

Schroeder S, Kim SH, Cheung WT, Sterflinger K, Breuil C, 2001. Phylogenetic relationship of *Ophiostoma piliferum* to other sapstain fungi based on the nuclear rRNA gene. *FEMS Microbiology Letters* **195**: 163-167.

Sczerbin-Parfenenko AL, 1953. Ra kovye a sasudistye bolezni listvennych porod. *Goslesbumizdat, Moskva-Leningrad* **49**: 3-89.

Seifert KA, 1993. Sapstain of commercial lumber by species of *Ophiostoma* and *Ceratocystis*, in: Wingfield MJ, Seifert KA, Webber J (Eds), *Ceratocystis and Ophiostoma: Taxonomy, Ecology and Pathogenicity*. The American Phytopathological Society, St Paul, Minnesota, pp. 141-151.

Simonin G, Cochard H, Delatour C, Granier A, Dreyer E, 1994. Vulnerability of young oak seedlings (*Quercus robur L*) to embolism: responses to drought and to an inoculation with *Ophiostoma querci* (Georgevitch) Nannf. *Annales des Sciences Forestieres* **51**: 493-504.

Spatafora JW, Blackwell M, 1994. The polyphyletic origins of ophiostomatoid fungi. *Mycological Research* **98**: 1–9.

Stipes RJ, Campana RJ, 1981. *Compendium of elm diseases* American Phytopathological Society, St. Paul, Minnesota.

Taylor JW, Jacobson DJ, Kroken S, Kasuga T, Geiser DM, Hibbett DS, Fisher MC, 2000. Phylogenetic species recognition and species concepts in fungi. *Fungal Genetics and Biology* **31**: 21-32.

Temple B, Horgen PA, 2000. Biological roles for cerato ulmin a hydrophobin secreted by the elm pathogens, *Ophiostoma ulmi* and *O. novo-ulmi*. *Mycologia* **92:** 1-9.

Thwaites JM, Farrell RL, Duncan SM, Reay SD, Blanchette RA, Hadar E, Hadar Y, Harrington TC, McNew D, 2005. Survey of potential sapstain fungi on *Pinus radiata* in New Zealand. *New Zealand Journal of Botany* **43:** 653-663.

Upadhyay HP, 1981. *A monograph of Ceratocystis and Ceratocystiopsis*. University of Georgia Press, Athens, Georgia, USA.

Uroševic B, 1987. Tracheomycotic Diseases in Oak. *Communicationes Instituti Forestalis Èechosloveniae* **13**: 85-100.

Uzunovic A, Seifert KA, Kim SH, Breuil C, 2000. *Ophiostoma setosum*, a common sapwood staining fungus from Western North America, a new species of the *Ophiostoma piceae* complex. *Mycological Research* **104**: 486-494.

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

Wingfield MJ, Kendrick B, Van Wyk PS, 1991. Analysis of conidium ontogeny in anamorphs of *Ophiostoma: Pesotum* and *Phialographium* are synonyms of *Graphium*. *Mycological Research* **95**: 1328-1333.

Wingfield MJ, Seifert KA, Webber J, 1993. *Ceratocystis and Ophiostoma: Taxonomy, Ecology and Pathology* APS Press, St. Paul, Minnesota.

Yamaoka Y, Masuya H, Ohtaka N, Kaneko S, Abe JP, 2004. Three new *Ophiostoma* species with *Pesotum* anamorphs associated with bark beetles infesting *Abies* species in Nikko, Japan. *Mycoscience* **45**: 277-286.

Zhang N, Castlebury LA, Miller AN, Huhndorf SM, Schoch CL, Seifert KA, Rossman AY, Rogers JD, Kohlmeyer J, Volkmann-Kohlmeyer B, Sung G, 2006. An overview of the systematics of the Sordariomycetes based on a four-gene phylogeny. *Mycologia* **98**: 1076-1087.

Zhou XD, De Beer ZW, Ahumada R, Wingfield BD, Wingfield MJ, 2004. *Ophiostoma* and *Ceratocystiopsis* spp. associated with two pine-infesting bark beetles in Chile. *Fungal Diversity* **15**: 261-274.

Zhou XD, De Beer ZW, Wingfield MJ, 2006. DNA sequence comparisons of *Ophiostoma* spp., including *Ophiostoma aurorae* sp. nov., associated with pine bark beetles in South Africa. *Studies in Mycology* **55:** 269-277.

Zink P, Fengel D, 1989. Studies on the colouring matter of blue-stain fungi. Part 2 Electron microscopic observations of the hyphae walls. *Holzforschung* **43**: 371-374.

Zink P, Fengel D, 1990. Studies on the colouring matter of blue-stain fungi. Part 3. Spectroscopic studies on fungal and synthetic melanins. *Holzforschung* **44**: 163-168.

Zipfel RD, De Beer ZW, Jacobs K, Wingfield BD, Wingfield MJ, 2006. Multigene phylogenies define *Ceratocystiopsis* and *Grosmannia* distinct from *Ophiostoma*. *Studies in Mycology* **55**: 75-97.

CHAPTER 2

DELIMITATION OF *OPHIOSTOMA QUERCUS* AND ITS SYNONYMS USING MULTIPLE GENE PHYLOGENIES

Abstract

Ophiostoma quercus* is a morphologically variable species that is isolated from mostly hardwood hosts worldwide. Several species have been suggested as synonyms of *O. quercus* in the past, including *O. fagi, O. roboris, O. valachicum, O. kubanicum*, and *O. catonianum*. A recent collection of isolates resembling *O. quercus* from Azerbaijan provided the opportunity to reconsider the accuracy of these synonymies based on morphology and DNA sequence data. Four gene regions, the ribosomal internal transcribed spacer regions including the 5.8s gene, part of the β–tubulin gene, translation elongation factor-1α, and histone gene, were used to determine the phylogenetic relationships between the various species and isolates of different origin. In all four resulting phylogenetic trees, isolates of *O. quercus, O. fagi* and *O. roboris* formed a single, well-supported cluster, but with some internal variation. All the other species in the analyses, including *O. piceae* and *O. catonianum*, grouped distinctly with good node support. These results thus support the synonymy of *O. fagi* and *O. roboris* with *O. quercus*, and confirm that *O. piceae* and *O. catonianum* are distinct taxa. *Ophiostoma valachicum* and *O. kubanicum* could not be considered due to the absence of cultures, but based on published descriptions we argue that *O. valachicum* should be regarded as a valid species in need of neotypification. *Ophiostoma kubanicum* was never validly described and should be excluded from the list of synonyms of *O. quercus.

Taxonomic novelties *Pesotum roboris* (Georgescu, Teodoru & Badea) Grobbelaar, Z.W. de Beer, M.J. Wingf.; *Sporothrix roboris* (Georgescu, Teodoru & Badea) Grobbelaar, Z.W. de Beer, M.J. Wingf.

Key words: Ascomycetes, phylogeny

Introduction

The blue-staining fungi *Ophiostoma piceae* and *O. quercus* have had a confused taxonomic history. The former species was described in 1907 (Münch 1907) from sap-stained pine and spruce in Germany while *O. quercus* was described in 1926 from oak in the former Yugoslavia (Georgevitch 1926, 1927) and has been implicated as a causal agent of oak decline in Central Europe (Cech *et al*. 1990). Hunt (1956) treated these species as synonyms and this remained the case for more than 30 years. However, a suite of studies including Morelet (1992), Przybyl & Morelet (1993), Brasier & Stephens (1993), Brasier & Kirk (1993), Halmschlager *et al*. (1994), Pipe *et al*. (1995) and Harrington *et al*. (2001) have treated these fungi in depth and they are now widely accepted to represent two distinct taxa. Most recently, DNA sequence analyses (Harrington *et al*. 2001) have confirmed that *O. piceae* is a predominantly conifer infesting fungus, while *O. quercus* is mostly isolated from a variety of hardwood hosts.

A number of recent studies have shown that *O. quercus* is much more widely distributed on woody substrates than previously recognized, with reports of the fungus coming from countries such as Australia (Harrington *et al*. 2001), Brazil and Japan (De Beer *et al*. 2003a), Ecuador (Geldenhuis *et al*. 2004), Chile (Zhou *et al*. 2004), Canada, (Brasier & Kirk 1993, Pipe *et al*. 1995, Kim et al. 1999), Korea (Harrington *et al*. 2001), New Zealand (Thwaites *et al*. 2005), Uruguay (Harrington *et al*. 2001), the USA (Kim *et al*. 1999), South Africa (De Beer *et al*. 2003; Zhou *et al*. 2006, Kamgan Nkuekam *et al*. 2008a) and Uganda (Kamgan Nkuekam *et al*. 2008b). These reports of the fungus have been from a large variety of non-native and native hardwood trees, and in some cases non-native plantation pines (De Beer *et al*. 2003, Zhou *et al*. 2004, 2006, Thwaites *et al*. 2005). In terms of biology, the fungus appears to be a casual associate of many different bark beetles and other tree-wound visiting insect species (Kirisits 2004, Zhou *et al*. 2004, 2006, Romón *et al*. 2007, Kamgan Nkuekam *et al*. 2008a, b, Linnakoski *et al*. 2008).

During the period that *O. quercus* was treated as a synonym of *O. piceae*, several similar hardwood-infesting species were also listed as synonyms of *O. piceae*. These included *O.*

catonianum (de Hoog 1974), *O. roboris* (de Hoog 1979), *O. fagi* (Hutchinson & Reid 1988), *O. valachicum,* and *O. kubanicum* (Przybyl & De Hoog 1989). Considering the almost exclusive association of *O. piceae* with conifers, all of these species, other than *O. catonianum,* have been suggested to be synonyms of *O. quercus* (Brasier 1993, Brasier & Kirk 1989, 1993, Harrington *et al*. 2001). Harrington *et al*. (2001) included the ex-type isolate of *O. fagi* and an authentic isolate of *O. catonianum* (deposited at CBS by Goidánich) in their study. Based on nuclear ribosomal internal transcribed spacer (ITS) region sequences and mating compatibility, *O. fagi* was suggested as a synonym of *O. quercus,* but *O. catonianum* was shown to be a distinct taxon (Harrington *et al*. 2001). No cultures representing *O. valachicum*, *O. kubanicum* and *O. roboris* were available for Harrington's (2001) study. They thus listed these three species as questionable synonyms of *O. quercus*, and the status of these species remains unresolved today.

In view of the increasing number of reports of *O. quercus* from many countries of the world, it has become necessary to delimit the species boundaries for this fungus. A collection of isolates resembling *O. quercus* and *O. roboris* from oak and chestnut in Azerbaijan prompted reconsideration of the synonyms of *O. quercus,* including the possible need to neo-typify *O. roboris*. Several recent studies of the genus *Ophiostoma* have shown that ITS sequences do not always distinguish between closely related fungal species, but that protein coding genes produce better resolution at the species level (Kim & Breuil 2001; Schroeder *et al*. 2001; Lim *et al*. 2004; De Meyer *et al*. 2008; Roets *et al*. 2008). The aim of this study was thus to identify the isolates tentatively treated as *O. querus* from Azerbaijan, but also to reconsider the species boundaries for taxa in the *O. piceae* / *O. quercus* complex using micromorphology together with phylogenetic analyses of DNA sequences derived from four nuclear gene regions.

Materials and methods

Isolates and herbarium specimens

Forty isolates were used for DNA sequence comparisons in this study (Table 1). These included 11 isolates resembling *O. quercus* and *O. roboris* from Azerbaijan. Isolates previously identified as *O. quercus* were also used and these included five from France together with an ex neo-type culture of *O. quercus* (Morelet 1992), one from the UK (Brasier & Kirk 1993; Brasier & Stephens 1993; Pipe *et al*. 1995; Harrington *et al*. 2001) and three from South Africa (De Beer *et al*. 2003a). A single isolate from Azerbaijan, previously referred to as *O. roboris* (Guseinov 1984; Brasier & Kirk 1989), and subsequently treated as *O. quercus* (Webber & Brasier 1991; Brasier & Kirk 1993; Brasier & Stephens 1993; Pipe *et al*. 1995), was made available to us for inclusion in the study by Dr Clive Brasier (Forestry Commission Research Agency, Alice Holt Lodge, Farnham, Surrey, UK). The ex-type isolate of *O. fagi* and an authentic isolate of *O. catonianum* collected by Goidánich (1935) were obtained from the CBS. Our efforts to obtain type material for *O. roboris*, *O. kubanicum* and *O. valachicum* were unsuccessful. However, with the assistance of Dr Vadim Mel'nik (Komarov Botanical Institut, St. Petersburg, Russia) we were able to obtain three dried culture herbarium specimens (Table 1) of *O. roboris* isolates used in inoculation tests by Potlajczuk (1957). The herbarium specimens of *O. roboris* consisted of three dried cultures on agar discs, one of which was labelled as *O. roboris*. The remaining two isolates had been obtained from re-isolations from *Acer* and *Betula* inoculated with the first isolate. A culture labeled as *O. kubanicum* (VKM-F 3181) received from the All Russian Collection of Micro-organisms in Moscow, was found to be contaminated with a *Fusarium* species and could not be salvaged. All attempts to obtain material of *O. valachicum* were unsuccessful, consistent with the experience of Przybyl & De Hoog (1989).

For phylogenetic comparisons sequences of the above-mentioned isolates were compared with those of other species in the larger *O. piceae* complex (Harrington *et al*. 2001).

These included *O. piceae, O. himal-ulmi, O. ulmi, O. novo-ulmi, O. floccosum, O. setosum* and *Pesotum australi*.

All isolates used in this study are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa. In addition, representative isolates have been deposited at the CBS. Dried cultures of the isolates that produced perithecia (CMW 9256, CMW 9262) have been deposited in the herbarium of the National Collection of Fungi, Pretoria, South Africa (PREM 57852, PREM 57851).

Morphology

For morphological comparisons, isolates from Azerbaijan and those of *O. quercus*, *O. catonianum, O. fagi* and the '*O. roboris*' isolate of Guseinov (Table 1) were grown on MEA (20 g malt extract agar (Biolab, Midrand, South Africa), 1000 ml deionised water) and WA (20 g agar, 1000 ml deionised water) amended with debarked oak twigs (40 x 5 mm). Fifty measurements were made for each taxonomically informative structure of the isolates that produced perithecia. Anamorphs of all isolates were studied, with 25 measurements made for each characteristic structure per isolate. Three-day-old slide cultures (Riddel 1950) of the mononematous anamorph structures were made and fixed in lactophenol for microscopy.

Scanning electron microscopy (SEM) was used to observe the anamorphs of isolates that produced perithecia in culture. Small agar blocks were cut from the sporulating colonies with a sterilized scalpel and fixed overnight in 2.5 % glutaraldehyde in 0.1 M sodium phosphate buffer (pH = 7.4). After three buffer rinses the specimens were post-fixed in 1.0 % aqueous osmium tetraoxide for 30 min, dehydrated in a graded ethanol series starting at 30 % and dried with a Polaron Critical Point Drier. Specimens were coated with gold and examined using a JEOL JSM 6400 scanning electron microscope.

Growth studies

Growth of the same set of isolates used for morphological studies was compared on 2 % MEA. Discs, 5 mm in diameter, were taken from the actively growing edge of colonies and placed in the centres of 90 mm Petri dishes. Three replicates were made for each isolate and these were incubated at temperatures ranging from 5 °C to 35 °C, with 5 °C intervals, for 10 days in the dark. Isolates were also grown at 32 °C, the best temperature for *O. quercus*. Two measurements of colony diameter, perpendicular to each other, were taken each day from the second day of the trial. Six measurements were thus made for each isolate at each assessment. The entire experiment was repeated.

DNA extraction, PCR and sequencing

Single conidium and ascospore cultures were prepared for all isolates and DNA extractions were performed as described by Aghayeva *et al*. (2004) using PrepMan Ultra Sample Preparation reagent (Applied Biosystems, Foster City, CA, USA). The ITS 1 and 2 regions of the ribosomal DNA operon, including the 5.8S gene, were amplified using primers ITS1-F (Gardes & Bruns 1993) and ITS4 (White *et al*. 1990). Part of the β-tubulin (BT) gene was amplified using primers Bt2A and Bt2B (Glass & Donaldson 1995). Bt2B was replaced in some cases with primer T10 (O'Donnell & Cigelnik 1997) to obtain a longer fragment. Amplicons were also obtained from part of the translation Elongation Factor-1α (TEF-1α) gene, with primers EF1-728F and EF1-986R (Carbone & Kohn 1999) or primers EF1F and EF2R (Jacobs *et al*. 2004). Part of the histone gene (HIS) was amplified with primers H3-1A and H3-1B (Glass & Donaldson 1995).

The reaction mixture (50 µl final volume) contained five units of Expand *Taq* Polymerase (Roche Biochemicals, Mannheim, Germany), 1x PCR reaction buffer, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.1 mM of each primer, and 1 µl DNA template. PCR reactions were performed in a thermal cycler (Hoffman-La Roche, Nutley, NJ, USA). PCR conditions for the ITS1/ITS2 regions were the same as those used by Aghayeva *et al*. (2004). The β-tubulin gene was amplified using an initial denaturation at 94 °C for 1 min,

followed by 30 cycles denaturation at 94 °C for 1 min, primer annealing at 53–55 °C (depending on the isolate) for 1 min, and elongation at 72 °C for 1 min. A final extension step at 72 °C for 5 min completed the program. The EF-1α was amplified using denaturation at 95 °C for 3 min followed by 30 cycles of denaturation at 95 °C for 1 min, primer annealing at 54–61 °C (depending on the isolate) for 1 min 30 s and elongation at 72 °C for 2 min. A final extension step was performed for 10 min at 72 °C. The histone gene region amplified with a denaturation step at 95 °C for 1 min, followed by 40 cycles of denaturation at 95 °C for 1 min 30 sec, primer annealing at 60 °C for 30 s, and elongation at 72 °C for 1 min. This was followed by a final extension step for 8 min at 72 °C.

PCR products were separated on a 1 % (w/v) agarose gel stained with ethidium bromide and visualized under UV illumination. PCR fragments were purified using Sephadex G50 (Sigma-Aldrich, Chemie Gmbh, Steinheim, Germany). Both strands of the PCR products were sequenced using amplification primers and the ABI PRISM® BigDye™ Terminator v 3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). DNA sequencing reactions were analyzed on an ABI PRISM® 3100 Genetic Analyzer or an ABI PRISM™ 377 DNA sequencer.

Analyses of sequence data

Sequence data from the four genes were analysed and contigs assembled using the Vector NTI Advance 10 software (Invitrogen). The assembled forward and reverse contigs were aligned in MAFFT v 5.731, option E-INS (Katoh *et al*. 2005, Morrison 2006), then edited manually where necessary. Gaps were treated as 5th characters. Exon positions were identified using the available sequences from GenBank. GenBank accession numbers for all sequences generated in this study, as well as some ITS sequences from previous studies that were obtained from GenBank, are listed in Table 1. Relationships between the isolates using sequences of the four gene regions (ITS, β-tubulin, EF-1α and HIS) were determined by Maximum Likelihood (ML) and Bayesian Inference (BI). *Ophiostoma setosum* was used as the outgroup taxon in all cases as it is closely related to

O. quercus but clusters in the 'coniferous group' within the *O. piceae* complex, thus avoiding confusion that might have arisen by choosing a fungus that could possibly have been a synonym of *O. quercus*.

The most appropriate model(s) of sequence evolution for the relevant genes were chosen using the Akaike Information Criterion (AIC) as selected from a set of 56 hierarchically nested contenders in MODELTEST v 3.7 (Posada & Crandall 1998) for each of the four genes. The ML analysis was conducted using the best model results in PHYML (Guindon & Gascuel 2003). Nonparametric bootstrap analyses (Felsenstein 1985) employed one heuristic search for the 1000 bootstrap replicates under maximum likelihood to estimate the reliability of nodes.

The BI analysis was performed in MRBAYES v 3.1.2 (Ronquist & Heulsenbeck 2003) using the best fitting model selected by the AIC test in MRMODELTEST v 2.2 (Nylander 2004). Model parameters were derived from the default prior distributions. The MRBAYES analysis was comprised of four independent runs of 1 000 000 generations using duplicate Monte Carlo Markov chain searches with four chains (one cold, three hot to improve mixing) and a sampling frequency of every 100 generations. Stationarity for LnL and nucleotide substitution parameters was determined and consequently the relevant numbers of trees sampled for each gene region were discarded as burn-in before the consensus topology and posterior probabilities were calculated.

Results

Morphology

Isolates of *O. quercus*, *O. fagi*, *O. catonianum* and the '*O. roboris'* isolate (CMW 12618), varied in culture morphology and colour. The colony morphology and colour of ten-day-old cultures of all isolates was similar, but differences became more pronounced after synnemata or perithecia had formed. The formation of rings or sectors of growth in culture was not consistent in different cultures of the same isolate and this feature was not considered taxonomically informative.

Only two isolates (CMW 9256, CMW 9262) formed perithecia on their own in culture (Figs 1 and 2). Morphological features of these isolates are summarized in Table 2. The most obvious differences in the sexual state of the two isolates were the neck length and ascospore shape (Figs 1 and 2).

Most isolates, including the Guseinov '*O. roboris*' isolate (CMW 12618), produced both *Pesotum* and *Sporothrix* anamorphs in culture. The herbarium specimens from Russia, labeled as *O. roboris*, contained *Pesotum* and *Sporothrix* structures similar to those of the *O. quercus* isolates, and perithecia were absent. Isolate CMW 9262 from Azerbaijan produced, in addition to the typical *Pesotum* and *Sporothrix* anamorphs, a third anamorph structure (Fig. 2g). This structure that is best described as *Hyalorhinocladiella*-like was less common than the other two types in both the original and single conidium cultures, and was not observed in any of the other isolates.

The ex-type isolate of *O. fagi* obtained from CBS formed *Pesotum* and *Sporothrix* states on MEA cultures stored at 4 °C. Although most of the synnemata were fused, mature, compacted synnemata similar to those of *O. quercus*, were also observed. The ex-type culture of *O. catonianum* (CMW 11535) formed only a *Sporothrix* anamorph in culture, with no synnematous structures present.

Growth studies

All isolates started to grow on the second day at 15 °C and above. At 10 °C, growth started on the third day and at 5 °C growth was only observed on the sixth day. For most of the isolates, optimum growth was observed at 25 °C (Table 1). Growth of the *O. quercus* isolates at 25 °C after 10 days varied between 41mm and 57mm. In the same period, the *O. fagi* isolate grew to 58.6mm, the '*O. roboris'* isolate (CMW 12618) to 42.1mm and CMW 9262 (with the *Hyalorhinocladiella*-like anamorph) to 52.5mm. The ex-type isolate of *O. catonianum* grew slower reaching an average colony diameter of 26.2mm in ten days at 25 °C.

Analyses of sequence data

All four data sets consisted of sequences obtained from 40 isolates grouping within the *O. piceae* complex. Aligned DNA sequence from ITS gene region yielded 592 characters, including gaps, while the BT gene region alignment had 331 characters, the TEF-1α gene region 495 characters, and the HIS region 294 nucleotides and no gaps. The phylogenetic information content varied across the four genes with most of the variability seen within the introns of the BT, HIS and ITS gene regions. However, the TEF-1α gene displayed a large amount of variation throughout the sequence and included numerous indels.

Results of the maximum likelihood and Bayesian analyses yielded trees with concordant topologies and similar levels of node support for all four genes. Stationarity was achieved after 100 000 generations across all four genes. We preferred not to combine the data for the four gene regions as each phylogeny represents a unique evolutionary history. Results are, therefore, presented independently of each other (Figs 3a-d).

In all four gene regions, *O. floccosum*, *O. setosum*, *O. piceae*, *O. novo-ulmi*, *O. himal-ulmi, Pesotum australi* and *O. catonianum* were separated from each other in distinct

clusters (Figs 3a-d). The rest of the isolates grouped in a single, well-supported lineage that included the ex-neotype of *O. quercus* (CMW 2467) from France, an isolate from the UK (CMW 7650), some South African isolates, the ex-type isolate of *O. fagi* (CMW 11532 from Germany) and an isolate from Azerbaijan previously treated as *O. roboris* (CMW 12618). This lineage also included all the other isolates from Azerbaijan, including the isolate (CMW 9262) producing the mononematous, *Hyalorhinocladiella*-like anamorph.

Analyses of sequences for the BT gene and TEF-1α showed the most strongly supported terminal nodes of all four genes studied, with posterior probabilities close to 1.00 at almost all of the internal nodes. The TEF-1α gene showed the most resolution and best defines the relationships between the forty sequences analysed. It is interesting to note that all the hardwood inhabiting species are more closely related to each other than to species found predominantly on other tree species (indicated as HW in Figs 3a-d).

Discussion

The four gene genealogies presented in this study show that *O. quercus* isolates represent a well supported monophyletic group (group Q, Figs 3a-d). In these analyses, *O. quercus* was typified by the ex-neotype isolate of the species (CMW 2467) from France (Morelet 1992, Przybyl & Morelet 1993). Group Q included other authentic *O. quercus* isolates used in previous studies, from France (Morelet, 1992), the UK (Brasier & Kirk, 1993; Brasier & Stephens, 1993; Pipe *et a*l. 1995, Harrington *et al*. 2001) and South Africa (de Beer *et al*. 2003a), as well as isolates obtained in this study from various hardwood hosts in Azerbaijan. Although several sub-groups were present within group Q in analyses for all four gene regions, these were not consistent between the various gene regions and did not have statistical support. However, these sub-groups are indicative of substantial genetic variation within the species.

The culture morphology and growth in culture of the *O. quercus* isolates, including those from Azerbaijan, showed considerable variability, similar to that which has been reported

in previous studies (Morelet, 1992; Brasier & Stephens, 1993; Halmschlager *et al*. 1994). All these isolates produced typical *Pesotum* and *Sporothrix* anamorphs in culture. Two of the isolates from Azerbaijan also formed teleomorphs in culture (Figs 1-2). Although the averages calculated for the perithecial and synnematal dimensions of these two isolates (CMW 9256 and CMW 9262) differed from each other, ranges of both isolates reside within the published ranges for *O. quercus* (Table 2). The one Azerbaijan isolate (CMW 9262) differed from the other isolates and produced unusual anamorph structures that can best be described as *Hyalorhinocladiella*-like.

Ophiostoma fagi was described from *Fagus sylvatica* in Germany by Loos (1932). During the period that *O. quercus* was treated a synonym of *O. piceae*, Upadhyay (1981), Hutchinson & Reid (1988), and Przybyl & De Hoog (1989) listed *O. fagi* as a synonym of *O. piceae*. Harrington *et al*. (2001) suggested that *O. fagi* should be treated as a synonym of *O. quercus* based on ITS sequence data and sexual compatibility using the ex-type isolate of *O. fagi*. We included the same isolate (CMW 11532) in the present study. In phylograms obtained from all four gene regions, this species clustered within in the *O. quercus* group (Q). We thus support the synonymy of *O. fagi* with *O. quercus* as proposed by Harrington *et al*. (2001). This is in contrast to the report of Melin and Nannfeldt (1934) who stated that the synnematal anamorph of this isolate had been lost and placed *O. fagi* in the '*pilifera* group' of *Ophiostoma* that contained species with only mononematous anamorphs.

Ophiostoma roboris was originally described from Romania (Georgescu *et al*. 1948), and together with *O. quercus*, was considered a synonym of *O. piceae* by De Hoog (1979) and Kowalski and Butin (1989). The synonymy was questioned by Przybyl and De Hoog (1989) based on a lack of authentic material and differences in anamorph morphology. Guseinov (1984) described an isolate from oak in Azerbaijan as *O. roboris* based on morphological similarities with the descriptions by Georgescu *et al*. (1948). In both publications (Georgescu *et al*. 1948, Guseinov 1984), a synnematal ('*Graphium'*) anamorph was described that is currently recognized as the *Pesotum* anamorph. The typical *Sporothrix* synanamorph that is usually associated with species in the *O. piceae*

complex, was often described as 'of the *Cladosporium*- or *Cephalosporium*-type' in older publications (e.g. Loos 1932). Georgescu *et al*. (1948), Guseinov (1984) and Georgiev (1986), however, described the synanamorph of *O. roboris* as a *Hyalodendron roboris*. They distinguished it from the '*Cladosporium'*- or '*Cephalosporium*-type' anamorphs of related species, because it formed a mononematous conidiophore that lacked the pronounced denticles characteristic of the form genus *Sporothrix*. Considering the illustrations of Georgescu *et al*. (1948, Figs 32-33), this mononematous anamorph broadly resembles the *Hyalorhinocladiella*–like anamorph observed in one of the isolates in the present study. Kowalski and Butin (1989) presented a similar illustration for the anamorph of *O. quercus* (as '*Ceratocystis piceae'*), and also described this as an *Hyalodendron*-type. However, the genus *Hyalodendron* is restricted to anamorphs of Basidiomycetes (de Hoog 1979) and is thus not available for *Ophiostoma* anamorphs.

The *'O. roboris'* isolate described by Guseinov (1984), was successfully crossed with authentic *O. quercus* isolates by Brasier and Kirk (1989, 1993), who then suggested *O. roboris* might be a synonym of *O. quercus*. The same isolate (CMW 12618) was included in the present study and could not be distinguished from *O. quercus* isolates based on morphology or sequence data comparisons (Figs 3a-d). Our results, therefore, confirm those of previous studies (Brasier & Kirk, 1989, 1993; Webber & Brasier, 1991; Brasier & Stephens, 1993; Pipe *et al*. 1995; Lin, 2003) suggesting that this isolate should be treated as *O. quercus*. However, we did not observe the characteristic *Hyalorhinocladiella*-like ('*Hyalodendron'*) anamorph described by Guseinov (1984) in this particular isolate (CMW 12618). One of our other isolates (CMW 9262) from Azerbaijan formed an anamorph that corresponded with the line drawings of the '*Hyalodendron roboris'* anamorph in the original description of *O. roboris* (Georgescu *et al*. 1948, pp. 208–209). Based on the morphological similarities, this isolate could have been considered to represent a neotype for *O. roboris*, but it also grouped within the *O. quercus* group (Q) in all four phylogenies. We thus conclude that *O. roboris* should be treated a synonym of *O. quercus*.

The presence of the *Hyalorhinocladiella*-like ('*Hyalodendron'*) anamorph in a species like *O. quercus* is not surprising. In the original definition for the anamorph genus

Pesotum, Crane and Schocknecht (1973) included a continuum of structures between the classical synnematous structure at the one extreme, and a typical mononematous, denticulate *Sporothrix* conidiophore, at the other extreme. Several intermediate structures and sizes had been recognized, and contributed to the confusing use of generic names such as *Cladosporium*, *Cephalosporium*, *Hyalodendron,* and *Rhinotrichum* to describe *Ophiostoma* anamorphs in many previous publications (Przybyl & De Hoog 1989; Benade *et al*. 1997, 1998). Following Harrington *et al*. (2001), we accept the broad definition of *Pesotum* by Crane and Schoknecht (1973), which includes the *Hyalorhinocladiella*-like structures described for a species like *O. roboris*.

Treating *O. roboris* as a synonym of *O. quercus* creates a technical taxonomic problem. The *Pesotum* and *Sporothrix* synanamorphs of *O. quercus* had never been supplied with binary names, while those of *O. roboris* were described as *Graphium roboris* and *Hyalodendron roboris,* respectively. However, neither of the latter genera are available for anamorphs of *Ophiostoma* (de Hoog 1979; Okada *et al*.1998). Furthermore, Article 59 of the ICBN (McNeill *et al*. 2006) allows the epithets of these two form species to be available for the anamorphs of *O. quercus*. The practice of assigning binary names to anamorphs, especially when the teleomorph is known and described, is becoming outdated. However, to avoid the use of an inappropriate anamorph genus name in future nomenclators listing *Ophiostoma* species, new combinations have been provided for the two *O. roboris* synanamorphs.

Ophiostoma catonianum has been listed as a synonym of *O. piceae* by several authors (de Hoog 1974, Upadhyay 1981, Hutchison & Reid 1988, Przybyl & De Hoog 1989). However, Morelet (1992) and Okada *et al*. (1998) treated *O. catonianum* as synonym of *O. quercus* because the species was described from *Pyrus,* a hardwood host (Goidánich 1935). The authentic isolate (CMW 11535) from the original study included in the present study consistently grouped in a lineage distinct from *O. quercus* in all four gene regions analysed, and it clearly represents a distinct species. Goidánich (1935) described the synanamorphs of *O. catonianum* as *Hyalodendron pirinum* and *Graphium pirinum*. Morelet (1992) and Okada *et al*. (1998) made the necessary new combinations, namely

Sporothrix pirinum and *Pesotum pirinum* respectively, but treated these as the anamorphs of *O. quercus*. Harrington *et al*. (2001), however, distinguished between *O. quercus* and *O. catonianum* based on ITS sequence data, and correctly listed *S. pirinum* and *P. pirinum* as the anamorphs of *O. catonianum*. According to the original description, the species has shorter perithecial necks and longer ositiolar hyphae than *O. quercus*, and also had a homothallic mating system rather than the heterothallic system of other species in the *O. piceae* complex (Goidánich 1935; Harrington *et al*. 2001).

Ophiostoma valachicum was treated as a *nomen dubium* by Upadhyay (1981), but Przybyl and De Hoog (1989) and Harrington *et al*. (2001) considered it as a possible synonym of *O. piceae* and *O. quercus* respectively. No material exists for this species, but it was originally isolated from oak in Romania and validly published in the same paper as *O. roboris* (Georgescu *et al*. 1948). The original morphological description mentions only *Rhinotrichum valachicum* as anamorph (Georgescu *et al*. 1948), but no synnematous anamorph that would be expected if it were closely related to *O. quercus*. Although Sczerbin-Parfenenko (1953) confirmed that no other conidial stages are known for *O. valachicum*, Potlajczuk and Schekunova (1985) and Georgiev (1986), mentioned a *Graphium* anamorph in addition to the *Rhinotrichum* anamorph for isolates of Russian and Bulgarian origin respectively. The genus *Rhinotrichum* was not validly published (Hawksworth *et al*. 1983), and is thus not available for use. Furthermore, Przybyl and De Hoog (1989) suggested the *Rhinotrichum*-type anamorphs of *Ophiostoma* spp. are simply one of three manifestations of the genus *Sporothrix,* a conclusion with which we concur. The absence of a synnematous anamorph from the original description, white colonies, crescent shaped ascospores, and curved conidia (Georgescu *et al*. 1948, this study), distinguishes *O. valachicum* from species in the *O. piceae* complex. We thus recognize *O. valachicum* as a distinct and valid species. Neotypification of this species would make it possible to determine its phylogenetic placement which would most likely be in the *O. stenoceras – S. schenckii* complex as defined by De Beer *et al.* (2003b).

Ophiostoma kubanicum was described from oak in the former Soviet Union (Sczerbin-Parfenenko 1953), but was invalidly published as no Latin description was given (Article

36.1, McNeill *et al*. 2006). Furthermore, no authentic material of this species is available for study, making validation impossible. For this reason, we suggest that it should be excluded from future treatments of the genus *Ophiostoma*.

The delimitation of *O. quercus* and clarification of its synonyms in the present study, have paved the way for future studies that will focus on population level questions pertaining to this species. *Ophiostoma quercus* isolates consistently display considerable phenotypic variation as well as variation in DNA sequences. The capacity to colonize wounds on a wide range of hosts, its association with many different insect vectors, and its world-wide distribution raises intriguing questions regarding its origin and ecology. A clear definition of the species boundaries for this fungus as presented in this study will make it possible to resolve some of those questions.

Taxonomy

Phylogenetic analyses of sequences for four gene regions were used to delimit *Ophiostoma quercus* sensu stricto and to clarify the status of those species that have in the past been listed as synonyms of this species. These data, together with morphological characteristics extracted from descriptions of *O. quercus* and its synonyms led to the conclusion that *O. fagi* and *O. roboris* are valid synonyms of *O. quercus*. In contrast, *O. catonianum* and O. *valachicum* should be considered as distinct taxa. We further argue that *O. kubanicum* was not validly published and that it should not be treated as a synonym of *O. quercus*.

Ophiostoma quercus **(Georgev.) Nannf.**, in Melin & Nannf., Svenska Skogsvårsför. Tidskr. 32: 408. 1934.

Basionym: *Ceratostomella quercus* Georgev., Compt. Rend. Hebd. Séances Acad. Sci. 183: 759. 1926. [as '*Querci*'] (*non Ceratostomella quercus* A.C. Santos & Sousa da Câmara, Agronomia Lusitania 17: 136. 1955., *nom. inval*.)

≡ *Ceratocystis querci* (Georgev.) C. Moreau, Rev. Mycol. (Paris) Suppl. Col. 17: 22. 1952.

= *Ceratostomella fagi* W. Loos, Arch. Mikrobiol. 3: 376. 1932.

≡ *Ophiostoma fagi* (W. Loos) Nannf., in Melin & Nannf., Svenska Skogsfor. Tidskr. 32: 408. 1934.

≡ *Ceratocystis fagi* (W. Loos) C. Moreau, Rev. Myc. (Paris) Suppl. Col. 17: 22. 1952.

= *Ophiostoma roboris* Georgescu & Teodoru, in Georgescu, Teodoru & Badea, Anal. Inst. Cerc. Exp. For. Rom., Ser 1. 11: 207. 1948.

≡ *Ceratocystis roboris* (Georgescu & Teodoru) Potl., in Potlajczuk & Schekunova, Nov. Sist. Niz. Rast. 22: 154. 1985.

Anamorph: ***Pesotum roboris* (Georgescu, Teodoru & Badea) Grobbelaar, Z.W. de Beer, M.J. Wingf. comb. nov.**

≡ *Graphium roboris* Georgescu, Teodoru & Badea, Anal. Inst. Cerc. Exp. For. Rom., Ser 1. 11: 212. 1948.

Synanamorph: ***Sporothrix roboris* (Georgescu, Teodoru & Badea) Grobbelaar, Z.W. de Beer, M.J. Wingf. comb. nov.**

≡ *Hyalodendron roboris* Georgescu & Teodoru, in Georgescu, Teodoru & Badea, Anal. Inst. Cerc. Exp. For. Rom., Ser 1. 11: 209. 1948.

***Ophiostoma catonianum* (Goid.) Goid.**, Boll Staz. Patol. Veg. Roma, n.s. 15: 125. 1935.

Basionym: *Ceratostomella catonianum* Goid., R.C. Accad. Lincei 21: 199. 1935. [as '*catoniana'*]

≡ *Ceratocystis catoniana* (Goid.) C. Moreau, Rev. Myc. (Paris) Suppl. Co. 17: 22. 1952.

Anamorph: *Pesotum pirinum* (Goid.) G. Okada & Seifert, in Okada et al. Can. J. Bot. 76: 1504. 1998.

≡ *Graphium pirinum* Goid., Boll. Staz. Patol. Veg. Roma, n.s. 15: 132. 1935.

Synanamorph: *Sporothrix pirina* (Goid.) Morelet, Extrait Annales S.S.N.A.T.V. 44: 110. 1992. [as '*pirinum'*]

≡ *Hyalodendron pirinum* Goid., Boll. Staz. Patol. Veg. Roma, n.s. 15: 136. 1935.

***Ophiostoma valachicum* Georgescu, Teodoru & Badea**, Anal. Inst. Cerc. Exp. For. Rom., Ser 1. 11: 198. 1948.

≡ *Ceratocystis valachicum* (Georgescu, Teodoru & Badea) Potl., in Potlajczuk & Schekunova, Nov. Sist. Niz. Rast. 22: 155. 1985.

Anamorph: *Sporothrix* (Przybyl & De Hoog 1989).

≡ *Rhinotrichum valachicum* Georgescu, Teodoru & Badea, Anal. Inst Cerc. Exp. For., Ser. 1, 11: 201. 1948

Nomen invalidum

***Ophiostoma kubanicum* Sczerbin-Parfenenko**, Rak. Sos. Bol. List. Porod (Moscow) p. 49. 1953.

= *Ceratocystis kubanica* (Sczerbin-Parfenenko) Potlajczuk, Nov. Sist. Niz. Rast. 22: 153. 1985.

Anamorph: *Graphium kubanicum* Sczerbin-Parfenenko, Rak. Sos. Bol. List. Porod (Moscow) p. 51. 1953.

Synanamorph: *Sporothrix* (Przybyl & De Hoog 1989).

≡ *Verticillium kubanicum* Sczerbin-Parfenenko, Rak. Sos. Bol. List. Porod (Moscow) p. 51. 1953.

Acknowledgements

We are deeply grateful to our colleagues and curators of herbaria for supplying cultures and specimens without which this study could not have been undertaken. We also thank Dr Danut Chira of the Forest Research and Management Institute (ICAS), Romania, for providing us with literature and the English translation of the species diagnoses of *O. roboris* from Romania, and Mr Chris van der Merwe of the Laboratory for Microscopy and Microanalysis, School of Physical Sciences, University of Pretoria, who assisted us with scanning electron microscopy. We are indebted to Dr Thomas Kirisits for advice on an earlier draft of the manuscript. We thank Dr Ursula Heiniger, Swiss Federal Institute and the SCOPES Programme 2000-2003 (Scientific Co-operation Programme between Eastern Europe and Switzerland, Swiss National Research Foundation, Grant: 7CO 064552) for support. We also acknowledge members of the Tree Protection Cooperative

Programme (TPCP), Centre for Tree Health Biotechnology (CTHB), the National Research Foundation (NRF) and the THRIP initiative of the Department of Trade and Industry, South Africa for financial assistance.

References

Aghayeva DN, Wingfield MJ, De Beer ZW, Kirisits T, 2004. Two new *Ophiostoma* species with *Sporothrix* anamorphs from Austria and Azerbaijan. *Mycologia* **96**: 866-878.

Benade E, Wingfield MJ, van Wyk PS, 1997. Conidium development in *Sporothrix* anamorphs of *Ophiostoma*. *Mycological Research* **101**: 1108-1112.

Benade E, Wingfield MJ, Van Wyk PS, 1998. Conidium development in *Hyalodendron* and *Allescheriella* anamorphs of *Ophiostoma* and *Ceratocystiopsis*. *Mycotaxon* **68**: 251-263.

Brasier CM, 1993. The genetic system as a taxonomic tool: gene flow, molecular variation and sibling species in the *Ophiostoma piceae* - *Ophiostoma ulmi* complex and its taxonomic and ecological significance. In: Wingfield MJ, Seifert KA, Webber J (eds) *Ceratocystis* and *Ophiostoma*: Taxonomy, Ecology and Pathogenicity. American Phytopathological Society, St. Paul Minnesota, pp77-92.

Brasier CM, Kirk S, 1989. European oak decline, identity of *Ophiostoma roboris*. In: Report on Forest Research: HMSO, London, pp47-48.

Brasier CM, Kirk S, 1993. Sibling species within *Ophiostoma piceae*. *Mycological Research* **97**: 811-816.

Brasier C, Stephens TM, 1993. Temperature-growth responses distinguish the OPC and OPH sibling species within *Ophiostoma piceae*. *Mycological Research* **97**: 1416-1418.

Carbone I, Kohn ML, 1999. A method for designing primer sets for speciation studies in filamentous ascomycetes. *Mycologia* **91**: 553-556.

Cech T, Donaubauer E, Tomiczek C, Leontovyc R, Yde-Anderson A, Delatour C, Wulf A, Toth J, Vajna L, Vammini A, Luisi N, Oosterbaan A, Kowalski T, Gibbs JN, Jurc D, 1990. Oak Decline and the Status of *Ophiostoma* spp. on oak in Europe. EPPO Bulletin 20: 405-422.

Crane JL, Schoknecht JD, 1973. Conidiogenesis in *Ceratocystis ulmi, Ceratocystis piceae* and *Graphium penicillioides*. *American Journal of Botany* **60**: 346-354.

Crous PW, Braun U, Schubert K, Groenewald JZ, 2007. Delimiting *Cladosporium* from morphologically similar genera. *Studies in Mycology* **58**: 33-56.

De Beer, ZW, Wingfield BD, Wingfield MJ, 2003a. The *Ophiostoma piceae* complex in the Southern Hemisphere: a phylogenetic study. *Mycological Research* **107**: 469-476.

De Beer ZW, Harrington TC, Vismer HF, Wingfield BD, Wingfield MJ, 2003b. Phylogeny of the *Ophiostoma stenoceras- Sporothrix schenkii* complex. *Mycologia* **95**: 434-441.

De Hoog GS, 1974. The genera *Blastobotrys*, *Sporothrix*, *Calcarisporium* and *Calcarisporiella* gen. nov. *Studies in Mycology* **7**: 1-84.

De Hoog GS, 1979. Taxonomic review of *Moniliella*, *Trichosporonoides* and *Hyalodendron*. *Studies in Mycology* **19**: 1-36.

De Meyer EM, De Beer ZW, Summerbell RC, Moharram AM, De Hoog GS, Vismer HF, Wingfield MJ, 2008. Taxonomy and phylogeny of new wood- and soil-inhabiting *Sporothrix* species in the *Ophiostoma stenoceras-Sporothrix schenckii* complex. *Mycologia* **100**: 647-661.

Felsenstein J, 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**: 783-791.

Gams W, 1968. Typiserung der Gattung *Acremonium*. *Nova Hedwigia* **16**: 141-145.

Gardes M, Bruns TD, 1993. ITS primers with enhanced specificity for basidiomycetes - application to the identification of mycorrhizae and rusts. *Molecular Ecology* **2**: 113-118.

Geldenhuis MM, Roux J, Montenegro F, De Beer ZW, Wingfield MJ, Wingfield BD, 2004. Identification and pathogenicity of *Graphium* and *Pesotum* species from machete wounds on *Schizolobium parahybum* in Ecuador. *Fungal Diversity* **15**: 135-149.

Georgescu CC, Teodoru I, Badea M, 1948. Uscarea in massa a stejarului. Ciupere de alteratie cromatica parazitara a lemnului de stejar. *Anàlele Institutului de Cercetari Forestiere al Românei* **11**: 185-217.

Georgevitch P, 1926. *Ceratostomella querci* n. sp. *Comptes rendus Académie des Sciences* **183**: 759-761.

Georgevitch P, 1927. *Ceratostomella quercus* n. sp. Ein Parasit der slawonischen Eichen. *Biologia Generalis* **3**: 245-252.

Georgiev D, 1986. Species composition, morphology and certain cultural properties of the agents of the tracheomycosis on the sessile and pedunculate oak in south Bulgaria. [In Bulgarian with English summary]. *Gorskostopanska Nauka* **23**: 62-69.

Glass NL, Donaldson GC, 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous Ascomycetes. *Applied and Environmental Microbiology* **61**: 1323-1330.

Glenn AE, Bacon CW, Price R, Hanlin RT, 1996. Molecular phylogeny of *Acremonium* and its taxonomic implications. *Mycologia* **88**: 369-383.

Goidánich G, 1935. Uno nuova specie di *Ophiostoma* vivente sul pero ed alcune osservazioni sull'esatta posizione sistematica della forma ascofora e delle forme metagenetiche del genere. *Bolletino della Stazione di Patología Vegetale di Roma* **15**: 122-168.

Guindon S, Gascuel O, 2003. A Simple, Fast, and Accurate Algorithm to Estimate Large Phylogenies by Maximum Likelihood. *Systematic Biology* **52**: 696-704.

Guseinov ES, 1984. [Vascular dieback of oaks in Azerbaijan. I.] *Mikologiya i Fitopatologiya* **18**: 144-149.

Halmschlager E, Messner R, Kowalski T, Prillinger H, 1994. Differentiation of *Ophiostoma piceae* and *Ophiostoma quercus* by morphology and RAPD analysis. *Systematic and Applied Microbiology* **17**: 554-562.

Harrington TC, McNew D, Steimel J, Hofstra D, Farrell R, 2001. Phylogeny and taxonomy of the *Ophiostoma piceae* complex and the Dutch elm disease fungi. *Mycologia* **93**: 111-136.

Hawksworth DL, Sutton BC, Ainsworth GC (eds) 1983. Dictionary of the Fungi. Commonwealth Mycological Institute, Kew Surrey UK.

Hunt J, 1956. Taxonomy of the genus *Ceratocystis*. *Lloydia* **19**: 1-58.

Hutchison LJ, Reid J, 1988. Taxonomy of some potential wood-staining fungi from New Zealand 1. *Ophiostomataceae*. *New Zealand Journal of Botany* **26**: 63-81.

Jacobs K, Bergdahl DR, Wingfield MJ, Halik S, Seifert KA, Bright DE, Wingfield BD, 2004. *Leptographium wingfieldii* introduced into North America and found associated with exotic *Tomicus piniperda* and native bark beetles. *Mycological Research* **108**: 411-418.

Kamgan Nkuekam G, Jacobs K, De Beer ZW, Wingfield MJ, Roux J, 2008a. *Ceratocystis* and *Ophistoma* species, including three new taxa, associated with wounds on native South African trees. *Fungal Diversity* **29**: 37-59.

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

Katoh K, Kuma K-i, Toh H, Miyata T, 2005. MAFFT version 5: improvement in accuracy of multiple sequence alignment. *Nucleic Acids Research* **33**: 511-518.

Kim SH, Uzunovic A, Breuil C, 1999. Rapid detection of *Ophiostoma piceae* and *O. quercus* in stained wood by PCR. *Applied and Environmental Microbiology* **65**: 287-290.

Kim SH, Breuil C, 2001. Common nuclear ribosomal internal transcribed spacer sequences occur in the sibling species *Ophiostoma piceae* and *O. quercus*. *Mycological Research* **105**: 331-337.

Kirisits T, 2004. Fungal associates of European bark beetles with special emphasis on the Ophiostomatoid fungi. In: Lieutier F, Day KR, Battisti A, Grégoire J-C, Evans H (eds), *Bark and Wood Boring Insects in Living Trees in Europe, a Synthesis*. Kluwer Academic Publishers, Dordrecht, pp 181-235.

Kowalski T, Butin H, 1989. Taxonomie bekannter und neuer Ceratocystis-Arten an Eiche (*Quercus robur* L.). *Journal of Phytopathology* **124**: 236-248.

Lehmann E, 1932. *Ceratostomella quercus* Georgevitch in der schwäbischen Alb. *Zentralblatt für Bakteriologie Parasitenkunde Infektionkrankheiten und Hygiene II.* **86**: 404-407.

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

Lin TC, Huang JW, Hsieh WH, 2003. Identification of ophiostomatoid fungi associated with Chinese fir wilt in Taiwan. [in Chinese]. *Plant Pathology Bulletin* **12**: 33-42.

Linnakoski R, De Beer ZW, Rousi M, Niemela P, Pappinen A, Wingfield MJ, 2008. Fungi including *Ophiostoma karelicum* sp. nov., associated with *Scolytus ratzeburgi* infesting birch in Finland and Russia. *Mycological Research* **112**: 1475-1488.

Loos W, 1932. Über eine buchenholzbewohnende *Ceratostomella*, *Ceratostomella fagi* nov. sp. *Archiv für Microbiologie* **3**: 370-383.

McNeill J, Barrie FR, Burdet HM, Demoulin V, Hawksworth DL, Marhold K, Nicolson DH, Prado J, Silva PC, Skog JE, Wiersema JH, Turland NJ, 2006. International Code of Botanical Nomenclature (Vienna Code) adopted by the Seventeenth International Botanical Congress Vienna, Austria, July 2005. *Regnum Vegetabile* 146.

Melin E, Nannfeldt JA, 1934. Researches into the blueing of ground wood-pulp. *Svenska Skogsvårdsföreningens Tidskrift* **32**: 397-616.

Moreau C, 1952. Coexistence des formes *Thielaviopsis* et *Graphium* chez une souche de *Ceratocystis major* (van Beyma) nov. comb. *Revue de Mycologie* (Paris) **17**: 17-25.

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

Morrison DA, 2006. Multiple sequence alignment for phylogenetic purposes. *Australian Systematic Botany* **19**: 479-539.

Münch E, 1907. Die Blaufäle des Nadelholzes. I. *Naturwissenschaftliche Zeitschrift für Forst- und Landwirtschaft* **5**: 531-573.

Nylander JAA, 2004. MrModeltest v.2. Program distributed by the author. Evolutionary Biology Centre Uppsala University Uppsala.

O'Donnell K. Cigelnik E, 1997. Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. *Molecular Phylogenetics and Evolution* **7**: 103-116.

Okada G, Seifert KA, Takematsu A, Yamaoka Y, Miyazaki S. Tubaki K, 1998. A molecular phylogenetic reappraisal of the *Graphium* complex based on 18S rDNA sequences. *Canadian Journal of Botany* **76**: 1495-1506.

Pipe ND, Buck KW, Brasier CM, 1995. Genomic fingerprinting supports the separation of *Ophiostoma piceae* into two species. *Mycological Research* **99**: 1182-1186.

Posada D, Crandall KA, 1998. MODELTEST: testing the model of DNA substitution. *Bioinformatics* **14**: 817-818.

Potlajczuk VI, 1957. On the biology of the causal agent of oak wilt. *Trudy Vsesojuz Naucno-Issled Inst Zascity Rasteniy* **8**: 227-237.

Potlajczuk VI, Schekunova EG, 1985. De distributione specierum generic *Ceratocystis* Ell. et Halst. emend. Bakshi in URSS. *Novosti Sistematiki nizsich rastieniy "Nauka" Akademia Nauk SSSR* **22**: 148-156.

Przybyl K, De Hoog GS, 1989. On the variability of *Ophiostoma piceae*. *Antonie van Leewenhoek* **55**: 177-188.

Przybyl K, Morelet M, 1993. Morphological differences between *Ophiostoma piceae* and *O. querci*, and among *O. querci* isolates. *Cryptogamie, Mycologie* **14**: 219-228.

Riddell RW, 1950. Permanent stained mycological preparations obtained by slide culture. *Mycologia* **42**: 265-270.

Roets F, De Beer ZW, Wingfield MJ, Crous PW, Dreyer LL, 2008. *Ophiostoma gemellus* and *Sporothrix variecibatus* from mites infesting *Protea* infructescences in South Africa. *Mycologia* **100**: 496-510.

Romón P, Zhou X, Iturrondobeitia JC, Wingfield MJ, Goldarazena A, 2007. *Ophiostoma* species (Ascomycetes: Ophiostomatales) associated with bark beetles (Coleoptera: Scolytinae) colonizing *Pinus radiata* in northern Spain. *Canadian Journal of Microbiology* **53**: 756-767.

Ronquist F, Heulsenbeck JP, 2003. MRBAYES 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**: 1572-1574.

Schroeder S, Kim SH, Cheung WT, Sterflinger K, Breuil C, 2001. Phylogenetic relationship of *Ophiostoma piliferum* to other sapstain fungi based on the nuclear rRNA gene. *FEMS Microbiology Letters* **195**: 163-167.

Sczerbin-Parfenenko AL, 1953. Rakovye i sosudistye bolezni listvennych porod. *Goslesbumizdat, Moskva-Leningrad*.

Thwaites JM, Farrell RL, Duncan SM, Reay SD, Blanchette RA, Hadar E, Hadar Y, Harrington TC, McNew D, 2005. Survey of potential sapstain fungi on *Pinus radiata* in New Zealand. *New Zealand Journal of Botany* **43**: 653-663.

Upadhyay HP (ed) 1981. A monograph of *Ceratocystis* and *Ceratocystiopsis*. University of Georgia Press, Athens Georgia USA.

Webber JF, Brasier CM, 1991. Status of *Ophiostoma piceae* on hardwoods and conifers. In: Report on Forest Research 1990. HMSO, London UK, pp 54-55.

White TJ, Bruns T, Lee S, Taylor, J, 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, and White TJ (eds) PCR protocols: a guide to methods and application. Academic Press San Diego, pp 315-322.

Zhou XD, De Beer ZW, Ahumada R, Wingfield BD, Wingfield MJ, 2004. *Ophiostoma* and *Ceratocystiopsis* spp. associated with two pine-infesting bark beetles in Chile. *Fungal Diversity* **15**: 261-274.

Zhou XD, De Beer ZW, Wingfield MJ, 2006. DNA sequence comparisons of *Ophiostoma* spp., including *Ophiostoma aurorae* sp. nov., associated with pine bark beetles in South Africa. *Studies in Mycology* **55**: 269-277.

Fig 1 Morphological characteristics of an isolate of *Ophiostoma quercus* from Azerbaijan (CMW 9256). a. Perithecium. b**.** Ostiolar hyphae. c. Allantoid ascospores in side view. d. *Pesotum* type synnematous conidiophore. e. Scanning electron micrograph (SEM) of a synnematous conidiophore (*Pesotum* type). f. SEM of conidia of *Pesotum* anamorph. g. Conidiogenous cells of *Sporothrix*-like anamorph. h. SEM of *Sporothrix*-like anamorph. i. Conidia of *Sporothrix*-like anamorph. Scale bars: Figs a, d, e = 100 μm; Figs b, g, h, i = 10 μm; Figs c, f = 5 μm.

Fig 2 Morphological characteristics of the so-called *Ophiostoma roboris* isolate from Azerbaijan (CMW 9262). a. Perithecium. b. Ostiolar hyphae. c. Reniform ascospores in side view. d. Scanning electron micrograph (SEM) of a synnematous conidiophore (*Pesotum* type). e. SEM of conidia of *Pesotum*. f. SEM of *Sporothrix*-like anamorph. g. SEM of *Hyalorhinocladiella*-like anamorph. h. Conidia of *Hyalorhinocladiella*-like anamorph. i. Conidia of *Sporothrix*-like anamorph. Scale bars: Figs a, d = 100 μm; Figs b, g, i = 10 μm; Figs c, f, h = 5 μm.

Fig 3 Phylograms resulting from Bayesian analyses of (a) the partial Histone (HIS) gene, (b) Translation Elongation Factor-1α (TEF-1α), (c) the rDNA Internal Subscribed Spacers (ITS) regions 1 and 2, and (d) the Bt2 region of the β-tubulin (BT) gene. The box delimiting *Ophiostoma quercus* is indicated with Q, and the lineage represeting hardwood species as HW. Species previously considered synonyms of *O. quercus* are indicated in coloured boxes, with the Azerbaijan isolate presenting the *O. roboris* morphotype (CMW 9262) in blue. The ex-neotype isolate of *O. quercus* (CMW 2467) are indicated with T. The posterior probability support values are given first followed by the ML bootstrap values (1000 replicates) for each node respectively (scores of 0.95 pp or 70 bs and less have not been included and are indicated with '-').

CHAPTER 3

DEVELOPMENT AND CHARACTERIZATION OF POLYMORPHIC MARKERS FOR THE SAP-STAIN FUNGUS *OPHIOSTOMA QUERCUS*

Abstract

Eight polymorphic markers were developed from South African isolates of *Ophiostoma quercus*. The genome was screened for repeat regions using the fast isolation by amplified fragment length polymorphism of sequences containing repeats protocol and 20 *de novo* primer pairs flanking putative microsatellite regions were designed. Eight loci were optimized and their polymorphisms evaluated by sequencing. The repeat and flanking regions were highly polymorphic containing both indels and base-pair substitutions revealing a total of 46 alleles in 14 isolates and an average heterozygosity of 0.68. Substantial sequence variability makes these markers useful for genotyping populations in order to calculate diversity and monitor global movement of *O. quercus*.

Keywords: blue-stain fungi, M-FIASCO, microsatellites, *Ophiostoma quercus,* polymorphic markers, sequence variation

Ophiostoma quercus is a wood-inhabiting, heterothallic ascomycete causing blue-stain. The fungus has been recorded in many countries on hardwood and coniferous trees although for many years it was treated collectively with the morphologically similar *O. piceae*. The advent of DNA sequence-based phylogenies has led to *O. quercus* being accepted as a discrete taxon, part of the *O. piceae* complex (Harrington *et al.* 2001). However, almost nothing is known regarding its population genetic structure.

Microsatellites display high levels of polymorphism and are ideal genetic markers to provide resolution in relatedness studies (Tautz & Renz 1984). The aim of this study was to develop polymorphic microsatellite markers specific for *O. quercus* in order to describe its population genetic structure and worldwide distribution.

A microsatellite-enriched library was made using the fast isolation by amplified fragment length polymorphism of sequences containing repeats (FIASCO) protocol (Zane *et al.* 2002) with modifications (M-FIASCO) as described by Cortinas *et al.* (2006). Genomic

DNA was pooled from six South African isolates of *O. quercus* (CMW 2520, CMW 2521, CMW 2534, CMW 3119, CMW 3117 and CMW 3116) to yield a total of 2 µg. Cultures were made from single, germinating conidia or ascospores and genomic DNA was extracted using the method of Jacobs *et al.* (2004).

Selections of biotinylated oligo probes representing di- to hexanucleotides in different combinations were used in the enrichment procedure. Enriched fragments were cloned using the TOPO 4 TA Kit (Invitrogen) and 576 colonies were selected and grown in 96-well plates containing 2 mL Luria-Bertani (LB) broth. M13 TOPO vector primers (Invitrogen) were used for colony PCR's and amplicons were cleaned with 1.25 U of Exonuclease I and 1 U Shrimp Alkaline Phosphatase (Fermentas Life Sciences) to digest excess primers and dNTP's.

Cloned products were sequenced using the ABI PRISM®BigDye™ Terminator version 3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems Inc.) and the M13 forward and reverse vector primers. Sequenced products were purified using the Applied Biosystems precipitation method and were separated on an ABI PRISM 3100 automated sequencer (Applied Biosystems).

Sequences were manually screened for microsatellite regions using Vector NTI Advance 10 software (Invitrogen). Of 576 clones, 121 contained highly repetitive regions with many displaying a CT bias. Primer pairs were designed (manually or with Primer Designer 5.0 [Sci. Ed. Central]) flanking 20 sequences containing putative microsatellite regions.

Twenty primer pairs were tested for polymorphisms using seven isolates. PCR's contained 20-100 ng DNA template, 2.5 mM of each dNTP's (Promega), 2 pmol of each primer, 0.1U of *Taq* polymerase (Roche Molecular Biochemicals), 1x buffer and $MgCl_2$ (concentration indicated in Table 1). Thermal conditions in a Bio-Rad iCycler were: 96 °C for 2 min, followed by 40 cycles of 94 °C for 30 s, T_a (as per Table 1) for 30 s, 72 °C for 30 s and a final extension at 72 °C for 10 min.

Eight primer pairs resulted in consistent amplification across all the isolates and amplicons were sequenced to confirm the presence of the repeat. The remaining 12 loci were discarded as they failed to consistently amplify due to incorrect amplicon size, or did not amplify or produced multiple bands.

Sequence data from the seven isolates revealed that all eight markers were polymorphic. Nucleotide variation was observed within the repeat and flanking regions of the markers and was a combination of repeat length differences, indels and base pair substitutions (Table 2). Marker Oqrc9 exhibited no length variation, but considerable sequence variation which conventional genotyping (PCR and electrophoresis) cannot distinguish. It is recognized that microsatellites in fungi are often short in length and homoplasy in the microsatellite regions have previously been observed (Bogale *et al*. 2005). We, therefore, chose to treat the repeat regions as sequence-based markers as opposed to PCR-based markers. The alleles were characterised as haplotypes based on sequence polymorphisms rather than length variation.

To test the efficacy of the markers, they were screened in 14 isolates collected in South Africa (*n*=9), Malawi (*n*=4) and Uganda (*n*=1). Forty six haplotypes were obtained across all eight loci (three-eight haplotypes per locus) as calculated using TCS software (Clement *et al*. 2000). Nei's gene diversity (Nei 1973) was calculated using POPGENE version 1.32 (Yeh & Boyle 1997) (Table 1) and all pairwise loci were tested for linkage disequilibrium with MultiLocus 1.3b (Agapow & Burt 2001) following 1000 randomizations. Departure from equilibrium was significant suggesting little recombination or non-random mating. This could be due to asexual reproduction or selfing which is common in ascomycetes. *Ophiostoma quercus* can reproduce both asexually and sexually in nature. The extent to which either mechanism is used, however, is not known.

In cross-species amplification, primers Oqrc2 and Oqrc18 amplified in four closely related *Ophiostoma* species namely: *O. floccosum, O. catonianum, O. novo-ulmi* and *O. himal-ulmi*. Primer Oqrc12 successfully amplified in *O. himal-ulmi*.

Eight sequence-based polymorphic markers were developed for *O. quercus* in this study. Isolates were found to have differences in repeat length and they also displayed sequence variation. In future studies, these loci will be used to assess the genetic diversity and worldwide patterns of distribution in *O. quercus*. A few markers were also shown to be applicable for studying related species in the *O. piceae* complex.

Acknowledgements

We thank the Centre of Excellence in Tree Health Biotechnology, the National Research Foundation and members of the Tree Protection Co-operative Program for financial support.

References

Agapow P-M, Burt A, 2001. Indices of multilocus linkage disequilibrium. *Molecular Ecology Notes* **1**: 101-102.

Bogale M, Wingfield BD, Wingfield MJ, Steenkamp ET, 2005. Simple sequence repeat markers for species in the *Fusarium oxysporum* complex. *Molecular Ecology Notes* **5**: 622-624.

Clement M, Posada D, Crandall KA, 2000. TCS: a computer program to estimate gene genealogies. *Molecular Ecology* **9**: 1657-1660.

Cortinas M-N, Barnes I, Wingfield BD, Wingfield MJ, 2006. Polymorphic microsatellite markers for the *Eucalyptus* fungal pathogen *Colletogloeopsis zuluensis*. *Molecular Ecology Notes* **6**: 780-783.

Harrington TC, McNew D, Steimel J, Hofstra D, Farrell R, 2001. Phylogeny and taxonomy of the *Ophiostoma piceae* complex and the Dutch elm disease fungi. *Mycologia* **93**: 111-136.

Jacobs K, Bergdahl DR, Wingfield MJ, Halik S, Seifer, KA, Bright DE, Wingfield BD, 2004. *Leptographium wingfieldii* introduced into North America and found associated

with exotic *Tomicus piniperda* and native bark beetles. *Mycological Research* **108**: 411-418.

Nei M, 1973. Analysis of gene diversity in subdivided populations. *Proceedings of the National Academy of Sciences* **70**: 3321-3323.

Tautz D, Renz M, 1984. Simple sequences are ubiquitous repetitive components of eukaryotic genomes. *Nucleic Acids Research* **12**: 4127-4138.

Yeh FC, Boyle TJB, 1997. Population genetic analysis of co-dominant and dominant markers and quantitative traits. *Belgian Journal of Botany* **129**: 157.

Zane L, Bargelloni L, Patarnello T, 2002. Strategies for microsatellite isolation: a review. *Molecular Ecology* **11**: 1-16.

CHAPTER 4

OPHIOSTOMA TSOTSI SP. NOV., A SAP-STAINING FUNGUS ON HARDWOOD TREES IN AFRICA

Abstract

Polymorphic sequence-characterised marker assays from a recent diversity study on the Ascomycete fungus *Ophiostoma quercus* from Africa showed that some isolates collected from hardwoods were genetically distinct from *O. quercus*. In the present study we compared these African isolates with authentic *O. quercus* isolates, including the neo-type of the species from oak in France, by evaluating morphological characters, growth in culture, mating compatibility, and DNA sequence data. The isolates from Africa exhibited substantial variability in culture and were morphologically similar to *O. quercus*. Phylogenetic analyses of the ITS, β-tubulin and Translation Elongation Factor 1-α gene regions confirmed that the African group represents a distinct species in the hardwood lineage of the *O. piceae*-complex, closely related to *O. ulmi* and *O. himal-ulmi*. Mating studies between *O. quercus* and the African isolates showed that isolates predominantly mated with those of their own group, although there were rare cases of fertile crosses between the groups. Isolates residing in the African lineage is described here as a new species, *O. tsotsi* sp. nov., and is characterized based on morphological characteristics, growth in culture and sequence comparisons.

Introduction

"Ex Africa semper aliquid novi – From Africa there is always something new"
Pliny the Elder (AD 23 – AD 79).

The Ophiostomatales is an order of the Ascomycete that includes primarily insect-vectored, wood-inhabiting fungi. Three teleomorph genera are currently recognized in the *Ophiostomatales* and these include *Ceratocystiopsis, Grosmannia* and *Ophiostoma* (Upadhyay 1981; Zipfel *et al*. 2006). The majority of the *ca.* 160 known species of *Ophiostoma* cause stain of conifer and angiosperm wood which significantly reduces its commercial value (Seifert 1993). The genus *Ophiostoma* also includes numerous tree pathogens such as the Dutch elm disease fungi that are well known for the devastation that they have caused to elm forests in the northern hemisphere (Brasier 1990; 1991).

Harrington *et al*. (2001) reported nine *Ophiostoma* spp.with *Pesotum* anamorphs in their treatment of the *Ophiostoma piceae* complex. In the hardwood clade of the complex, they recognised *O. quercus* (Georgevitch 1926; 1927), *O. catonianum*, *O. ulmi*, *O. novo-ulmi* and *O. himal-ulmi*. The remaining species in the complex, *O. piceae, O. canum*, *O. setosum* and *O. floccosum,* were associated predominantly with coniferous hosts. In recent years, several species that are phylogenetically closely related to members of the *O. piceae* complex have been described. These include *O. distortum, O. triangulosporium* (Hausner & Reid 2003), *O. kryptum* (Jacobs & Kirisits 2003)*, O. tetropii* (Jacobs *et al*. 2003), *O. subalpinum, O. ssiori* (Masuya *et al*. 2003), *O. flexuosum, O. bacillisporum* (Villarreal *et al*. 2005), *O. arduennense* (Carlier *et al*. 2006), *O. breviusculum* (Chung *et al*. 2006), *O. ainoae, O. araucariae* (Zipfel *et al.* 2006)*, O. karelicum* (Linnakoski *et al*. 2008) and *P. australi* (Kamgan *et al*. 2008a). Interestingly, not all of these species have *Pesotum* anamorphs, which was one of the morphological characters used to define the *O. piceae* complex (Harrington *et al*. 2001). It has further been shown that sequences of the ribosomal internal transcribed spacer (ITS) region that is widely used for species definition, does not always allow for differentiation between closely related species in the *O. piceae* complex. Thus performing additional phylogenetic reconstruction with β-tubulin gene sequences improves the resolution between closely related *Ophiostoma* species (Chung *et al*. 2006; Kamgan *et al*. 2008b).

Most *Ophiostoma* species are believed to have their origins in the northern hemisphere (Przybyl & Morelet 1993; Harrington *et al*. 2001). However, as interest in these fungi has increased in the southern hemisphere, many known and new species have been reported or described from this part of the world (De Beer *et al*. 1995; De Beer *et al*. 2003b; Geldenhuis *et al*. 2004; Kamgan *et al*. 2008b; Thwaites *et al*. 2005; Zhou *et al*. 2006; Zhou *et al*. 2004). Some of these have been accidentally introduced, but many are also likely to be native to the southern hemisphere, which is far from completely sampled.

Recently, we developed polymorphic sequence characterised markers for the saprophyte, *O. quercus* (Grobbelaar *et al*. 2009a). These markers were tested on a collection of *O.*

quercus-like isolates from Africa and the results showed a great number of polymorphisms in the repeat and flanking sequences, resulting in many additional haplotypes. Further analyses of the data showed two distinct groups within the collection of what was believed to represent only *O. quercus* isolates. One group consisted of isolates including the ex-neotype isolate and were subsequently found to be the authentic *O. quercus* isolates. The second group of isolates consisted only of those originating from sub-Saharan Africa. The aim of this study was to use DNA sequence comparisons for three gene regions, mating compatibility studies, and morphological comparisons to resolve the inconsistency between these two groups of *O. quercus* isolates.

Materials and Methods

Fungal isolates

Ophiostoma quercus-like isolates were collected from wounds on trees and from cut timber of various types in South Africa, Uganda and Malawi **(Table 1)**. For growth and mating compatibility studies, as well as DNA sequencing, single spore cultures were prepared for all isolates from germinating conidia or ascospores. All isolates used in this study are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI). Herbarium specimens were deposited in the National Collection of Fungi, Pretoria, South Africa (PREM), and representative cultures including those used in mating compatibility tests have been deposited in the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands.

DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from cultures using PrepMan Ultra (Applied Biosystems). Genomic DNA was diluted 1 part in 10 parts deionised water. Primer pairs ITS1F (Gardes & Bruns 1993) and ITS4 (White *et al*. 1990) were used to amplify the internal transcribed spacer (ITS) regions 1 and 2, including the ribosomal 5.8S gene. For the β-tubulin region, the primers T10 (O'Donnell & Cigelnik 1997) and Bt2b (Glass &

Donaldson 1995) were used, and for the partial translation elongation factor 1-α (TEF1-α), the primers EF1F and EF2R (Jacobs *et al*. 2004). Conditions for the PCR amplification and sequencing reactions were the same as those used by Grobbelaar *et al*. (2009b). The sequenced products were purified and fragments run on an ABI PRISM^{TM} 3100 automated sequencer (Applied Biosystems).

Phylogenetic analyses

Sequence data were analysed and contigs assembled using the Vector NTI Advance 10 software (Invitrogen). Sequences generated in this study (**Table 1**) were supplemented with published sequences of related species from GenBank (accession numbers in **Figs 1** and **2**). The data sets were aligned using the E-INS option in MAFFT v. 5.731 (Katoh & Toh 2008). Data for three gene regions were analysed separately because data were not available from GenBank for similar sets of reference isolates.

Three different methods of phylogenetic analysis were used for each of the data sets. These included Maximum Parsimony (MP), Maximum Likelihood (ML) and Bayesian Inference (BI). The ITS and β-tubulin data sets were rooted against sequences of *Ophiostoma piliferum*. In the case of TEF 1-α, phylogenetic analyses were performed without an outgroup as no sequence data were available for an appropriate root. Un-weighted MP analyses were calculated using PAUP* v. 4.0 beta 10 (Swofford 2002). The heuristic search algorithm was utilised to find the most parsimonious trees using tree bisection-reconnection (TBR) branch-swapping. One thousand bootstrap replicates (Felsenstein 1985) were conducted to calculate confidence levels.

For ML, the most appropriate substitution models were selected using the Akaike Information Criterion in Modeltest v. 3.7 (Posada & Crandall 1998) for each of the three gene regions. ML analyses were conducted in PAUP*. Nonparametric bootstrap analyses (Felsenstein 1985) employed one heuristic search for 1000 bootstrap replicates to estimate the reliability of nodes. Bayesian analyses were performed in MrBayes v. 3.1.2 (Ronquist & Heulsenbeck 2003) using the best fitted model selected by the AIC test in

MrModeltest v. 2.2 (http://www.abc.se/~nylander/). Model parameters were derived from the default prior distributions. The Bayesian analyses comprised four independent runs of 1 000 000 generations, using duplicate Monte Carlo Markov chain searches with four chains (one cold, three hot to improve mixing) and a sampling frequency of every 100 generations. The average standard deviation of split frequencies was 0.01. Stationarity for LnL and nucleotide substitution parameters was achieved within the first 100 000 generations. Consequently, the first 1000 trees sampled were discarded as burn-in.

Growth in culture

Three isolates of *O. quercus* and three from the African lineage were selected for comparative studies of growth in culture. Mycelium-covered agar plugs (7mm diameter) were transferred from actively growing colonies to the centres of three 90mm diameter Petri dishes containing 2% malt extract agar (MEA, Biolab, Midrand, South Africa) for each isolate and each temperature to be tested. The plates were incubated in the dark at temperatures ranging from 5 °C to 35 °C at 5 °C intervals. Two colony diameter measurements at right angles to each other were made every other day for 10 days. The mean diameter was calculated in mm (± standard deviation) for each isolate.

Mating compatibility

Isolates representing the authentic *O. quercus* (9) and one from the African lineage were selected to determine whether the individuals of these two groups were capable of mating with each other. Single spore cultures were transferred to 2% MEA and incubated for 14 days at 25 C. Agar blocks (ca. 4 x 4 mm) covered in mycelium of two isolates were placed adjacent to each other on plates containing WA (20g agar, 1000ml distilled water), supplemented with debarked, autoclaved oak or eucalyptus twigs. As a control, each isolate was also paired against itself. All isolates were crossed against two *O. quercus* tester strains of opposite mating type (CMW2520 and CMW2521) defined in a previous study (De Beer *et al*. 2003b). Plates were incubated at 22 C for 2 to 4 weeks and were inspected weekly for the presence of ascomata.

Crosses were considered positive when ascomata produced droplets of viable ascospores and also where no ascomata were formed on the control plates of the parental strains. Positive crosses were scored with a (+) and mating types of either "a" or "b" were assigned based on the positive crosses with the two tester strains. Crosses that produced no ascomata were considered negative and scored with a (-). Crosses that produced ascomata without ascospores were labelled as sterile (S). In positive crosses between isolates of the *O. quercus* and African groups, drops containing spores were lifted from the apices of the ascomata with a sterile needle, suspended in distilled water, shaken and spread onto 2% MEA plates. After 12-48 hours of incubation, plates were inspected for germinating ascospores to determine the viability of the F1 progeny. Successive subculturing also confirmed the viable nature of the isolates.

Morphology

Ascomata and anamorph structures were taken from the wood surface from a positive cross between isolates residing in the African group, mounted on glass slides in lactophenol and studied with a Zeiss Axiovision 2 plus light microscope (Carl Zeiss, Jena, Germany). Twenty five measurements were taken and the means and standard deviations calculated for each of the sexual and asexual structures. The measurements are presented as (minimum-) mean minus standard deviation – mean plus standard deviation (-maximum).

Scanning electron microscopy (SEM) was used for detailed examination of the ascomata and ascospores of *O. quercus*, the isolates of the African group, as well as a hybrid obtained *in vitro* between the two groups of isolates. Blocks of agar (1 cm^2) were cut from actively growing cultures and fixed overnight in 2.5% glutaraldehyde in 0.075 M sodium phosphate buffer (pH = 7.4). After three buffer rinses, the specimens were held in 1 % aqueous osmium tetroxide for 60 min, dehydrated in a series of ethanol dilutions (50% - 100%), and dried with a Polaron Critical Point Drier. Thereafter, specimens were

coated with 23 carat gold and examined using a JEOL JSM 6400 scanning electron microscope.

Results

PCR and sequence analyses

Amplicons of approximately 620, 435 and 711 base pairs long were produced for the ITS, β-tubulin and the TEF 1-α gene regions respectively. Forward and reverse contigs were assembled respectively and each data set was trimmed and aligned.

Phylogenetic analyses

Analyses of the ITS data set (**Fig 1**) showed two clusters present within what was traditionally considered as one group of species, however it lacked resolution to distinguish between the authentic *O. quercus* and African isolates. The topology of the consensus MP tree, and those of the phylograms obtained from ML and Bayesian analyses, gave similar results. MP analyses yielded 211 parsimony-informative characters, a consistency index (CI) of 0.87 and a retention index (RI) of 0.93 with four trees being retained.

Bayesian (**Fig 2**) and ML analyses of the β-tubulin data generated phylograms that separated the isolates sequenced in this study into two well-supported lineages referred to as Lineage A and Lineage B. Lineage A represented authentic *O. quercus* isolates, and Lineage B the African isolates. Cladistic analysis based on parsimony resulted in 116 parsimony informative characters and a CI = 0.66 and RI = 0.87. The cladogram (bootstrap values presented in **Fig 2**) also confirmed that each of the taxa grouped in distinct, well supported clades.

All three phylogenetic methods of analysis applied to the TEF1-α sequences confirmed the divergence within what was traditionally regarded as one group into the authentic *O.*

quercus (Lineage A) and the African isolates (Lineage B) with strong bootstrap support values and posterior probabilities (**Fig 3**). Maximum parsimony analysis yielded 431 informative characters, a CI = 0.76 and RI = 0.89.

The datasets all confirmed the inclusion of the African lineage into the hardwood clade of the *O. piceae* complex. The new species is closely related to *O. quercus* as well as to the Dutch Elm disease fungi. *O. novo-ulmi, O. ulmi* and *O. himal-ulmi*, and can easily be distinguished from these well known species using the β-tubulin gene or TEF 1-α.

Growth in culture

Results of the growth study (**Fig 4**) showed that the African cultures grew slightly slower than the *O. quercus* isolates at all temperatures, apart from 30 °C. At this temperature the African isolates had a mean colony diameter of 45 mm (± 8.3mm), while *O. quercus* had a mean colony diameter of 34 mm (± 6.8mm). None of the isolates grew at 35 °C.

Mating compatibility

Fully fertile crosses were seen in most instances (**Table 2**) between the *O. quercus* tester strains and other authenticated isolates of *O. quercus*. All isolates could clearly be segregated into two mating types based on their reactions with the tester strains, and behaved in a heterothallic fashion with none of the control crosses, where only one isolate was used, producing ascomata. Positive crosses between *O. quercus* and an isolate representing the African group (CMW5943) occurred but were infrequent, with some of these crosses producing sterile perithecia or only a few perithecia. Culture morphology of the progeny from those positive crosses between authentic *O. quercus* and the African group isolate (CMW5943) was highly variable, similar to that of their parent isolates.

Morphology

The African isolates showed considerable variation in culture morphology, which is similar to what is observed amongst *O. quercus* isolates. The African isolates produced *Pesotum* and *Sporothrix* synanamorphs (**Fig 5**) with structures similar to those of *O. quercus*. Ascomata of the African isolates were also similar to those of *O. quercus*, however the ascospore morphology was slightly different for the two groups of isolates (**Fig 5**). Thus, while the ascospores of *O. quercus* were distinctly reniform in side view (Upadhyay 1981; Kowalski & Butin 1989), those of the African isolates were orange segment shaped and less distinctly curved.

Taxonomy

β-tubulin and TEF1-α DNA sequence data, together with morphological differences, showed that the African group of isolates treated in this study represent a species distinct from *O. quercus*. The fungus is consequently described as a novel taxon as follows:

Ophiostoma tsotsi Grobbelaar, Z.W. de Beer & M.J. Wingf **sp. nov. Fig 5.**
Anamorphs: *Pesotum* and *Sporothrix*

Etym: tsotsi, from the Sesotho language meaning "to con" and describing the cryptic nature of the African fungus that resembles *O. quercus* that was consequently not recognised as a distinct taxon (http://en.wikipedia.org/wiki/Tsotsitaal).

Ascomata: Bases nigrae vel brunneae, globosae (72.2-)91.1-119.2-147.3(-180.1) µm diametro; hyphae ornamentales (16.7-)26.7-47.6-68.5(-94.6) µm longae. *Colla* laevia, atra, apice pallida, recta vel parum curvata, (351.8-)737-1047.7-1358.4(-1647) µm longa, basi (17.6-)21.6-26.3-31(-36.1) µm, apice (8.5-)9.1-11.6-14.1(-14.9) µm lata. *Hyphae ostiolares* hyalinae, divergentes, non septatae, (1.1-)13.2-21-28.8(-33.81) µm longae. *Asci* non visi. *Ascosporae* hyalinae, unicellulares, forma segmenti citri, (2.8-)3.2-3.7-4.2(-4.6) µm longae, (1.5-)1.6-1.8-2(-2.5) µm latae, in massa luteola in apice colli.

Anamorpha *Pesotum*: in cultura dominans. Synnemata brunnea vel nigra, (394.7-)438.2-658.3-878.4(-1049.7) µm longa, basi (28.3-)29.3-32.9-36.5(-37.7) µm, apice (21-)26.2-33.4-40.6(-47.9) µm lata, basi cum rhizoideis brunneis septatis (53.8-)50.1-90-129.90(-211.7) µm longis; cellulae conidiogenae (0.8-)0.9-1.2-1.5(-2) x (4.9-)6.5-12.9-19.3(-29.6) µm; conidia hyalina unicellularia elliptica (1.1-)1.3-1.5-1.7(-1.8) x (2.7-)3.1-3.4-3.7(-4.1) µm in guttula mucosa eburnea portata.
Anamorpha Sporothrix micronema; cellulae conidiogenae proxime ex hyphis orientes (7.7-)23.1-38-53(-78.6) x (0.9-)1.5-1.9-2.4(-2.6) µm, conidia hyalina laevia (3-)5.3-13-20.7(-24) µm longa (0.5-)0.9-2-2.9(-3.4) µm lata.

Teleomorph: Ascomata emerging after 20 d on sterilized oak twigs (Fig. 5{1-3}) or partially embedded in media when isolates of opposite mating type are paired. *Bases* black or brown, globose, (76.4-) 79.4-100.1-120.8(-156) µm diam longitudinally, (72.2-)91.1-119.2-147.3(-180.1) µm diam wide transversely; ornamented with non-septate hyphae, (16.7-)26.7-47.6-68.5(-94.6)µm long, (1.0-)1.3-2.0-2.7(-3.54) µm wide at apex. *Ascomatal necks* smooth, dark, light at apex, straight or slightly curved, (351.8-)737-1047.7-1358.4(-1647) µm long, (17.6-)21.6-26.3-31(-36.1) µm wide at base, (8.5-)9.1-11.6-14.1(-14.9) µm at the apex. *Ostiolar hyphae* hyaline, divergent, non-septate, (1.1-)13.2-21-28.8(-33.81) µm x (0.6-)1-1.3-1.6(-1.6) µm. *Asci* not observed. *Ascospores* hyaline, one-celled, narrowly ellipsoidal, orange segment-shaped in side view, (2.8-)3.2-3.7-4.2(-4.6) µm x (1.5-)1.6-1.8-2(-2.5) µm, accumulated in a light yellow mass at the tip of the neck.

Pesotum anamorph: synnemata,(Fig. 5{4-9}) brown or black, (394.7-)438.2-658.3-878.4(-1049.7) µm long, (28.3-)29.3-32.9-36.5(-37.7) µm wide at base and (21-)26.2-33.4-40.6(-47.9) µm wide at apex, attached to substrate with brown, septate rhizoids (53.8-)50.1-90-129.90(-211.7) µm long and (1.5-)1.8-2.2-2.6(-3.1) µm wide; conidiogenous cells (0.8-)0.9-1.2-1.5(-2) x (4.9-)6.5-12.9-19.3(-29.6) µm; conidia hyaline, smooth, one-celled, elliptical (1.1-)1.3-1.5-1.7(-1.8) x (2.7-)3.1-3.4-3.7(-4.1) µm, aggregating into an ivory coloured mucilaginous spore drop.

Sporothrix anamorph: micronematous, conidiogenous cells arising directly from hyphae with multiple denticles, (7.7-)23.1-27-53(-78.6) x (0.9-)1.5-2.1-2.4(-2.6) µm; conidia hyaline, smooth, (3-)5.3-13-20.7(-24) x (0.5-)0.9-2-2.9(-3.4) µm.

Colonies reaching 56.4 mm diameter on 2% MEA in 10 days at 25 °C in the dark. *Pesotum* anamorph dominant in cultures. Mycelium hyaline, superficial on the agar.
Sexuality: heterothallic.

Host range: Associated with wounds on native and exotic hardwood tree species in southern Africa infested by bark beetles, flies and nitidulid beetles.

Distribution: Presently known from sub-Saharan African countries, however this fungus most likely occurs elsewhere in the world, where *Eucalyptus* spp. are planted.

Specimens examined: **Holotype** (PREM 59820) consisting of a dried culture representing a cross between isolate CMW 3117/ CBS 122287 from South Africa on *Eucalyptus* sp. collected by Z.W. de Beer and isolate CMW 15239/ CBS 122288 from Malawi isolated from *Eucalyptus grandis* collected by R.N. Heath.
Additional specimens: Anamorph cultures examined PREM 60015, 60016 and CBS 123599.

Discussion

Ophiostoma quercus is a well-known species, commonly found on angiosperm wood in many parts of the world. In this study, we have shown that isolates from Africa that closely resemble *O. quercus* represent a distinct phylogenetic lineage for which the new name *O. tsotsi* has been provided. The first evidence for the existence of a cryptic species amongst *O. quercus* isolates emerged from a genetic sampling using polymorphic markers (Grobbelaar *et al.* 2009a) designed for *O. quercus* isolates from southern Africa. Results established differences between the genotypes, separating the isolates into two groups, the unique nature of which was confirmed in this study.

Phylogenetic analyses for β-tubulin and TEF 1-α gene regions confirmed that the group of isolates from sub-Saharan African initially treated as *O. quercus* represents a species distinct from authentic *O. quercus* based on the neotype of that fungus. Interestingly the new species, *O. tsotsi,* groups within the hardwood clade of the *O. piceae*-complex. The tree topologies also show that it is closely related *O. quercus* and to the Dutch Elm disease pathogens, *O. ulmi* and *O. himal-ulmi.*

The morphology of *O. tsotsi* is similar to that of *O. quercus* and the phenotypes of isolates observed in culture are highly variable. However, *O. tsotsi* appears to have ascospores of a shape distinct from those of authentic *O. quercus*. In addition, cultures of *O. tsotsi* grew more slowly than those of *O. quercus* at 25 °C, and faster than the latter fungus at 30 °C. Although these morphological characteristics appear stable, speciation does not necessarily involve morphological changes. Therefore, genetic diversity and the use of molecular sequence data is especially important to map boundaries for species delineation and to recognize closely related species (Bickford *et al*. 2006; Noor & Feder 2006) such as those in this study.

Mating studies between authentic *O. quercus* isolates and the African isolates now known as *O. tsotsi* showed that isolates predominantly mated with those of their own group. However, a rare case of interfertility was observed where one isolate from each of the distinct taxa mated, but the resulting ascospore progeny had reduced germination capacity. This is not uncommon in the fungi where there are other examples of speciation, without full reproductive isolation (Kohn 2005; Taylor *et al*. 2000; Engelbrecht & Harrington 2005). This system of partial interfertility, can exist between species (Brasier 1993), however, they do have a lower ascospore germination percentage with fewer ascospores produced than that seen at intraspecific level (Harrington & McNew 1998).

Ophiostoma tsotsi discovered in this study commonly occurs on cut timber throughout southern Africa. Interestingly, this new taxon is frequently found in association with *O.*

quercus and it is not surprising that it remained hidden from recognition as a discrete entity until molecular genetic comparisons of isolates could be undertaken. *O. quercus* is considered to be a species of northern hemisphere origin (Harrington *et al.* 2001) and its presence on lumber in Africa would imply that it has been introduced into this region. This could easily have occurred with wood and wood products transported from the northern hemisphere to Africa. Although movement of wood products from Africa to northern hemisphere countries would have been less common, it is also possible that *O. tsotsi* has a distribution more extensive than is currently known. Population genetic studies on *O. quercus* and *O. tsotsi* will contribute to a deeper understanding of the worldwide distribution and origin of these fungi. In addition, the biology, pathogenicity, and ecological role of *O. tsotsi* is unknown. In view of its relatedness to the Dutch Elm disease fungi, which have caused significant losses to elm species in forest ecosystems, these aspects of its ecology deserves serious consideration.

Acknowledgements

We thank Prof. Jolanda Roux and Ronald Heath and various colleagues who assisted us in collecting isolates used in this study. We would like to acknowledge our African collaborators; Gerald Meke from the Forestry Research Institute of Malawi, Aza Mbaga from the Tanganyika Wattle Company, Tanzania and the Forestry Department, Uganda and Makerere University, Uganda. We are also grateful to Dr Seonju Marincowitz for assisting with light microscopy and Mr Alan Hall for assistance with the scanning electron microscopy. We acknowledge Dr Hugh Glen for providing the Latin diagnosis. Funding for this study was kindly provided by the National Research Foundation (NRF), the Tree Protection Co-operative Programme (TPCP), the THRIP initiative of the Department of Trade and Industry (DTI), and the Department of Science and Technology (DST)/ NRF Centre of Excellence in Tree Health Biotechnology, South Africa.

References

Bickford D, Lohman DJ, Sodhi NS, Ng PKL, Meier R, Winker K, Ingram KK, Das I, 2006. Cryptic species as a window on diversity and conservation. *Trends in Ecology & Evolution* **22**: 148-155.

Brasier CM, 1990. China and the origins of Dutch elm disease: an appraisal. *Plant Pathology* **39**: 5-16.

Brasier CM, 1991. *Ophiostoma novo-ulmi* sp. nov., causative agent of current Dutch elm disease pandemics. *Mycopathologia* **115**: 151-161.

Brasier CM, 1993. The genetic system as a fungal taxonomic tool: gene flow, molecular variation and sibling species in the '*Ophiostoma piceae* - *Ophiostoma ulmi*' complex and its taxonomic and ecological significance. *Ceratocystis and Ophiostoma: Taxonomy, Ecology and Pathogenicity*. (Wingfield MJ, Seifert KA, Webber JF, eds), The American Phytopathological Society, St. Paul, Minnesota pp. 77-92.

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

Carlier F-X, Decock C, Jacobs K, Maraite H, 2006. *Ophiostoma arduennense* sp. nov. (Ophiostomatales, Ascomycota) from *Fagus sylvatica* in southern Belgium. *Mycological Research* **110:** 801-810.

De Beer ZW, Wingfield MJ, Kemp GHJ, 1995. First report of *Ophiostoma querci* in South Africa. *South African Journal of Science* **91**: 6.

De Beer ZW, Wingfield BD, Wingfield MJ, 2003b. The *Ophiostoma piceae* complex in the Southern Hemisphere: a phylogenetic study. *Mycological Research* **107:** 469-476.

Engelbrecht CJB, Harrington TC, 2005. Intersterility, morphology and taxonomy of *Ceratocystis fimbriata* on sweet potato, cacao and sycamore. *Mycologia* **97**: 57-69.

Felsenstein J, 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **39**: 783-791.

Gardes M, Bruns TD, 1993. ITS primers with enhanced specificity for basidiomycetes - application to the identification of mycorrhizae and rust. *Molecular Ecology* **2**: 113-118.

Georgevitch Y, 1926. *Ceratostomella querci* n.sp. *Comptes Rendus Hebdomadaires des Séances de l'Académie des Sciences* **183** : 759-761.

Georgevitch Y, 1927. *Ceratostomella quercus* n. sp. Ein Parasit der slawonischen Eichen. *Biologia Generalis* **3:** 245-252.

Geldenhuis MM, Roux J, Montenegro F, de Beer ZW, Wingfield MJ, Wingfield BD, 2004. Identification and pathogenicity of *Graphium* and *Pesotum* species from machete wounds on *Schizolobium parahybum* in Ecuador. *Fungal Diversity* **15**: 135-149.

Glass NL, Donaldson GC, 1995. Development of primer sets designed for use with PCR to amplify conserved genes from filamentous Ascomycetes. *Applied and Environmental Microbiology* **61**: 1323-1330.

Grobbelaar JW, Barnes I, Cortinas M-N, Bloomer P, Wingfield MJ, Wingfield BD, 2009a. Development and characterization of polymorphic markers for the sap-stain fungus *Ophiostoma quercus*. *Molecular Ecology Resources* **9:** 399-401.

Grobbelaar JW, Aghayeva D, de Beer ZW, Bloomer P, Wingfield MJ, Wingfield BD, 2009b. Delimitation of *Ophiostoma quercus* and its synonyms using multiple gene phylogenies. Chapter Two.

Harrington TC, McNew D, Steimel J, Hofstra D, Farrell R, 2001. Phylogeny and taxonomy of the *Ophiostoma piceae* complex and the Dutch elm disease fungi. *Mycologia* **93**: 111-136.

Harrington TC, McNew DL, 1998. Partial interfertility among the *Ceratocystis* species on conifers. *Fungal Genetics and Biology* **25**: 44-53.

Hausner G, Reid J, 2003. Notes on *Ceratocystis brunnea* and some other *Ophiostoma* species based on partial ribosomal DNA sequence analysis. *Canadian Journal of Botany* **81:** 865-876.

Jacobs K & Kirisits T, 2003. *Ophiostoma kryptum* sp.nov. from Larix decidua and Picea abies in Europe, similar to *O. minus*. *Mycological Research* **107:** 1231-1242.

Jacobs K, Seifert KA, Harrison KJ, Kirisits T, 2003. Identity and phylogenetic relationships of ophiostomatoid fungi associated with invasive and native *Tetropium* species (Coleoptera: Cerambycidae) in Atlantic Canada. *Canadian Journal of Botany* **81:** 316-329.

Jacobs K, Bergdahl DR, Wingfield MJ, Halik S, Seifert KA, Bright DE, Wingfield BD, 2004. *Leptographium wingfieldii* introduced into North America and found associated with exotic *Tomicus piniperda* and native bark beetles. *Mycological Research* **108**: 411-418.

Kamgan NG, Jacobs K, de Beer ZW, Wingfield MJ, Roux J, 2008a. *Pesotum australi* sp. nov. and *Ophiostoma quercus* associated with *Acacia mearnsii* trees in Australia and Uganda, respectively. *Australasian Plant Pathology* **37**: 406-416

Kamgan NG, Jacobs K, De Beer ZW, Wingfield MJ, Roux J, 2008b. *Ceratocystis* and *Ophistoma* species, including three new taxa, associated with wounds on native South African trees. *Fungal Diversity* **29**: 37-59..

Katoh K, Toh H, 2008. Recent developments in the MAFFT multiple sequence alignment program. *Briefings in Bioinformatics* **doi:10.1093/bib/bbn013, bbn013**.

Kohn LM, 2005. Mechanisms of Fungal Speciation. *Annual Review of Phytopathology* **43**: 279-308.

Kowalski T, Butin H, 1989. Taxonomie bekannter und neuer *Ceratocystis*-Arten an Eiche (*Quercus robur* L.). *Journal of Phytopathology* **124**: 236-248.

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

Masuya H, Kubono T, Ichihara Y, 2003. *Ophiostoma ssiori* sp. nov. (Ophiostomatales, Ascomycetes) isolated from a bark beetle in *Prunus* species. *Bulletin of the National Science Museum, Tokyo,* **29:** 35-43.

Noor MAF, Feder JL, 2006. Speciation genetics: evolving approaches. *Nature Reviews* **7**: 851-861.

O'Donnell K, Cigelnik E, 1997. Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. *Molecular Phylogenetics and Evolution* **7:** 103-116.

Posada D, Crandall KA, 1998. MODELTEST: testing the model of DNA substitution. *Bioinformatics* **14:** 817-818.

Przybyl K, Morelet M, 1993. Morphological differences between *Ophiostoma piceae* and *O. querci*, and among *O. querci* isolates. *Cryptogamie, Mycologie* **14**: 219-228.

Ronquist F, Heulsenbeck JP, 2003. MRBAYES 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19:** 1572-1574.

Seifert KA, 1993. Sapstain of commercial lumber by species of *Ophiostoma* and *Ceratocystis*. *Ceratocystis and Ophiostoma: Taxonomy, Ecology and Pathogenicity*, (Wingfield MJ, Seifert KA, Webber J, eds), The American Phytopathological Society, St Paul, Minnesota pp. 141-151.

Swofford DL, 2002. *PAUP* Phylogenetic Analysis Using Parsimony (*and other methods),* version 4. *Sinauer Associates, Sunderland, Massachusetts.*

Taylor JW, Jacobson DJ, Kroken S, Kasuga T, Geiser DM, Hibbett DS, Fisher MC, 2000. Phylogenetic Species Recognition and Species Concepts in Fungi. *Fungal Genetics and Biology* **31:** 21-32.

Thwaites JM, Farrell RL, Duncan SM, Reay SD, Blanchette RA, Hadar E, Hadar Y, Harrington TC, McNew D, 2005. Survey of potential sapstain fungi on *Pinus radiata* in New Zealand. *New Zealand Journal of Botany* **43**: 653-663.

Upadhyay HP, 1981. *A monograph of Ceratocystis and Ceratocystiopsis*. University of Georgia Press, Athens, Georgia, USA.

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

White TJ, Bruns TD, Lee S, Taylor JW, 1990. *Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics*. New York Academic Press, New York.

Zhou XD, De Beer ZW, Ahumada R, Wingfield BD, Wingfield MJ, 2004. *Ophiostoma* and *Ceratocystiopsis* spp. associated with two pine-infesting bark beetles in Chile. *Fungal Diversity* **15:** 261-274.

Zhou X, De Beer ZW, Wingfield MJ, 2006. DNA sequence comparisons of *Ophiostoma* spp., including *Ophiostoma aurorae* sp. nov., associated with pine bark beetles in South Africa. *Studies in Mycology* **55**: 269-277.

Zipfel RD, De Beer ZW, Jacobs K, Wingfield BD, Wingfield MJ, 2006. Multigene phylogenies define *Ceratocystiopsis* and *Grosmannia* distinct from *Ophiostoma*. *Studies in Mycology* **55:** 75-97.

Fig 1 Phylogram resulting from a Bayesian Monte Carlo Markov chain (MCMC) analyses of the ITS sequences. The posterior probability (pp) support values are given first followed by the ML bootstrap (bs) values (1000 replicates) for each node respectively (scores of 0.90 pp or 70 bs and less have not been included and are indicated with '-'. Bootstrap support values (1000 replicates) above 75% for Maximum parsimony are indicated with bold lines at the relevant branching points. Isolate numbers of unique sequences obtained in this study are printed in bold type.

Fig 2 Phylogram resulting from a Bayesian Monte Carlo Markov chain (MCMC) analyses of the β-tubulin sequences. The posterior probability (pp) support values are given first followed by the ML bootstrap (bs) values (1000 replicates) for each node respectively (scores of 0.90 pp or 70 bs and less have not been included and are indicated with '-'. Bootstrap support values (1000 replicates) above 75% for Maximum parsimony are indicated with bold lines at the relevant branching points. Isolate numbers of unique sequences obtained in this study are printed in bold type.

Fig 3 Phylogram resulting from a Bayesian Monte Carlo Markov chain (MCMC) analyses of the TEF1-α sequences. The posterior probability (pp) support values are given first followed by the ML bootstrap (bs) values (1000 replicates) for each node respectively (scores of 0.90 pp or 70 bs and less have not been included and are indicated with '-'. Bootstrap support values (1000 replicates) above 75% for Maximum parsimony are indicated with bold lines at the relevant branching points. Isolate numbers of unique sequences obtained in this study are printed in bold type.

Fig 4 Comparison of mean growth in 10 days on MEA (three isolates per tested species, (± standard deviation) of *O.quercus* (purple) and *O. tsotsi* (grey) held at different temperatures (grown 10 d in the dark).

Fig 5 Morphological characteristics of *Ophiostoma tsotsi* (from holotype) (CMW 3117 x CMW15239) 1. Perithecium (bar = 100 µm), 2. Scanning electron micrograph (SEM) of ostiolar hyphae at the end of a perithecial neck (bar = 10 µm), 3. Ascospores, side view above and face view below (bar = 10 µm), 4. Synnematous *Pesotum* (bar = 100 µm) 5. SEM of a young conidiophore (bar = 10 µm), 6. SEM of conidia (bar = 5 µm), 7. *Sporothrix* conidiogenous cell (bar = 100 µm), 8. SEM of a *Sporothrix* anamorph (bar = 10 µm), 9. SEM of a *Sporothrix* conidiogenous cell with emerging conidia (bar = 10 µm).

CHAPTER 5

DISCOVERY OF *OPHIOSTOMA TSOTSI* ON *EUCALYPTUS* WOOD CHIPS IN CHINA

Abstract

Ophiostoma **species such as** ***O. quercus*** **are the most frequent causal agents of sapstain of freshly felled hardwood timber and pulpwood. Many species are regarded as economically important agents of wood degradation. The aim of this study was to identify a collection of** ***Ophiostoma*** **isolates, resembling** ***O. quercus*****, found on stained** ***Eucalyptus*** **pulpwood chips in China. Culture morphology and DNA sequences of the β-tubulin region revealed that the isolates were not** ***O. quercus*****. Surprisingly, they represented** ***O. tsotsi*****, a wound-infesting fungus recently described from hardwood in Africa. The study also confirms the occurrence of** ***O. quercus*** **from native** ***Tsuga*** **in China and native** ***Quercus*** **in Bhutan. A high level of genetic variability was observed among isolates of both** ***O. quercus*** **and** ***O. tsotsi.*** **This was unexpected and suggests that both species have been present in Asia for a significant amount of time.**

Keywords: *Ophiostoma tsotsi*, *Ophiostoma quercus*, blue-stain fungi, *Ophiostoma piceae* complex, new species

Introduction

Eucalyptus spp. are becoming increasingly widely planted for sustainable commercial plantations in many countries due to their complementary wood qualities, adaptability to a wide range of different environments and their rapid growth potential (Turnbull 2000). They are planted extensively in South-East Asia where the timber is mainly used for paper, oil and pulp production. About 30% of China's 1.75 million ha of forests are commercial plantations, which consist of *Eucalyptus* and *Corymbia* species, hybrids and clones (Annonymous 2006).

A number of known and novel forest pathogens have emerged from recent surveys on *Eucalyptus* in China (Butterworth & Lei 2005; Zhou *et al*. 2007, 2008). However, these

lists include only a single ophiostomatoid fungus, an uncharacterized *Ceratocystis* sp. (Zhou *et al.* 2008). This, despite the fact that in recent years numerous *Ceratocystis* and *Ophiostoma* species have been associated with disease and blue-stain on commercial *Eucalyptus* trees, timber and pulpwood (De Beer *et al.* 2003a, b; Roux *et al.* 2004; Van Wyk *et al.* 2007; Rodas *et al.* 2008). These fungal infections most often occur through wounds in the bark and sapwood of trees caused by commercial harvesting practices or animal damage. The exposed sapwood is susceptible to colonization of ophiostomatoid fungi, vectored by a large variety of relatively non-specific insects (Seifert 1993).

One of the ophiostomatoid fungi most frequently isolated from exposed sapwood or *Eucalyptus* pulpwood chips is *Ophiostoma quercus* (De Beer *et al.* 2003a, b). This species is a ubiquitous sap-stain fungus of primarily hardwoods, and to a lesser extent conifers, with a global distribution (Brasier & Kirk 1993; Harrington *et al.* 2001; Geldenhuis *et al*. 2004; Thwaites *et al.* 2004; Zhou *et al*. 2004; Kamgan Nkuekam *et al*. 2008a, b; Linnakoski *et al.* 2008). It is perhaps surprising that this common fungus was only recently reported from East Asia (Lin *et al.* 2003; Kim *et al.* 2005; Chung *et al.* 2006; Paciura *et al.* 2009).

As part of an ongoing survey of fungi infecting *Eucalyptus* and *Corymbia* species in China (Zhou *et al*. 2008), *Eucalyptus* pulpwood chips collected in the Guangdong province in the southern part of China mainland, were screened for the presence of ophiostomatoid fungi. A collection of cultures with *Pesotum* anamorphs reminiscent of the anamorph of *O. quercus* was isolated from the chips. The aim of this study was to determine the identity of these isolates using culture morphology and DNA sequence comparisons of part of the β-tubulin gene region, shown to be adequate for distinguishing between *O. quercus* and closely related species (Grobbelaar *et al.* 2009a, c).

Methods and Materials

Collection and isolation of fungi

Eucalyptus pulpwood chips were collected from a small commercial chipping factory in LeiZhou, China. The wood chips were incubated in moist chambers at 25 °C until fruiting structures appeared. Isolations were made and purified as described by Kamgan Nkuekam *et al.* (2008a). For reference purposes, several additional isolates, tentatively identified as *O. quercus,* from other hosts and countries were included (Table 1). All of the isolates sequenced in this study are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI) and a duplicate set in the China Eucalypt Research Centre (CERC).

Culture and anamorph morphology

Single spore cultures from germinating ascospores or conidia were prepared for all isolates obtained in this study. Isolates were grown on 2% malt extract agar, MEA (Biolab. Midrand, South Africa) at room temperature for 10 days. Culture morphology was compared to descriptions of those of *O. quercus* and closely related species. Fruiting structures were mounted in lactophenol and examined using a compound microscope.

DNA extraction, PCR and sequencing

Genomic DNA was extracted from actively growing fungal mycelium using the method described by Linnakoski *et al.* (2008). To amplify part of the β-tubulin region, the primers T10 (O'Donnell & Cigelnik 1997) and Bt2b (Glass & Donaldson 1995) were used. The PCR reaction mixture (50 µl final volume) contained five units of Expand *Taq* Polymerase (Roche Biochemicals, Mannheim, Germany), 1x PCR reaction buffer, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.1 mM of each primer, and 1 µl DNA template. PCR reactions were performed in a thermal cycler (Hoffman-La Roche, Nutley, NJ, USA) using an initial denaturation at 94 °C for 1 min, followed by 30 cycles denaturation

at 94 °C for 1 min, primer annealing at 53–55 °C (depending on the isolate) for 1 min, and elongation at 72 °C for 1 min. A final extension step at 72 °C for 5 min completed the program.

DNA sequence analyses and phylogenetic reconstruction

Sequence data were analysed and contigs assembled using the Vector NTI Advance 10 software (Invitrogen, Carlsbad, CA). For reference purposes, published sequences of species closely related to *O. quercus* were obtained from GenBank. These included of *O. piceae* (Münch) H. & P. Sydow, *O. himal-ulmi* Brasier & M.D. Mehrotra, *O. novo-ulmi* Brasier, *O. floccosum* Mathiesen, *O. setosum* Uzunovic, Seifert, Kim &Breuil, *Pesotum australi* Kamgan-Nkuekam, Jacobs & M.J. Wingf., the ex-type isolate of *O. catonianum*, and the ex-neotype of *O. quercus*. For the *O. tsotsi* isolates, cultures and sequence data from the ex-holotype and -paratype cultures were included (Grobbelaar *et al.* 2009c). Genbank accession numbers of published sequences are listed in Fig. 1. The complete data set was aligned in MAFFT v 5.731, option E-INS (Katoh *et al.* 2005), then edited manually.

Relationships among isolates were examined using two different methods of phylogenetic analysis based on the β-tubulin sequence data: Maximum Likelihood (ML) (Aldrich 1997) and Bayesian Inference (BI) (Heulsenbeck & Ronquist 2001), rooted against *O. piceae* as outgroup. Gaps were treated as 5th characters.

For ML, the most appropriate model of sequence evolution (GTR+I+G) was selected from a set of 56 hierarchically nested models using the AIC test in MODELTEST v 3.7 (Posada & Crandall, 1998). The ML analysis was conducted using the best model results in PHYML (Guindon & Gascuel 2003). Nonparametric bootstrap analyses (Felsenstein 1985) employed one heuristic search for each of the 1000 bootstrap replicates under maximum likelihood.

The BI analysis was performed in MRBAYES v 3.1.2 (Heulsenbeck *et al.*, 2001) using the best model (GTR+I+G) selected by the AIC test in MRMODELTEST v 2.2

(http://www.abc.se/~nylander/). Model parameters were derived from the default prior distributions. The MRBAYES analysis comprised two independent runs of 5 million generations using duplicate Monte Carlo Markov chain searches and a sampling frequency of every 100 generations. Stationarity for LnL and nucleotide substitution parameters was achieved within the first 590 000 generations. Consequently, the first 5900 trees sampled were discarded as burn-in before the consensus topology and posterior probabilities were calculated.

Results

Culture and anamorph morphology

There was substantial variability in culture and anamorph morphology among the isolates from China and elsewhere, but all corresponded relatively to culture descriptions for *O. quercus* (Morelet, 1992; Halmschlager *et al*., 1994; Harrington *et al.* 2001), *Pesotum australi* (Kamgan Nkuekam see refs *et al.* 2008b), and *O. tsotsi* (Grobbelaar *et al.,* 2009c). However, none of the Chinese or other isolates could be conclusively assigned to one of the three species based on phenotypic characters.

DNA sequence analyses and phylogenetic reconstruction

Amplicons from the partial β-tubulin gene region had a length of approximately 580 base pairs. The aligned data set consisted of 41 isolates. Results from the ML and BI yielded concordant topologies with respect to the composition of the clades. The *Tsuga* isolates from China and *Quercus* isolates from Bhutan grouped with *O. quercus* isolates from various countries and hosts (group A, Fig. 1), including the ex-neotype isolate (CBS 117913) for the species. The *Eucalyptus* isolates from China all grouped in a monophyletic lineage (group B) with the African isolates of *O. tsotsi.* Both groups (A and B) exhibited good statistical support and the Bayesian topology revealed considerable variation within both *O. quercus* and *O. tsotsi*. The various taxa studied separated from

one another in distinct groups supported with high posterior probabilities and bootstrap values.

Discussion

In this study, the occurrence of *O. tsotsi* in China on wood of exotic *Eucalyptus* trees was observed. This is the first time the fungus has been reported outside of Africa. In addition the presence of *O. quercus* is reported on native *Tsuga* in China and native *Quercus* in Bhutan. Sequence data of the partial β-tubulin gene region also showed substantial variability within both species.

Ophiostoma tsotsi is known only from Africa, both from exotic *Eucalyptus* and native hardwoods (Grobbelaar *et al.* 2009c). The discovery of the fungus on *Eucalyptus* in China alters our perceptions regarding a possible African origin for the fungus. The genetic variability among isolates from both Africa and China is indicative of widespread sexual recombination. In addition, if the fungus was in fact introduced into a region, it is likely that there were multiple introductions. However, only more extensive sampling from native eucalypts in Australasia and exotic eucalypt plantations in areas such as South America could provide conclusive answers to questions regarding the origin of the fungus.

The origin of *O. quercus* has briefly been considered in previous studies. Brasier and Kirk (1993) mentioned the possibility that *O. quercus* was introduced from Europe to North America based on the prevalence of a single mating type in North America. Harrington *et al.* (2001) speculated regarding a Northern Hemisphere origin for the fungus based on its presence on exotics in the Southern Hemisphere and North America. De Beer *et al.* (2003b) suggested the occurrence of the fungus on various native hosts in several Southern Hemisphere countries, and its ability to grow at higher temperatures than *O. piceae*, might indicate that it is native to the Southern Hemisphere. The reports of *O. quercus* from native hardwoods in Azerbaijan (Grobbelaar *et al.* 2009a), Bhutan (the present study), China (Lin *et al.* 2003; Paciura *et al.* 2009), Korea (Kim *et al.* 2005),

Japan (Chung *et al.* 2006), and Australia (Kamgan Nkuekam *et al.* 2008), confounds all previous suggestions regarding its origin. The high level of morphological and genetic variability within localized collections of *O. quercus* (De Beer *et al*. 2003; Grobbelaar *et al.* 2009a, b; Paciura *et al.* 2009) also contributes to confusion regarding the origin of this widely distributed fungus.

The phenotypic and genotypic variability amongst isolates of both *O. tsotsi* and *O. quercus* deserves further investigation. The influence of host specialization and geographical location also needs consideration, as does a long history of human movement of timber across and between continents. Numerous unanswered questions thus remain regarding the biology and ecological role of these economically important fungi to the global forestry industry.

Acknowledgements

We thank Prof. Jolanda Roux, Gilbert Kamgan and Ronald Heath for sharing the African isolates with us. We would like to acknowledge our African collaborators; Gerald Meke from the Forestry Research Institute of Malawi, Aza Mbaga from the Tanganyika Wattle Company, Tanzania and the Forestry Department, Uganda and Makerere University, Uganda. We also thank Dr Thomas Kirisits for providing cultures from Bhutan and various colleagues who assisted us in collecting isolates used in this study. We acknowledge the National Research Foundation (NRF), members of the Tree Protection Co-operative Programme (TPCP), the THRIP initiative of the Department of Trade and Industry (DTI), and the Department of Science and Technology (DST) South Africa for financial support. This work forms part of on-going cooperation between South Africa and China, and is funded through the projects of 2007DFA31190 and 2006BAD08A11 from Ministry of Science and Technology of China (MOST).

References

Aldrich J, 1997. R.A. Fisher and the making of maximum likelihood 1912-1922. *Statistical Science* **12:** 162-176.

Anonymous, 2006. The sixth survey of Chinese forestry resources. China State Forestry Admistration. [in Chinese].

Brasier CM, Kirk SA, 1993. Sibling species within *Ophiostoma piceae*. *Mycological Research* **97**: 811-816.

Butterworth J, Lei Z, 2005. China, Peoples Republic of Solid Wood Products. China's Sixth Forest Resource Inventory Report. *USDA Foreign Agricultural Service, Global Agricultural Information Network Report Number: CH5027. 6p*.

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

De Beer ZW, Wingfield BD, Wingfield MJ, 2003a. The occurrence of *Ophiostoma piliferum*-like fungi on pulpwood chips and other wood sources in South Africa. *South African Journal of Science* **99**: 34-36.

De Beer ZW, Wingfield BD, Wingfield MJ, 2003b. The *Ophiostoma piceae* complex in the Southern Hemisphere: a phylogenetic study. *Mycological Research* **107**: 469-476.

Felsenstein J, 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **39:** 783-791.

Geldenhuis MM, Roux J, Montenegro F, De Beer ZW, Wingfield MJ, Wingfield BD, 2004. Identification and pathogenicity of *Graphium* and *Pesotum* species from machete wounds on *Schizolobium parahybum* in Ecuador. *Fungal Diversity* **15**: 135-149.

Glass NL, Donaldson GC, 1995. Development of primer sets designed for use with PCR to amplify conserved genes from filamentous Ascomycetes. *Applied and Environmental Microbiology* **61**: 1323-1330.

Grobbelaar JW, Aghayeva DN, De Beer ZW, Bloomer P, Wingfield MJ, Wingfield BD, 2009a. Delimitation of *Ophiostoma quercus* and its synonyms using multiple gene phylogenies. Chapter Two.

Grobbelaar JW, Barnes I, Cortinas M-N, Bloomer P, Wingfield MJ, Wingfield BD, 2009b. Development and characterization of polymorphic markers for the sap-stain fungus *Ophiostoma quercus*. *Molecular Ecology Resources* **9:** 399-401.

Grobbelaar JW, De Beer ZW, Bloomer P, Wingfield MJ, Wingfield BD, 2009c. *Ophiostoma tsotsi* sp. nov., a new sap-staining agent from hardwoods in Africa. Chapter Four.

Guindon S, Gascuel O, 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by Maximum Likelihood. *Systematic Biology* **52**: 696-704.

Halmschlager E, Messner R, Kowalski T, Prillinger H, 1994. Differentiation of *Ophiostoma piceae* and *Ophiostoma quercus* by Morphology and RAPD Analysis. *Systematic and applied Microbiology* **17**: 554-562.

Harrington TC, McNew D, Steimel J, Hofstra D, Farrell R, 2001. Phylogeny and taxonomy of the *Ophiostoma piceae* complex and the Dutch elm disease fungi. *Mycologia* **93**: 111-136.

Heulsenbeck JP, Ronquist F, 2001. MRBAYES: Bayesian inference of phylogeny. *Bioinformatics* **17:** 754-755.

Heulsenbeck JP, Ronquist F, Nielsen R, Bollback JP, 2001. Bayesian Inference of Phylogeny and its impact on Evolutionary Biology. *Science* **294**: 2310-2314.

Kamgan Nkuekam G, Jacobs K, de Beer ZW, Wingfield MJ, Roux J, 2008a. *Ceratocystis* and *Ophiostoma* species including three new taxa, associated with wounds on native South African trees. *Fungal Diversity* **29**: 37-59.

Kamgan Nkuekam G, Jacobs K, de Beer ZW, Wingfield MJ, Roux J, 2008b. *Pesotum australi* sp. nov. and *Ophiostoma quercus* associated with *Acacia mearnsii* trees in Australia and Uganda, respectively. *Australasian Plant Pathology* **37**: 406-416.

Katoh K, Kuma K-I, Toh H, Miyata T, 2005. MAFFT version 5: improvement in accuracy of multiple sequence alignment. *Nucleic Acids Research* **33**: 511-518.

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

Lin TC, Huang JW, Hsieh WH, 2003. Identification of ophiostomatoid fungi associated with Chinese fir wilt in Taiwan. [in Chinese] *Plant Pathology Bulletin* **12**: 33-42.

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

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

O'Donnell K, Cigelnik E, 1997. Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. *Molecular Phylogenetics and Evolution* **7**: 103-116.

Paciura D, Zhou XD, De Beer ZW, Jacobs K, Wingfield MJ, 2009. Characterisation of synnematous bark beetle-associated fungi from China, including *Graphium carbonis* sp. nov. *Fungal Diversity*. In Press.

Posada D, Crandall KA, 1998. MODELTEST: testing the model of DNA substitution. *Bioinformatics* **14**: 817-818.

Rodas CA, Roux J, van Wyk M, Wingfield BD, Wingfield MJ, 2008. *Ceratocystis neglecta* sp. nov., infecting *Eucalyptus trees* in Colombia *Fungal Diversity* **28**: 73-84.

Roux J, Van Wyk M, Hatting H, Wingfield MJ, 2004. *Ceratocystis* species infecting stem wounds on *Eucalyptus grandis* in South Africa. *Plant Pathology* **53**: 414-421.

Seifert KA, 1993. Sapstain of commercial lumber by species of *Ophiostoma* and *Ceratocystis*. In: *Ceratocystis and Ophiostoma: Taxonomy, Ecology and Pathogenicity* (eds. Wingfield MJ, Seifert KA, Webber J). The American Phytopathological Society, St Paul, Minnesota, pp. 141-151.

Thwaites JM, Farrell RL, Hata K, Carter P, Lausberg M, 2004. Sapstain fungi on *Pinus radiata* logs – from New Zealand Forest to export in Japan. *Journal of Wood Science* **50**: 459-465.

Turnbull JW, 2000. Economic and social importance of eucalypts. In: *Diseases and Pathogens of Eucalypts* (eds. Keane PJ, Kile GA, Podger FD, Brown BN), CSIRO Publishing, Australia, pp. 1-9.

Van Wyk M, Pegg G, Lawson S, Wingfield MJ, 2007. *Ceratocystis atrox* sp. nov. associated with *Phoracantha acanthocera* infestations on *Eucalyptus grandis* in Australia. *Australasian Plant Pathology* **36**: 407-414.

Zhou XD, de Beer ZW, Ahumada R, Wingfield BD, Wingfield MJ 2004. *Ophiostoma* and *Ceratocystiopsis* spp. associated with two pine-infesting bark beetles in Chile. *Fungal Diversity* **15**: 261-274.

Zhou XD, De Beer ZW, Xie YJ, Pegg GS, Wingfield MJ, 2007. DNA-based identification of *Quambalaria pitereka* causing severe leaf blight of *Corymbia citriodora* in China. *Fungal Diversity* **25**: 245-254.

Zhou XD, Xie Y, Chen S, Wingfield MJ, 2008. Diseases of eucalypt plantations in China: challenges and opportunities. *Fungal Diversity* **32**: 1-7.

Fig 1 Phylogram resulting from a Bayesian Monte Carlo Markov chain (MCMC) analysis of the β-tubulin sequences. The posterior probability (pp) support values are given first followed by the ML bootstrap (bs) values (1000 replicates) in bold type for each node respectively (scores of 0.95 pp or 70 bs and less have not been included). T indicates ex-type isolates of species and NT points out the ex-neotype.

CHAPTER 6

INTRASPECIFIC VARIATION IN A GENERALIST ASCOMYCETE FUNGUS, *OPHIOSTOMA QUERCUS*, REVEALS GENETIC STRUCTURE AT DIFFERENT GEOGRAPHIC SCALES

Abstract

In order to appreciate the evolution and biology of a species, it is important to understand the extent to which its genetic variation is geographically structured at different levels. This is also of practical relevance to the study and control of fungi including the wood-inhabiting fungus, *Ophiostoma quercus*. Here we report on variation at five independent nuclear loci (SSR and β-tubulin) sequenced for 122 isolates representative of a large proportion of the global range of *O. quercus*. These isolates were widely collected from various tree hosts to assay allelic variation and to determine structuring at regional, continental and global scales. Sequence and repeat variation at the SSR loci yielded 62 alleles, while 16 haplotypes were observed for the β-tubulin gene region. A collection of Norwegian isolates showed moderate levels of regional genetic diversity, especially in southern Norway. At a continental scale, it was interesting that inferred ancestral SSR alleles were found at high frequencies among the African isolates where the fungus is thought to have been introduced. Bayesian inference to establish the number of sub-populations revealed four clusters and assignment of individual isolates to these groups showed a clear signal of admixture in some of the sampled localities. At a global scale, the sampled regions were not equally variable and neither were they equally connected via gene flow. These patterns of differentiation are interpreted as resulting from long distance movement of forest products carrying *O. quercus* with them.

Keywords: *Ophiostoma quercus*, blue-stain fungi, *O. piceae* complex, intraspecific genetic diversity, sequence variation, geographic scale

Introduction

An understanding of the extent to which intraspecific genetic variation is geographically structured at different levels (e.g. continental, global) is needed to fully appreciate the evolution and biology of a species. Introduced species are predicted to experience reduced levels of genetic diversity in their non native ranges (Bossdorf *et al.* 2005) and in

the case of fungi, very little is known about the invasive potential of this group of organisms (Desprez-Loustau *et al.* 2007) or their impact on biodiversity and agriculture (Palm 2001). Biological invasions of fungi in new forest ecosystems are increasing as a result of globalization, with changes in natural and human mediated movement and climate change (Anderson *et al.* 2004). Introductions of new organisms, whether endophytic or pathogenic, may be unintended, for example via the introduction of non-native tree species in forestry (Wingfield *et al.* 2001). These introductions may be facilitated by different factors including insect dissemination (Barras & Perry 1975), aerial dispersion (Brown & Hovmøller 2002) or anthropogenic movement.

Ongoing human-mediated introductions and habitat modification, as a result of deforestation or the development of commercial plantations have an impact on plant-pathogen interactions by disturbing the natural populations of fungi (Brasier 2000; Brasier *et al.* 1998). In the case of the ophiostomatoid fungi (Wingfield *et al.* 1993), the genus *Ophiostoma* accommodates more than 100 fungi including human pathogens (Mariat *et al.* 1968), saprophytes colonizing tree sapwood (Jacobs & Wingfield 2001; Wingfield *et al.* 1993) and serious tree pathogens, such as the fungi that cause Dutch elm disease (Brasier 1990; 1991). *Ophiostoma quercus* is a weakly pathogenic, yet commercially important species of *Ophiostoma* with a cosmopolitan distribution and that is highly adaptable in new and existing niches (Harrington *et al*. 2001; Morelet 1992). This fungus typically infects freshly cut or wounded angiosperm wood, although it is sometimes also found on conifers (Thwaites *et al*. 2005; Webber & Brasier 1991). In this niche, it causes a blue/black-stain of the sapwood reducing the economic value of lumber (Seifert 1993).

Most *Ophiostoma* spp. are vectored by bark beetles and mites, particularly those that infest conifers (Bridges & Moser 1983; Upadhyay 1981). In contrast, *O. quercus* usually occurs in a non-specific relationship with its vectors that are typically sap-feeding insects that visit freshly wounded timber. *O. quercus* is a haploid fungus with a heterothallic mating system (Harrington *et al.* 2001) that promotes outbreeding because two opposite mating types are necessary for the production of fertile progeny.

Most wood-inhabiting *Ophiostoma* species appear to have their origins in the northern hemisphere, where many of their host trees are native (Harrington *et al.* 2001; Przybyl & Morelet 1993; Wingfield *et al*. 1993). However, as interest in the blue-stain fungi has increased in the southern hemisphere, many known and new species have been reported or described from this part of the world (de Beer *et al.* 1995; 2003; Geldenhuis *et al.* 2004; Thwaites *et al.* 2005). Some of these fungi have been accidentally introduced, but many are also likely to be native to the southern hemisphere, where novel species have recently been isolated from native hosts (Grobbelaar *et al.* 2009b; Kamgan Nkuekam *et al.* 2008).

Wood products are known to have been extensively moved around the world since the late 1800's (Elton 1958) and this has also led to the emergence of devastating tree diseases. It is thus likely that common wood-inhabiting fungi such as *O. quercus* might also have been introduced into many new environments in a similar way. It may also be possible that the saprophytic nature of *O. quercus* has resulted in its cosmopolitan distribution without barriers to its dispersal over very long periods of time. It would then occur naturally on freshly cut angiosperm wood in all parts of the world where the climate is suitable for it to be present.

The distribution and degree of genetic diversity over large geographical scales can reflect the identity of populations with the most variable area potentially representing the geographic origin of the species (Banke & McDonald 2005; Crandall & Templeton 1999). Many studies have referred to the high degree of phenotypic variability in *O. quercus*, noting its varied morphological characteristics (Halmschlager *et al.* 1994; Morelet 1992; Przybyl & Morelet 1993). However, nothing is known regarding its genetic diversity based on genomic data or the possible influence of host specialization and geographical location on intraspecific variation.

The purpose of the study was primarily to assess the intraspecific variation in *O. quercus* isolates and to investigate the levels of connectivity of the collection of samples at three different geographical scales. Specifically we questioned whether the isolates represent part of a single global population or whether genetic structuring by means of

introductions is evident amongst the samples. Sequence data for five variable loci were used to generate multilocus haplotypes and allele-based information revealing high levels of haplotype and nucleotide diversity in *O. quercus*.

Methods and Materials

Sample collections and identification

Cultures were made from single, germinating conidia or ascospores for all isolates of *O. quercus* (Table 1). The isolates were grown on 2% malt extract agar (MEA) (Biolab, Midrand, South Africa) in Petri plates and incubated at room temperature. A limited number of *O. quercus* isolates were collected in southern hemisphere countries including those from Australia, Chile, Malawi, New Zealand, South Africa, Tanzania, Uganda and Uruguay and in the northern hemisphere from Austria, Azerbaijan, Bhutan, France, Japan and Norway. A larger collection of isolates was made in Norway (N=46) including 29 samples collected in the south of the country and 17 collected at a site 1000 km north, within the Arctic Circle. The latter collection represents isolates from natural stands of oak, poplar and birch as well as from felled logs gathered by local foresters and farmers.

All isolates were identified based on their molecular characteristics using comparisons of sequences for part of the β-tubulin gene region. Thus, the identities of all the samples included in this study were confirmed by genetic identification using NCBI-BLAST. A total of 122 *O. quercus* isolates were sequenced and characterized and these are preserved in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI).

DNA sequence variation

Genomic DNA was extracted by suspending freshly collected fungal mycelium in 200 ml PrepMan Ultra Sample Preparation reagent (Applied Biosystems), incubated for 10 min at 95 °C and centrifuged at 13 000 rpm for 5 min. Ten µl supernatant of the aqueous

phase was diluted 1:10 with deionised water. The diluted DNA was used in all subsequent Polymerase Chain Reactions.

Two sources of sequence data were used to evaluate the genetic variation in *O. quercus* including part of the β-tubulin gene region (330bp) and four SSR allele polymorphisms. To amplify part of the β-tubulin region, the primers T10 (O'Donnell & Cigelnik, 1997) and Bt2b (Glass & Donaldson, 1995) were used. The reaction mixture contained 1 µL DNA template (20-90 ng), 1x PCR reaction buffer, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.1mM of each primer and 5 U of FastStart *Taq* polymerase (Roche Biochemicals, Germany) and was made up to a final volume of 50 µL with sterile water. Reactions were carried out in an iCycler (Bio-Rad) with conditions as follows: initial denaturation at 95 °C for 2 min, followed by 35 cycles of denaturation at 95 °C for 30 seconds, primer annealing at 53-55 °C (depending on the isolate) for 1 min and elongation at 72 °C for 1 min. A final extension step at 72 °C for 8 min completed the program.

Four repeat markers including their flanking regions were amplified and sequenced to assess DNA sequence variation and to provide optimal resolution in identifying differences between the *O. quercus* isolates. To amplify the repeat loci, unlabelled primer pairs developed for *O. quercus* (Grobbelaar *et al.* 2009a) were used in 25 µL reactions containing 20-100 ng DNA template, 2.5 mM of each dNTP's (Promega), 2 pmol of each primer, 0.1 units of *Taq* polymerase (Roche Molecular Biochemicals), 1x buffer and $MgCl_2$ (as indicated for each primer pair) supplied with the enzyme. Thermal conditions were: 96 °C for 2 min, followed by 40 cycles of 94 °C for 30 s, Ta (as indicated for each primer pair) for 30 s and extension at 72 °C for 30 s and a final extension at 72 °C for 10 min terminated the PCR.

All amplified PCR products were cleaned using Sephadex G-50 (Sigma-Aldrich) and were sequenced using their respective primers and the ABI PRISM®BigDye™ Terminator v 3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems Inc., Foster City, California). It was not necessary to clone the PCR products as all the isolates are haploid and have only one allele per individual. Standard reaction conditions were 96°C

for 10s, 56°C for 30s and 60°C for 4 minutes for a total of 25 cycles. The cycle sequencing products were precipitated and fragments were run on an ABI PRISM™ 3100 automated sequencer (Applied Biosystems).

Data analysis

The β-tubulin sequence data were analysed and contigs assembled using the Vector NTI Advance 10 software (Invitrogen). The assembled forward and reverse contigs were aligned in MAFFT v 5.731 (Katoh *et al.* 2005; Morrison 2006), then edited manually. Gaps were treated as a 5th character and the exon positions were identified using homolog sequences from GenBank.

Network analyses

To investigate the relationships amongst *O. quercus* isolates, haplotype networks (Excoffier *et al.* 1992) were calculated for each of the four SSR markers (Oqrc2, 12, 18 and 19) using parsimony analysis (Templeton *et al.* 1992) conducted in TCS version 1.21 (Clement *et al.* 2000) and defined by a 95% confidence limit. The sampling localities (countries) were treated as populations. To correct for the sample bias, the average number of isolates per population was used and a random number, equal to the population average, drawn from the Norwegian samples. This was repeated three times to confirm the results.

Intraspecific variation amongst *O. quercus* isolates at a smaller scale was considered using the Norwegian collection of isolates, to determine the level of connectedness between samples from the south and those isolated from northern Norway. Haplotype networks using the four SSR markers (Oqrc2, 12, 18 and 19) were applied to these isolates.

Gene diversity

Genetic polymorphism across all the isolates was measured by calculating the frequency of alleles for each of the SSR loci. Nei's gene diversity (Nei 1973) was calculated using POPGENE version 1.32 (Yeh & Boyle 1997) across the four repeat loci by treating the samples of *O. quercus* as a single population. To compare gene diversity at a global scale with regional scale diversity, the same test was conducted using the 46 Norwegian isolates only. Allelic diversity in the β-tubulin gene region was calculated across all the isolates including an additional three sequences from GenBank (789155, 789156 and 789157) for comparative purposes.

Clustering and assignment of samples

The clonal fraction was calculated by regarding the multilocus haplotypes that were repeated multiple times, as clones. All pairwise loci for the clone corrected data were tested for linkage disequilibrium with MultiLocus 1.3b (Agapow & Burt 2001) following 1000 randomizations. A Bayesian clustering method was applied to this dataset to infer population structure and to assign the *O. quercus* isolates to populations using the program, STRUCTURE version 2.2 (Pritchard *et al.* 2000). The method clusters individuals into *K* distinct populations and permits mixed ancestry. Both models of allele frequency (independent or correlated) were tested, however, given the reproductive biology of *O. quercus* and the results from the allele networks, the samples were expected to have correlated allele frequencies.

STRUCTURE was run for *K* ranging from 1 to 10 and 20 iterations of each were performed to confirm the convergence of the likelihood values for each value of *K*. The Monte Carlo Markov Chain (MCMC) was run for 100 000 replicates after an initial burn-in of 10 000. The optimum number of populations was determined by maximizing the estimated Ln likelihood of the data for each of the *K* values and by calculating delta *K* as suggested in

Evanno *et al.* (2005). A second round of longer runs was repeated at *K*=2, 3 and 4 for 1 000 000 replications over three runs at each *K* to verify assignment consistency.

To calculate the average assignment proportions and to identify admixed isolates, a cut off of 80 % assignment was used to determine cluster identity. After excluding the admixed isolates, the average assignment proportions for 13 localities were geographically depicted to determine overall trends in clustering over a broad geographic scale. Japan was not considered in this analysis since the collection included only a single isolate.

Analysis of Molecular Variance

Alternative hypotheses for geographical structuring of the data were compared through an analysis of molecular variance (AMOVA) conducted using the program GENALEX version 6.1 (Peakall & Smouse, 2006). The percentages of variance were calculated at three levels of partitioning: within populations, among populations within regions and among regions following 9999 permutations. The following hypotheses were considered: (i) all sampled *O. quercus* isolates represent one region / group (ii) isolates are genetically structured at a continental scale (i.e. five regions) and (iii) isolates are structured into six regions based on the observed global trend in allele frequences. The Japanese isolate was excluded from all three AMOVA runs.

Results

Sample collections and identification

Culture morphology of the collection of isolates in this study was highly variable. This was evident not only when comparing isolates from the different countries, but also for isolates from within a single country and even in subcultures of the same isolate transferred onto several new plates. This is a typical characteristic of *O. quercus* (Przybyl & Morelet, 1993) and does not reflect species differences. Morphology of the fruiting

structures of *O. quercus* isolates varied in culture morphology and colour and could not be relied upon for verification of the species. However, the NCBI-BLAST identification tool, established that all the samples included in this study represented *O. quercus*.

DNA sequence variation

Amplicons from the partial β-tubulin gene region were aligned and the sequences resulted in 327 characters with most variation found within the introns. Sixteen β-tubulin haplotypes were observed in our collection of *O. quercus*. The haplotype definition profile with population frequencies found within the five continental partitions including their respective host distribution is presented in Table 2.

Analyses of data

Network analyses

Multiple alleles identified at each of the SSR loci and statistical parsimony networks showed clear differences in haplotype and nucleotide diversity between the four loci (Fig. 1a-d). The most common haplotype (and inferred ancestral haplotype) at each locus was found in isolates from Africa, Europe, Asia, Australasia and South America. The common alleles were sampled at high frequency (~ 35%) from the African localities, while this was also the continent from which most unique haplotypes were recorded (Fig 1a-d blue). Most of the haplotypes were distributed across Asia, Africa, Europe and South America, whereas the Australasian haplotypes were not found to extend as widely (Fig 1a-d).

Ophiostoma quercus isolates from the southern collection sites in Norway showed higher haplotype and nucleotide diversity than those from the northern site (Figure 2 a-d). The northern site (Fig 2 a-d) harboured a high proportion of the most common allele found in the south (Fig 2 a-d) at each locus. In addition, the Norwegian samples included one private allele when compared to the rest of the samples.

Gene diversity

Sequence variation was evident within the repeat and flanking regions of the SSR loci (Fig 1a-d). Across the 122 isolates of *O. quercus* included in this study, a total of 62 haplotypes in total based on DNA sequence variation were identified across the four SSR loci, ranging from 13 to 17 haplotypes per locus (results not shown). The average gene diversity (H) for the total clone corrected dataset calculated using POPGENE was 0.70. When the Norway isolates were considered alone, a total of 21 alleles were obtained, ranging from 5 to 6 alleles per locus with an average H of 0.53 (results not shown). The intraspecific diversity observed within the conserved β-tubulin gene was high and yielded 16 haplotypes (Table 2) with alleles Oq2, Oq3 and Oq6 the most frequently found amongst the samples.

Clustering and assignment of samples

The number of population clusters (*K*) was based on the rate of change in the estimated log probability of the data between successive *K* values for *K* equalling 1 to 10. This method identified population clusters *K*=4 as the most likely when no prior information was known for the geographic location of the isolates (Fig. 3a and b). The average assignment for each isolate was compared at *K*=2, 3 and 4 (Fig 3c-e). The first division (*K*=2) was between the hemispheres with isolates from the southern hemisphere residing in population 1 (Fig 3c) and those from the northern hemisphere assigning to population 2. Further sub-structuring (*K*=3) indicates a subsequent division of isolates mostly from the north into population 3 (Fig 3d).

At *K*=4 most samples (Fig 3e) were unambiguously assigned to one of the four population clusters with an individual probability of assignment q=0.8. Of the 122 samples of *O. quercus*, 46 appeared to be admixed, revealing mixing between two or more of the clusters. Several of the isolates of *O. quercus* resided in three or four of the clusters with origins from both the northern and southern hemisphere (Figs 3 d and e).

However, isolates from some countries were more genetically homogeneous (e.g. New Zealand) than those from the other countries (Figs 3 c-e [9]).

For *K*=4 population clusters, the average assignment frequencies of isolates from 13 localities were plotted to summarise the geographic location of each cluster (Fig. 4)**.** A strong signal of co-occurrence of clusters was found for some countries such as Austria, Azerbaijan, Malawi, Norway, Tanzania and Uganda, while isolates from Australia and Bhutan appeared to be homogeneous. Isolates from New Zealand and South Africa also appeared to share cluster assignments. The assignment frequencies of isolates in Norway revealed a unique pattern, different to that from the other countries with only France sharing a smaller proportion of the dominant cluster seen in Norway (A). At a continental scale, the average assignments of the isolates varied between the countries (Fig 4).

No clear geographical patterns were observed. However, isolates from the African continent showed a clinal change in cluster frequencies from the south (South Africa) to the north (Uganda). At a global scale, some common haplotypes were shared between isolates from certain countries for example, between South Africa and New Zealand or between Australia and Bhutan. There were also haplotypes observed in isolates from both Norway and France, and to a lesser degree in Azerbaijan and Austria, but not seen in those from Southern Hemisphere countries.

Analysis of Molecular Variance

Where all the isolates of *O. quercus* were considered as a single group, significant overall variance of 30% was found among populations (Table 3). The five continental groups accounted for 5% of the overall variance among regions. Where the isolates were subjectively partitioned into six groups based on the geographical correlations of their assignment frequencies (Fig. 4), significant overall variance of 12 % among regions was calculated. The groups were represented as (1) Norway and France, (2) Austria, Azerbaijan, Uganda, Tanzania and Uruguay, (3) Bhutan and Australia, (4) South Africa and Chile, (5) Malawi and (6) New Zealand.

Discussion

Intraspecific genetic variation for a global collection of 122 *O. quercus* isolates revealed some degree of geographic partitioning at a global, continental and regional level. Haplotype networks calculated to detect fine scale differences (Posada &, Crandall 2001) revealed significant variability in all five loci with the non-coding loci showing higher levels of variation than the functional gene. The observed genetic polymorphisms and the previously well recognized phenotypic diversity suggest a history of recombination events for this fungus. This is likely for an out-crossing fungus (Milgroom & Brasier 1997) and has been documented in the closely related species *Ophiostoma piceae* (Gagne *et al.* 2001).

Patterns of genetic diversification were observed for *O. quercus* at three hierarchical levels. Samples from Norway offered the opportunity to consider differentiation at a local and regional scale (i.e. over a thousand km or less). The diversity among these isolates revealed that the isolates from the southern region were more diverse than those from the northern region of Norway. This might suggest that the samples from the northern part of Norway originated from the southern part of the country. Because the insect vectors of *O. quercus* are not likely to move over very large distances (> 1000 km), it is more likely that the fungus was introduced into the northern region with the movement of wood products from the south. Such a pathway of introduction would explain the shared haplotypes found in isolates from the northern and southern parts of Norway. An alternative hypothesis for the shared haplotypes would be that the southern part of Norway may have acted as a refugial region when the Artic ice sheets forced species southwards, a survival strategy hypothesised for many Boreal species (Hewitt 2004). With the retreat of the ice cap, the species would then have been able to expand in distribution to the northern part of Noway. It would thus appear that *O. quercus* is well established in Norway where previous studies too have reported the isolation of *Ophiostoma* spp. (Solheim 1992).

At a continental scale, the high levels of variation found in isolates from South Africa (SA), Malawi, Tanzania and Uganda were surprising. This was in view of the belief (Przybyl & Morelet 1993) that *O. quercus* is a northern hemisphere fungus that has subsequently spread to other parts of the world. If this were true, reduced diversity in the introduced populations would be expected (Allendorf & Lundquist 2003; Nei *et al*. 1975). In contrast, isolates from continental Africa contained a large proportion of the ancestral haplotypes as well as continent-specific haplotypes that were not observed in any other isolates sampled on the other four continents. It has previously been suggested that *O. quercus* might have been introduced into SA by the early Dutch settlers in the 16th century bringing oak timber from Europe for the production of wine barrels (de Beer *et al.* 2003). *O. quercus* has also previously been reported from South Africa on native *Olinia* sp. and on exotic *Eucalyptus grandis* and *Quercus robur* L. (de Beer *et al.* 2003). More recently, it has also been found on other native hardwood trees (Kamgan Nkuekam *et al.* 2008), which could either suggest a strong capacity of the fungus to colonise new environments successfully subsequent to introduction or alternatively, it could reflect variation in a native population of *O. quercus*.

At a global scale, isolates from Europe, Africa and South America showed high levels of diversity, however Australasian isolates (Australia, New Zealand and Asia) showed less variation. This may signify that *O. quercus* was first present in the west and introduced into the east by trade and movement of forest products. This may also explain why the isolates from Bhutan and Australia (Fig. 4) shared very similar assignment frequencies of their isolates. However, isolates from New Zealand showed a unique cluster combination, with most similarity to South African isolates and could suggest an independent introduction from there. It is interesting that the most common haplotype found in New Zealand (Table 2) on *Pinus radiata* was also collected from *Eucalyptus* spp. and *P. radiata* in South Africa. Given that the South African isolates display higher levels of variability, the results may also support the view that the connection between the two countries was as a result of an introduction of the haploytpe from South Africa into New Zealand.

No correlation was observed between host tree and haplotype when comparing the total number of β-tubulin haplotypes to its host distribution at a global scale. Although the majority of isolates were collected from *Eucalyptus* and *Quercus* spp., two popular and widely planted hardwoods, one β-tubulin haplotype (Oq3) occurred from isolates found on *Pinus radiata* in New Zealand, an introduced conifer to that country. The variability in the haplotypes highlights the cosmopolitan nature of *O. quercus* and provides further evidence that the fungus is not limited to any particular host tree species, also observed in other fungal examples (Slippers *et al*. 2005).

Populations in fungi are generally difficult to define because of their different modes of reproduction and their global movement (Wingfield *et al.* 2001). The four populations treated in this study were based on the proportion of their allele frequencies and likely defined as a result of the admixture indicator from the various isolates. The results of the AMOVA indicate high variance within the populations suggesting possible gene flow between the isolates (Templeton 1998). Twelve percent of the genetic differences are as a result of the six regions subjectively distinguished by the geography. There appeared to be a trend between the allele frequencies in the southern hemisphere isolates as compared with those identified in the northern hemisphere. One population cluster is common in the south while in Europe one cluster appears limited to that continent. The differences seen in our collection of isolates reveal a complicated life history and demography of *O. quercus*. They emphasize the role that population level studies can play in understanding genetic diversity (Fisher 1930) and the value of knowing the ecology of both the exotic and native host species which the fungus colonizes.

Most of the genetic variation in *O. quercus* is generated by sexual recombination between genetically different isolates.Substitution patterns in the DNA sequences are complex and poorly understood in this fungus. The geographic differences in the assignment frequencies cannot account for the origin of the observed variation. Although it is not possible to reject the hypothesis that *O. quercus* represents a single global population, alternative hypotheses to describe the avenues of *O. quercus* movement should be considered. The fungus has reproductive structures that are adapted to insect dispersion

and these characteristics may account for movement over short distances (Juzwik *et al.* 1998) which generate some of the variation seen. However, the long distance dispersal would most likely be due to the human-mediated movement of wood and forest products (Chornesky *et al.* 2005; Skarpaas & Økland 2008) creating opportunities for multiple introductions of *O. quercus* and driving the diversity in the intraspecific genetic variation observed.

Acknowledgements

We are very grateful to Dr Jane Wright, Ms Irene Barnes, Dr Treena Burgess, Dr Celeste Linde, and Dr Bernard Slippers for their advice and assistance in preparing the manuscript. We also thank Dr Thomas Kirisits, Dr Dilzara Aghayeva, Prof. Jolanda Roux and Mr Ronald Heath for sharing isolates of *O. quercus* with us. We are grateful to Ms Renate Zipfel for assistance in sequencing the isolates and the National Research Foundation (NRF), members of the Tree Protection Co-operative Programme (TPCP), the THRIP initiative of the Department of Trade and Industry (DTI), and the Department of Science and Technology (DST) South Africa for financial support.

References

Agapow P-M, Burt A, 2001. Indices of multilocus linkage disequilibrium. *Molecular Ecology Notes* **1**: 101-102.

Allendorf FW, Lundquist LL, 2003. Introduction: population biology, evolution and control of invasive species. *Conservation Biology* **17**: 24-30.

Anderson PK, Cunningham AA, Patel NG, Morales FJ, Epstein PR, Daszak P, 2004. Emerging infectious diseases of plants: pathogen pollution, climate change and agrotechnology drivers. *Trends in Ecology & Evolution* **19**: 535-544.

Banke S, McDonald BA, 2005. Migration patterns among global populations of the pathogenic fungus *Mycosphaerella graminicola*. *Molecular Ecology* **14**: 1881-1896.

Barras SJ, Perry TJ, 1975. Interrelationships among microorganisms, bark or ambrosia beetles, and woody host tissue: an annotated bibliography. *USDA Forest Service General Technical Report SO-10, Southern Forest Experiment Station, New Orleans, USA* pp.1956-1974.

Bossdorf O, Auge H, Lafuma L, Rogers WE, Siemann E, Prati D, 2005. Phenotypic and genetic differentiation between native and introduced plant populations. *Oecologia* **144**: 1-11.

Brasier CM, 1990. China and the origins of Dutch elm disease: an appraisal. *Plant Pathology* **39**: 5-16.

Brasier CM, 1991. *Ophiostoma novo-ulmi* sp. nov., causative agent of current Dutch elm disease pandemics. *Mycopathologia* **115**: 151-161.

Brasier CM, 2000. The rise of the hybrid fungi. *Nature* **405**(6783): 134-135.

Brasier CM, Kirk SA, Pipe ND, Buck KW, 1998. Rare interspecific hybrids in natural populations of the Dutch elm disease pathogens *Ophiostoma ulmi* and *O. novo-ulmi*. *Mycological Research* **102**: 45-57.

Bridges JR, Moser JC, 1983. Role of two phoretic mites in transmission of the blue-stain fungus, *Ceratocystis minor*. *Ecological Entomology* **8**: 9-12.

Brown JKM, Hovmøller MS, 2002. Aerial dispersal of pathogens on the global and continental scales and its impact on plant disease. *Science* **297**(5581): 537-547.

Chornesky EA, Bartuska AM, Aplet GH, Britton KO, Cummings-Carlson J, Davis FW, Eskow J, Gordon DR, Gottschalk KW, Haack RA, Hansen AJ, Mack RN, Rahel FJ, Shannon MA, Wainger LA, Wigley TB, 2005. Science priorities for reducing the threat of invasive species to sustainable forestry. *BioScience* **55**: 335-348.

Clement M, Posada D, Crandall KA, 2000. TCS: a computer program to estimate gene genealogies. *Molecular Ecology* **9**: 1657-1660.

Crandall KA, Templeton AR, 1999. The zoogeography and centres of origin of the crayfish subgenus *Procericambarus (Decapoda: Cambaridae)*. *Evolution* **53**: 123-124.

De Beer ZW, Wingfield BD, Wingfield MJ, 2003. The *Ophiostoma piceae* complex in the Southern Hemisphere: a phylogenetic study. *Mycological Research* **107**: 469-476.

De Beer ZW, Wingfield MJ, Kemp GHJ, 1995. First report of *Ophiostoma querci* in South Africa. *South African Journal of Science* **91**: vi.

Desprez-Loustau ML, Robin C, Buee M, Courtecuisse R, Garbaye J, Suffert F, Sache I, Rizzo DM, 2007. The fungal dimension of biological invasions. *Trends in Ecology & Evolution* **22**: 472-480.

Elton CS, 1958. *The Ecology of Invasions by Animals and Plants.* University of Chicago Press, Chicago, USA.

Evanno G, Regnaut S, Goudet J, 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular Ecology* **14**: 2611-2620.

Excoffier L, Smouse PE, Quattro JM, 1992. Analyses of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics* **131**: 479-491.

Fisher RA, 1930. *The genetical theory of natural selection* Clarendon Press, Oxford, United Kingdom.

Gagne P, Yang D-Q, Hamelin RC, Bernier L, 2001. Genetic variability of Canadian populations of the sapstain fungus *Ophiostoma piceae*. *Phytopathology* **91**: 369-376.

Geldenhuis MM, Roux J, Montenegro F, de Beer ZW, Wingfield MJ, Wingfield BD, 2004. Identification and pathogenicity of *Graphium* and *Pesotum* species from machete wounds on *Schizolobium parahybum* in Ecuador. *Fungal Diversity* **15**: 135-149.

Glass NL, Donaldson GC, 1995. Development of primer sets designed for use with PCR to amplify conserved genes from filamentous Ascomycetes. *Applied and Environmental Microbiology* **61**: 1323-1330.

Grobbelaar JW, Barnes I, Cortinas M-N, Bloomer P, Wingfield MJ, Wingfield BD, 2009a. Development and characterization of polymorphic markers for the sap-stain fungus *Ophiostoma quercus*. *Molecular Ecology Resources* **112**: 399-401.

Grobbelaar JW, De Beer ZW, Bloomer P, Wingfield MJ, Wingfield BD, 2009b. *Ophiostoma tsotsi* sp. nov., a sap-staining fungus on hardwood trees in Africa. Chapter four.

Halmschlager E, Messner R, Kowalski T, Prillinger H, 1994. Differentiation of *Ophiostoma piceae* and *Ophiostoma quercus* by Morphology and RAPD Analysis. *Systematic and Applied Microbiology* **17**: 554-562.

Harrington TC, McNew D, Steimel J, Hofstra D, Farrell R, 2001. Phylogeny and taxonomy of the *Ophiostoma piceae* complex and the Dutch elm disease fungi. *Mycologia* **93**: 111-136.

Hewitt GM, 2004. The structure of biodiversity – insights from molecular phylogeography. *Frontiers in zoology* **1**.

Jacobs K, Wingfield MJ, 2001. *Leptographium species: Tree Pathogens, Insect Associates, and Agents of blue–stain.* APS Press, St. Paul, Minnesota.

Juzwik J, Cease KR, Meyer JM, 1998. Acquisition of *Ophiostoma quercus* and *Ceratocystis fagacearum* by Nitidulids from *O.quercus* - Colonized Oak Wilt Mats. *Plant Disease* **82**: 239-243.

Kamgan Nkuekam G, Jacobs K, De Beer ZW, Wingfield MJ, Roux J, 2008. *Ceratocystis* and *Ophistoma* species, including three new taxa, associated with wounds on native South African trees. *Fungal Diversity* **29**: 37-59.

Katoh K, Kuma K-i, Toh H, Miyata T, 2005. MAFFT version 5: improvement in accuracy of multiple sequence alignment. *Nucleic Acids Research* **33**: 511-518.

Mariat F, Escudie A, Gaxotte P, 1968. Isolement de souches de *Ceratocystis* sp. a` forme conidienne Sporotrichum, secuirs chevelus humains et de poils de rats. Comparaison avec l'espe`ce pathoge`ne *Sporotrichum schenckii*. *Comptes rendus Hebdomadaires des Seances de la'Academie des Sciences,, Paris* **167**: 974-976.

Milgroom MG, Brasier CM, 1997. Potential diversity in vegetative compatability types of *Ophiostoma novo-ulmi* in North Amercia. *Mycologia* **89**: 722-726.

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

Morrison DA, 2006. Multiple sequence alignment for phylogenetic purposes. *Australian Systematic Botany* **19**: 479-539.

Nei M, 1973. Analysis of gene diversity in subdivided populations. *Proceedings of the National Academy of Sciences* **70**: 3321-3323.

Nei M, Maruyama T, Chakraborty R, 1975. The bottleneck effect and genetic variability in populations. *Evolution* **29**: 1-10.

O'Donnell K, Cigelnik E, 1997. Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. *Molecular Phylogenetics and Evolution* **7**: 103-116.

Palm M, (2001. Systematics and the impact of invasive fungi on agriculture in the United States. *BioScience* **51**: 141-147.

Peakall R, Smouse PE, 2006. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes* **6**: 288-295.

Posada D, Crandall KA, 2001. Intraspecific gene genealogies: Trees grafting into networks. *Trends in Ecology & Evolution* **16**: 37-45.

Pritchard JK, Stephens M, Donnelly P, 2000. Inference of Population Structure Using Multilocus Genotype Data. *Genetics* **155**: 945-959.

Przybyl K, Morelet M, 1993. Morphological differences between *Ophiostoma piceae* and *O. querci*, and among *O. querci* isolates. *Cryptogamie, Mycologie* **14**: 219-228.

Seifert KA, 1993. Sapstain of commercial lumber by species of *Ophiostoma* and *Ceratocystis*. In: *Ceratocystis and Ophiostoma: Taxonomy, Ecology and Pathogenicity* (eds. Wingfield MJ, Seifert KA, Webber J), APS Press, St Paul, Minnesota, pp. 141-151.

Slippers B, Stenlid J, Wingfield MJ, 2005. Emerging pathogens: fungal host jumps following anthropogenic introduction. *Trends in Ecology & Evolution* **20**: 420-421.

Skarpaas O, Økland B, 2008. Timber import and the risk of forest pest introductions. *Journal of Applied Ecology* **doi: 10.1111/j.1365-2664.2008.01561.x**.

Solheim H, 1992. The early stages of fungal invasion in Norway spruce infested by the bark beetle *Ips typographus*. *Canadian Journal of Botany* **70** : 1-5.

Templeton AR, 1998. Nested clade analyses of phylogeographic data: testing hypotheses about gene flow and population history. *Molecular Ecology* **7**: 381-397.

Templeton AR, Crandall KA, Sing CF, 1992. A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation. *Genetics* **132**: 619-633.

Thwaites JM, Farrell RL, Duncan SM, Reay SD, Blanchette RA, Hadar E, Hadar Y, Harrington TC, McNew D, 2005. Survey of potential sapstain fungi on *Pinus radiata* in New Zealand. *New Zealand Journal of Botany* **43**: 653-663.

Upadhyay HP, 1981. *A monograph of Ceratocystis and Ceratocystiopsis* University of Georgia Press, Athens, Georgia, USA.

Webber JF, Brasier CM, 1991. Status of *Ophiostoma piceae* on hardwoods and conifers. In: Report on Forest Research 1990. HMSO, London UK, pp 54-55.

Wingfield MJ, Seifert KA, Webber J, 1993. *Ceratocystis and Ophiostoma: Taxonomy, Ecology and Pathology* APS Press, St. Paul, Minnesota.

Wingfield MJ, Slippers B, Roux J, Wingfield BD, 2001. Worldwide movement of exotic forest fungi, especially in the tropics and Southern Hemisphere. *BioScience* **51**: 141-147.

Yeh FC, Boyle TJB, 1997. Population genetic analysis of co-dominant and dominant markers and quantitative traits. *Belgian Journal of Botany* **129**: 157.

Figs. 1a, b, c and d Haplotype networks for *O. quercus* isolates for each of the independent SSR markers. Oqrc2**(a)**, Oqrc12**(b)**, Oqrc18**(c)** and Oqrc19**(d)**. Each circle represents a unique sequence-based haplotype. One mutational step (one base pair difference) between haplotypes is represented by a line. The size of the haplotype circle represents the frequencies of the haplotypes and is colour-coded by continent. The largest haplotype has the highest frequency in each network and is presumably the ancestral haplotype. The smallest coloured circle represents one occurrence of a haplotype. Nodes on the lines indicate unsampled or extinct haplotypes. Sample areas for each haplotype were grouped according to continents: Europe (green) [AU, Austria; FR, France; NO, Norway], Asia (red) [AZ, Azerbaijan; BH, Bhutan; JAP, Japan], Africa (blue) [MA, Malawi; SA, South Africa; TZ, Tanzania; UG, Uganda], South America (pink) [CH, Chile; UR, Uruguay], Australasia (yellow) [AUS, Australia; NZ, New Zealand]

Figs. 2a, b, c and d Haplotype networks for the 46 Norwegian *O. quercus* isolates for each of the independent SSR markers. Oqrc2 **(a)**, Oqrc12 **(b)**, Oqrc18 **(c)** and Oqrc19 **(d)**. Each circle represents a unique sequence-based haplotype. One mutational step (one base pair difference) between haplotypes is represented by a line. The size of the haplotype circle represents the frequencies of the haplotypes and is colour-coded by continent. The largest haplotype has the highest frequency in each network and is presumably the ancestral haplotype. The smallest coloured circle represents one occurrence of a haplotype. Nodes on the lines indicate unsampled or extinct haplotypes. Sample areas for each haplotype were grouped according to Northern Norway (blue) and Southern Norway (green).

Figs. 3a and b Absolute values of the rate of change of the likelihood distribution (mean ± SD) (a) and the rate of change in the log probability of data between successive *K* values (b).

Figs. 3c, d and e Bar graphs showing the average population assignments when *K*=2 (c), 3 (d) and 4 (e) respectively. Population 1 (green), population 2 (blue), population 3 (yellow) and population 4 (pink). Each country is indicated by a number, 1-Australia, 2-Austria, 3-Azerbaijan, 4-Bhutan, 5-Chile, 6-France, 7-Japan, 8-Malawi, 9-New Zealand, 10-Norway, 11-South Africa, 12-Tanzania, 13-Uganda, 14-Uruguay.

Figs 4 World map showing the samples of *O. quercus* used in this study with an average assignment of 80% or higher assignment to a cluster at *K*=4 for each country. Correlated allele frequencies amongst the isolates are assumed as a result of shared ancestry.

SUMMARY

Ophiostoma quercus is a wood-inhabiting ascomycete that causes blue-stain in logs and lumber. It has a cosmopolitan distribution and is highly adaptable in new as well as existing niches. In the past, there has been much uncertainty regarding the taxonomy of *O. quercus*. To address this confusion, four independent nuclear loci were used for phylogenetic comparison of *O. quercus* and those species previously treated as its synonyms, to other species in the *O. piceae* complex. The results clarified the taxonomic status of those species that are distinct taxa and supported the synonymy of certain species with *O. quercus*. Accordingly, two taxonomic novelties are also presented in this thesis. A collection of *O. quercus* isolates from Africa displaying substantial variability in comparison to other *O. quercus* isolates were re-evaluated using phylogenetic analyses, growth and mating studies. This resulted in the African group being described as a distinct species, *O. tsotsi* sp. nov., characterized based on morphology, growth in culture, and sequence comparisons. In addition, a co-operative project between the South African and Chinese governments provided an opportunity for isolates of *O. quercus* to be collected in China. Morphology and DNA sequences revealed that some of the Chinese isolates were the same as the newly described *O. tsotsi* from Africa. The study also confirms the presence of *O. quercus* on native hosts in both China and Bhutan.

Little is known regarding the global distribution and diversity of *O. quercus*. Sequence-based polymorphic markers were developed from South African isolates of *O. quercus* in order to describe its diverse genetic structure and study its distribution on a global scale. The variation of 122 *O. quercus* isolates was evaluated at three levels of geographic partitioning, including at a global, continental and regional level. Haplotype networks constructed to detect the differences between each of the isolates found ancestral SSR alleles among the African isolates where the fungus is thought to have been introduced while Bayesian-based inference showed a clear signal of admixture in some of the sampled localities. Therefore, the observed patterns of differentiation are interpreted as resulting from long distance movement of forest products carrying *O. quercus* with them and is a first study to consider the variability of this fungus at an intraspecific level.