# Molecular studies on the taxonomy, host-associations and viruses of the *Diplodia*-like anamorphs of the Botryosphaeriaceae

By

**Juanita de Wet**

Submitted in partial fulfilment of the requirements for the degree

***PHILOSOPHIAE DOCTOR***

In the Faculty of Natural and Agricultural Science
Department of Microbiology and Plant Pathology
University of Pretoria
Pretoria

**August 2008**

**Supervisor: Prof. M.J. Wingfield**
**Co-supervisors: Prof. B.D. Wingfield**
**Dr. O. Preisig**

# DECLARATION

I, the undersigned, hereby declare that the thesis submitted herewith for the degree Philosophiae Doctor (PhD) to the University of Pretoria contains my own independent work and has hitherto not been submitted for any degree at any other University.

_____

Juanita de Wet

August 2008

# TABLE OF CONTENTS

## CHAPTER 2

**Multiple gene genealogies and microsatellite markers reflect relationships between the morphotypes of *Sphaeropsis sapinea* and identify a new species of *Diplodia*.**



## CHAPTER 3

**Molecular and morphological characterization of *Dothiorella casuarini* sp. nov. and other Botryosphaeriaceae with *Diplodia*-like conidia.**

## CHAPTER 4

**Phylogeny of the Botryosphaeriaceae reveals patterns of host association.**

## CHAPTER 5

**Patterns of multiple virus infections in the conifer pathogenic fungi, *Diplodia pinea* and *D. scrobiculata.***

# CHAPTER 6

**Characterization of a novel dsRNA element associated with the pine endophytic fungus, *Diplodia scrobiculata*.**

# ACKNOWLEDGEMENTS

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

FABI (Forestry and Agricultural Biotechnology Institute), CTHB (Centre of Excellence in Tree Health Biotechnology), TPCP (Tree Protection Co-operative Programme) and the University of Pretoria for funding, a science-inducing, multidisciplinary work environment and state of the art facilities in which this research was conducted.

A special thanks to my promoters, Mike, Brenda and Oliver for their mentorship, guidance and passion, as well as Bernard Slippers for his valuable contribution.


I also want to acknowledge The National Research Foundation (NRF), The Technology and Human Resources for Industry Programme (THRIP) and members of the TPCP for funding.

A big thank you to all the Fabians but especially:

- My colleagues in the Shaw lab for their friendship and valuable discussions.
- Rose, Helen, Martha, Eva, Martie and the rest of the Culture Collection staff for never hesitating to assist me with whatever I needed.

To those who kept me going outside FABI, my husband, family and friends, thanks for your continued love, support and encouragement through the years.

Above all, praise to my Father in Heaven for giving me Faith, perseverance and always listening to my prayers.

# PREFACE

The Botryosphaeriaceae represents a family of fungi that includes important pathogens of agricultural and forestry crops. *Diplodia pinea* is one of the best-known members of this family causing serious die-back and canker diseases mainly on *Pinus* spp. but also other conifers. The *D. pinea* species complex includes three morphologically similar forms that have been referred to as the A, B and C morphotypes of the fungus. Management of *Diplodia*-associated diseases is difficult as various biotic and abiotic factors influence the initiation and severity of the diseases. Hypovirulence (attenuation of virulence)-inducing dsRNA elements could provide an alternative mode of control against this fungus in a manner that has been shown for the chestnut blight fungus, *Cryphonectria parasitica*. An overall theme during studies conducted as part of this dissertation was to consider the taxonomy, host associations and viruses in isolates of the *D. pinea* species complex. Associated with this objective, I also considered the phylogenetic relationships of this fungus with other *Diplodia*-like anamorphs of the Botryosphaeriaceae.

The first chapter represents a literature review with a particular focus on members of the *D. pinea* species complex and their associated viruses. It reflects on various aspects of the taxonomy, pathogen biology, population genetics and disease management of members of this species complex. The potential of using dsRNA elements, which occur naturally in members of this species complex as biocontrol agents, is also considered. Detailed knowledge of members of the *D. pinea* species complex, closely related species, and their associated mycoviruses form the foundation for the research questions addressed in this dissertation.

The second chapter deals with the phylogenetic relationships between the morphotypes of *D. pinea*. The A, B and C morphotypes of the fungus had previously been shown to be

distinguishable based on morphology, RAPD (randomly amplified polymorphic DNA) banding profiles and SSR (short sequence repeats) markers. They also differ with regards to their distribution, population genetic structure and virulence. The aim of the study was to generate a multiple gene genealogy for isolates representing these morphotypes and closely related species from which more accurate phylogenetic inferences could be drawn. This was achieved using partial sequences of five protein-coding gene regions and microsatelite markers.

In the third chapter of this dissertation, *Diplodia*-like isolates from hosts other than *Pinus* spp. are characterized based on morphology and DNA sequences. These isolates all have conidia that are similar in size and shape, they are thick-walled and often become pigmented with age. For this reason some of these isolates have been treated as synonyms. Host association has also been used to provide an indication of identity. Like *D. pinea* f.sp. *cupressi* causing a canker disease of *Cupressus sempervirens* similar to that of *D. pinea* on *Pinus* spp. In this study, I characterized a set of *Diplodia*-like isolates by combining phylogenetic analysis of DNA sequences with morphological characteristics in an attempt to reveal their phylogenetic status as part of the Botryosphaeriaceae.

In the fourth chapter, I conducted a phylogenetic study to resolve relationships between morphologically similar species of the *Diplodia*-like anamorphs of the Botryosphaeriaceae (*Diplodia*, *Lasiodiplodia* and *Dothiorella*). The availability of sequence data for most genera of the Botryosphaeriaceae made it possible to extend the phylogeny and to explore host association patterns. The hope was that knowledge of these host association patterns and factors driving them would contribute to a better understanding of the evolution of the Botryosphaeriaceae, their co-evolution with their hosts and also help in the prediction of new diseases.

The fifth chapter of this dissertation treats the distribution and frequency of multiple virus infections in a collection of isolates belonging to the *D. pinea* species complex. Various dsRNA elements have previously been reported from the *D. pinea* species complex. Two of these, isolated from a South African *D. pinea* isolate were characterized and are known as *Sphaeropsis sapinea* RNA virus 1 and 2 (SsRV1 and SsRV2). A third dsRNA element was found to be more commonly associated with the B morphotype of *D. pinea*. Using Real-time PCR and three virus-specific primers, virus infections were genotyped to assess their frequency and distribution patterns in isolates of the *D. pinea* species complex.

In chapter six, the previously identified, undescribed dsRNA element most commonly associated with the B morphotype of *D. pinea* was characterized and its full nucleotide sequence determined. The genome was assembled by overlapping contigs obtained through RT-PCR and virus-specific primers. The open reading frames (ORFs) were analyzed for homologies to other viruses and phylogenetic relationships with other virus families were assessed.

All studies presented in this dissertation concern the *D. pinea* species complex and associated viruses. They were conducted independently and have been written as separate publishable units. Some repetition between chapters may, therefore, occur as it represents a progression of knowledge obtained over a relatively long period of time. I, nonetheless hope these studies will contribute to a deeper understanding of the *D. pinea* species complex, viruses associated with them and their interaction.

# SUMMARY

The Botryosphaeriaceae is a family of fungi that includes many species, which are well-known as pathogens, saprophytes and endophytes of plants and especially of trees. As a result of their pathogenic nature and potential threat to plantations and agricultural crops, much research has been devoted to their identification. The main focus of studies that make up this thesis has been on the fungal complex referred to as *Diplodia pinea sensu lato*. These fungi are members of the Botryosphaeriaceae and studies have specifically concentrated on their taxonomy, host associations and mycovirus infections associated with them.

*Diplodia pinea sensu lato* represents a species complex of highly similar morphological types that mainly infect *Pinus* spp., world-wide. The species complex includes what have in the past been known as the A, B and C morphological types of *D. pinea*. Multiple gene genealogies based on sequences of partial protein-coding genes and microsatellite markers were used to resolve the species complex into two genera, *D. pinea* and *D. scrobiculata* (= B morphotype).

Diplodia-like isolates from Australia, Greece and Cyprus were characterized using both morphological and molecular characteristics. Morphologically, these isolates all have dark, thick-walled conidia (Diplodia-like) but phylogenetically, they could belong to three distinct genera of the Botryosphaeriaceae namely *Diplodia*, *Lasiodiplodia* and *Dothiorella*. Results of this study led to the description of *Dothiorella casuarini* from *Casuarina* spp. in Australia and they highlight the fact that similar morphological characteristics and disease etiology does not necessarily provide a true reflection of the evolutionary history of a pathogen.

Phylogenetic studies on species of the Botryosphaeriaceae with Diplodia-like anamorphs revealed intriguing host association patterns. The availability of sequence data for many species of the Botryosphaeriaceae made it possible to extend the phylogeny to include six of the ten lineages as previously described for the Botryosphaeriaceae. Angiosperms appeared to be the most common, and possibly ancestral, host group of the Botryosphaeriaceae, with the exception of *Macrophomina*, *Guignardia*, *Saccharata* and "*Botryosphaeria*" *quercuum*. Infection of gymnosperms most likely occurred more recently, only in specific groups (*Diplodia* and *Lasiodiplodia*) via host shifts.

Three distinct viruses have now been characterized from isolates of *D. pinea sensu lato*. Two of these were previously characterized and are known as *Sphaeropsis sapinea* RNA virus 1 and 2 (SsRV1 and SsRV2). The third dsRNA element more commonly found in association with *D. scrobiculata* was characterized in this dissertation and named *Diplodia scrobiculata* RNA virus 1 (DsRV1). It has a genome of 5018 bp with a unique genome organization characterized by two open reading frames (ORFs). One ORF codes for a putative polypeptide similar to proteins of the vacuolar protein-sorting (VPS) machinery and the other one for a RNA dependent RNA polymerase (RdRp). The hypothetical protein probably has a role in transport or protection of this unencapsulated virus into membranous vesicles. Phylogenetically, DsRV1 groups closest to a dsRNA element from *Phlebiopsis gigantea* (PgV2) and they both group separately from other families in which fungal viruses have been classified.

The frequency and distribution of DsRV1, SsRV1 and SsRV2 were determined in a collection of *D. pinea* and *D. scrobiculata* isolates using Real-time PCR. Infections with SsRV1 and SsRV2 occurred in both *D. pinea* and *D. scrobiculata*, while DsRV1

was mainly found in *D. scrobiculata*. DsRV1 was also found to always occur in combination with SsRV1 and/or SsRV2. Therefore, DsRV1 probably selected against a coat protein as the result of a fitness trade-off. Although earlier studies indicated that these viruses have no effect on the phenotype or virulence of *D. pinea* and *D. scrobiculata* isolates, the presence of specific viruses in their host populations serve as a useful marker in studying movement of fungal pathogens.

The ultimate aim of studies making up this dissertation was to expand the base of knowledge regarding species in the *D. pinea* species complex. This was justified by the fact that *D. pinea* is one of the most important tree pathogens in South Africa and that an expanded knowledge might contribute to reducing diseases caused by it. Clearly understanding the identity of the fungus must clearly underpin many elements of a management strategy and this was one of the aims of the suite of studies conducted. Furthermore, I attempted to augment the knowledge base regarding dsRNA elements in *D. pinea sensu lato*. These studies were of a basic nature and relatively far removed from the practical application level. Nonetheless, it is my hope that they have pushed ahead knowledge barriers and that in some way they will contribute to reducing the impact of *Diplodia*-associated diseases in the future.