# MOLECULAR PHYLOGENETIC STUDIES ON *ARMILLARIA* WITH SPECIFIC REFERENCE TO SOUTHERN HEMISPHERE SPECIES

BY

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Submitted in partial fulfillment of the requirements for the degree

***PHILOSOPHIAE DOCTOR***

in the Faculty of Natural and Agricultural Science

University of Pretoria

Pretoria

November 2003

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# DECLARATION

I the undersigned hereby declare that the thesis submitted herewith for the degree *Philosophiae Doctor* to the University of Pretoria, contains my own independent work as hitherto not been submitted for any degree at any university or faculty.

Martin Petrus Albertus Coetzee

November 2003

# TABLE OF CONTENTS

# ACKNOWLEDGMENTS

I wish to express my sincere gratitude towards the following people and institutions that have made this work possible:

- My study leaders Profs. Brenda Wingfield, Mike Wingfield and Paulette Bloomer for their guidance, advice, patience (when needed) and endless support;
- Drs. Jolanda Roux, Pedro Crous, Sandra Denman, Geoff Ridley, Glen Kile, Thomas Kirisits and B. Chhetri who have, as co-authors, contributed to some of the studies;
- The members of the Tree Pathology Co-operative Programme (TPCP); the National Research Foundation (NRF); the THRIP initiative of the Department of Trade and Industry, South Africa; and the Mellon Foundation for financial support;
- The Department of Genetics for providing the facilities to undertake this study;
- Mark Banik and Drs. Kari Korhonen, Ottmar Holdenrieder and Caroline Mohammed who provided me with cultures of *Armillaria*;
- My brother Marius-Gert Coetzee who wrote and re-wrote the code for the computer program "RFLP Identification Tool for *Armillaria* species";
- Leo van Straten for translating to English species descriptions published in French;
- Vivienne Clarence for ensuring that all my needs in the laboratory were being provided for;
- My family, friends and colleagues;
- My parents for their unconditional love and support to fulfil my dreams;
- Jan Perold for challenging my ideas, reading my manuscripts, pointing out problems, and providing bottomless cups of coffee along with emotional support.

# PREFACE

*Armillaria* spp. are plant pathogenic fungi that cause the disease known as Armillaria root rot. Species in the genus are distributed throughout temperate and tropical regions of the world and have a broad range of plant hosts. *Armillaria* spp. from the Northern Hemisphere have received much attention in the past and their phylogenetic relationships are well resolved. There is, however, a dearth of information pertaining to phylogenetic relationships between *Armillaria* spp. from the Southern Hemisphere and their relationships with those from the Northern Hemisphere. This lack of knowledge has prompted the studies presented in this thesis.

The first chapter of this thesis presents a review of literature regarding *Armillaria*. The review is arranged in three sections. The first of these deals with the taxonomic history of *Armillaria*. This is followed by a broad discussion on species concepts and their application in *Armillaria* taxonomy. The last section is concerned mainly with the known phylogenetic relationships between species from the Northern Hemisphere and Africa; but also includes discussion on possible relationships between Australasian *Armillaria* spp.

Chapter Two of this thesis deals with the phylogenetic relationships between Australian and New Zealand *Armillaria* spp. Various species have previously been identified based on their basidiocarp morphology. A large volume of information about the distribution and the host ranges of these species is currently available. However, information pertaining to the phylogenetic relationships among these species has been conspicuously absent in systematic literature.

Chapter Three concerns the identity and phylogeny of *Armillaria* isolates from South America and Indo-Malaysia. Armillaria root rot is well known in South America and Indo-Malaysia but very little is known regarding the *Armillaria* spp. responsible for the disease. Studies presented in this chapter were, therefore, intended to add to the limited information regarding the species in these areas. Isolates were collected from infected trees with symptoms of Armillaria root rot in Chile, Indonesia and Malaysia. Basidiocarps were not present at the time of collection and field identification was, therefore, not possible. Isolates from basidiocarps with uncertain taxonomic status but resembling *A. novae-zelandiae* and *A. limonea* from Chile and Argentina, respectively, but with uncertain taxonomic status were also included in this study. The identities of all isolates

were determined by means of sequence comparisons, with those available for known *Armillaria* spp. The phylogenetic relationships among isolates of *Armillaria* spp. from the Southern Hemisphere were also determined.

Chapter Four of this thesis addresses the possible origin of *Armillaria* by investigating the phylogeny of *Armillaria* spp. from various floral kingdoms of the world. Earlier investigations revealed the phylogenetic relationships among *Armillaria* spp. from the Holarctic floral kingdom. Likewise, those from the Australian, Indo-Pacific and South American floral kingdoms were considered in chapters two and three of this thesis. The global phylogeny of the species in the genus, however, remained unresolved. The work presented in this chapter was, therefore, designed to determine the phylogenetic relationships between the *Armillaria* spp. from Africa, Australia, Europe, New-Zealand, South America and North America. In addition, the hypothesis that the genus originated in Gondwana was tested by estimating the date of divergence between non-Holarctic and Holarctic *Armillaria* spp.

Chapter Five deals with the phylogeny of African *Armillaria* isolates. One of my earlier studies showed that isolates thought to represent *A. fuscipes* reside in two monophyletic groups. The one group was suggested to represent *A. fuscipes* and the second group either *A. heimii* or an unknown species. Some isolates from the two monophyletic groups have, however, been found to represent the same biological species based on mating studies. The contradiction observed between phylogenetic studies and mating tests rendered the taxonomic position of the two African monophyletic groups refutable. The research presented in this chapter attempts to resolve the taxonomic status of isolates within two monophyletic groups.

Chapter Six deals with the identity of *Armillaria* isolates found on native *Protea* spp. and *Leucadendron* spp. in Kirstenbosh Botanical Gardens in the Western Cape Province of South Africa. Root rot disease on Proteaceae in this botanic garden is generally ascribed to *Phytophthora cinnamomi* infection. However, an investigation in 2000 revealed the presence of white mycelial fans between the bark and wood of dying plants, which are typical symptoms of Armillaria root rot. Basidiocarps were not found in the vicinity of the infected plants; consequently identification of the causal species based on morphology was not possible. The identity of the species was, therefore, determined based on DNA sequence data, their phylogenetic relationships with other *Armillaria* spp. and sexual compatibility tests.

Chapter Seven deals with identification of *Armillaria* isolates from Bhutan. Armillaria root rot is commonly encountered in fir and mixed forests in this mountain Kingdom. With the exception of one unsubstantiated record of *A. ostoyae,* virtually nothing is known about the species causing the disease in Bhutan. During a survey of tree diseases in 2001, isolates were collected from infected conifers showing typical symptoms of Armillaria root rot at four locations. Basidiocarps of the species causing the disease were, however, not found during the course of the survey. The identity of these isolates was therefore determined based on RFLP and DNA sequence data, their phylogenetic relationships with other Northern Hemisphere *Armillaria* spp. and sexual compatibility tests.

The last chapter of this thesis describes the development of an electronic RFLP identification tool for *Armillaria* spp. RFLP based methods provide a rapid and highly effective means for identification of *Armillaria* spp. The extensive use of this method has yielded a large number of PCR-RFLP profiles for various species. These profiles are currently available from a substantial and continuously growing set of publications. Identification using RFLP profiles, therefore, usually requires a cumbersome procedure of comparing profiles from unknown isolates with those that have been published. The software application described in this chapter circumvents this difficulty by providing all this information in a single database and employing an automated procedure for comparing RFLP profiles. The programme also allows for the addition of new information as it becomes available.

This thesis presents a collection of studies conducted over six year period that treat various aspects of *Armillaria* systematics. Three chapters deal with identification and phylogenetic relationships of unknown isolates from different locations in the world. Two chapters are specifically focussed on phylogenetic relationships among species from the Southern Hemisphere. One chapter deals with the global phylogeny of species from the Northern and the Southern Hemispheres. Each chapter is written in such a way that it can be read independently of the others; some repetition has, therefore, been unavoidable. It is my sincere hope that the work presented in thesis will advance our knowledge regarding *Armillaria* spp., their distribution and their relationships with one another.

*Dedicated to my grandparents, Ouma Marietjie and Oupa Herman*

*"It is a strange fate that we should suffer so much fear and doubt over so small a thing."*

BOROMIR

THE LORD OF THE RINGS,

THE FELLOWSHIP OF THE RING ( THE MOVIE)

# SUMMARY

Species of *Armillaria* are plant pathogens that cause the disease known as Armillaria root rot. Studies on the taxonomy and systematics of these fungi render a vital contribution to our ability to accurately identify them, as well as to our understanding of their distribution and ecology. This thesis represents an assemblage of studies that pertain to the taxonomy and phylogenetics of *Armillaria* species. The literature review presents an overview of the taxonomic history of the genus, the species concepts employed in fungal taxonomy, and the relevance of these concepts to *Armillaria*. It also discusses the phylogenetic relationships among *Armillaria* spp., to the extent that these were known prior to the studies constituting this thesis.

The major focus of the studies presented in this thesis was an investigation of the phylogenetic relationships between *Armillaria* spp. *Armillaria hinnulea* from Australia and New Zealand was shown to be closely related to the Northern Hemisphere *Armillaria* spp. The remainder of the *Armillaria* spp. from the two countries form a monophyletic group, thus confirming their separation based on morphology. A subsequent phylogenetic analysis for a global collection of *Armillaria* spp. suggests that species from the non-Holarctic floral kingdoms may be the ancestors of those from the Holarctic. Results also suggest that the genus probably originated in Gondwana. Phylogenetic and genetic analyses of isolates from Africa, which were previously considered to represent the same biological species, revealed two distinct phylogenetic species: *A. fuscipes* and an undescribed species.

Mating tests as well as RFLP and DNA sequence analyses were employed to determine the identity of vegetative isolates obtained during disease surveys from infected trees and shrubs. Isolates from South America and Indo-Malaysia were identified as *A. novae-zelandiae* and *A. luteobubalina* based on phylogenetic analyses. The Northern Hemisphere species, *A. gallica* and *A. mellea*, were shown to be the causal agents of Armillaria root rot on Proteaceae in Kirstenbosch Botanical Gardens, South Africa. Isolates from Bhutan were identified as *A. mellea* subsp. *nipponica* and an apparently undescribed species that we have referred to as Bhutanese Phylogenetic Species (BPS I).

Identification of *Armillaria* isolates is increasingly based on DNA sequence and RFLP data. There are currently a number of separate studies that present a confusing array of data for this

genus. To resolve this problem, a computer program was developed to provide an electronic database for managing RFLP profiles. It also includes an automated search algorithm for rapid identification of *Armillaria* isolates.

This thesis includes seven research chapters in addition to a comprehensive literature review. The collection of studies undoubtedly represents one of the most intensive efforts ever undertaken to identify *Armillaria* spp. This has been made possible through opportunities to collect isolates in many different countries and the availability of isolates from the collections of colleagues. New species have thus been recognised and intriguing patterns pertaining to the phylogeography of these fascinating fungi have begun to emerge. Additional evidence has emerged that, contrary to expectation, these soil-borne fungi have at least to some extent been dispersed across the globe by humans. This information and a considerably enhanced knowledge of the identity of *Armillaria* spp. should improve quarantine procedures to prevent their further spread.

# CHAPTER ONE

## LITERATURE REVIEW

## *ARMILLARIA* (FR.:FR.) STAUDE: TAXONOMY, SPECIES CONCEPTS AND PHYLOGENETIC RELATIONSHIPS

# *ARMILLARIA* (FR.:FR.) STAUDE: TAXONOMY, SPECIES CONCEPTS AND PHYLOGENETIC RELATIONSHIPS

## INTRODUCTION

Species of *Armillaria* (Fr.:Fr.) Staude (Basidiomycotina, Agaricales, Tricholomataceae) are best known as pathogens that cause the disease Armillaria root rot. These are widely distributed throughout the tropical, sub-tropical and temperate regions of the world (Hood *et al.* 1991). The impact of *Armillaria* spp. in these areas is intensified by their ability to survive as pathogens, saprobes or necrotrophs on a wide variety of woody plants (Gregory *et al.* 1991, Hood *et al.* 1991, Kile *et al.* 1991, Fox 2000).

*Armillaria* has had a confused and controversial taxonomic history. Much of this confusion arose from the historical use of a morphological species concept to delineate species. In many cases, the paucity of clear morphological discontinuities between isolates made it difficult for taxonomists to decide whether or not they should be classified as different species. *Armillaria mellea*, for example, was assumed to be a single, highly pleomorphic species, subsuming many isolates currently known to represent distinct species (Singer 1956). This controversy was largely resolved by the adoption of the biological species concept and the subsequent identification of various biological species in Europe, North America and Asia (Korhonen 1978, Anderson and Ullrich 1979, Ota *et al.* 1998b). Most of the biological species are now also equated with taxonomic species defined in terms of their basidiocarp morphology.

The study of *Armillaria* taxonomy is particularly important because of the relevance of Armillaria root rot to commercial forestry and agriculture. Analysis of the phylogenetic relationships among *Armillaria* spp. is also important for a number of reasons. First, knowledge of the evolutionary lineages of these species often yields valuable insights into their taxonomy. Phylogenetic analysis can also be used to determine whether or not species were introduced or are native to a region or continent. Finally, from a basic science perspective, understanding the evolutionary history of the genus is an important goal in itself.

The aim of this review is to provide an overview of the taxonomic history of *Armillaria*. In addition, species concepts that have been applied to *Armillaria* taxonomy are discussed and

current knowledge pertaining to the phylogenetic relationships among species in the genus is reviewed. Overall, the intention is to provide a foundation for studies that follow in this thesis.

## TAXONOMIC HISTORY OF THE GENUS *ARMILLARIA*

The taxonomy of *Armillaria* has plagued many fungal taxonomists ever since its recognition as tribe within *Agaricus*. The taxonomic history of *Armillaria* dates back to the 1700's with reference to *Agaricus melleus* by Danish botanist Martin Vahl (Vahl 1787), now accepted as *Armillaria mellea* (Vahl.: Fr.) P. Kummer and the type species of the genus. In the following Century, Swedish mycologist Elias Fries first introduced *Armillaria*, in his *Systema Mycologicum*, by subdividing the genus *Agaricus* into various tribes (sub-genera) that included *Agaricus* tribus *Armillaria* (Fries 1821). At this stage Fries included twelve *Agaricus* species, one of them being *Ag. melleus*. Four years later Fries abandoned *Armillaria* and transferred the species to the tribe *Lepiota* (Fries 1825). However, in 1838 Fries again re-established the tribe *Armillaria* in *Agaricus* but sub-divided it into three groups: Tricholomata subannulatae, Clitocybae annulatae, and Collybiae annulatae; with 24 species in total (Fries 1838). In 1854 Fries again abandoned the tribe *Armillaria* (Fries 1854). Fries later re-established the tribe in 1874 and maintained the 1838 arrangement (despite the fact that several authors had raised *Armillaria* to genus level) but added six additional species (Fries 1874).

Three independent authors accepted Fries's tribe, *Armillaria*, at the generic level in the mid 1800's. Staude (1857) was first to raise the tribe to genus level but did not transfer the species epithets to *Armillaria;* instead, he maintained the name *Agaricus* for the four species that were included. Later in 1871, Kummer gave *Armillaria* genus status and included eight species with their species epithets transferred to *Armillaria* (Kummer 1871). Quélet (1872) was thought to be the authority for *Armillaria* and authors for many years cited *Armillaria* (Fr.) Quél as the generic name. Quélet's status as authority was, however, rejected based on the fact that Staude (1857) and Kummer (1871) preceded him (Singer 1951, Donk 1962).

The validity of *Armillaria* (Fr.:Fr.) Staude (Staude 1857) versus *Armillaria* Kummer (Kummer 1871) has caused much debate in the past. Singer (1951, 1955a,b, 1986) proposed Kummer as the legitimate authority by arguing that Staude was unaware of difference between tribe and genus, and that he did not intend to give *Armillaria* genus status, and did not make any

combinations in *Armillaria*. According to Singer (Singer 1955b), the wording of Kummer (1871) led to the establishment of a genus rather than just raising the Friesian tribe to generic status. Various authors rejected Singer's interpretation, arguing that Staude had met all the requirements for a valid description (Donk 1962, Watling *et al.* 1982, Volk and Burdsall 1995). *Armillaria* (Fr.:Fr.) Staude is, therefore, accepted as legitimate and *A. mellea* (Vahl.:Fr.) Kummer {= *Agaricus melleus* Vahl} serves as the type species for the genus (Watling *et al.* 1982).

The genus name *Armillariella* (Karst.) Karst. is frequently encountered in older taxonomic and plant pathology literature. Karsten introduced this name in 1879 when he erected *Armillaria* section *Armillariella* and later, in 1881, raised it to generic rank (Karsten 1879, Karsten 1881). Three Finnish species were included in this genus with *Arm. mellea* (Vahl:Fr.) Karst. {= *Ag. melleus* Vahl} assumed to be the type species (Karsten 1881, Donk 1962, Watling *et al.* 1982). *Agaricus melleus* Vahl (as *A. mellea* (Vahl.:Fr.) Kummer) is, however, widely accepted as the type species for *Armillaria* (Fr.:Fr.) Staude (Watling *et al.* 1982). The genus name *Armillariella* Karst. was, therefore, considered as an obligate synonym of *Armillaria* (Fr.:Fr.) Staude (Watling *et al.* 1991). However, according to Burdsall and Volk (1993) the genus name *Armillariella* can be ignored and replaced by the name *Armillaria*.

## SPECIES CONCEPTS

A species concept represents an abstract idea regarding the variables that delimit species. From such an idea a set of operational criteria can be derived that enable investigators to categorise organisms. These criteria may include morphological similarity, ability to interbreed and reproduce, ecological adaptation, ancestry and descent relationships, or genetic cohesion (Rojas 1992). The application of such criteria to distinguish among species is complicated by the fact that organisms often differ on some of these dimensions but not in others (e.g. they display morphological discontinuity but no reproductive isolation). Decisions as to which criteria should be given preference are often a function of an investigator's philosophical predisposition. However, philosophical preferences must sometimes be set aside in view of the fact that some criteria are not applicable to all organisms (e.g. asexual organisms can not be differentiated based on their ability to interbreed). A single universal species concept can, therefore, not be uniformly imposed in taxonomy (Endler 1989, Davis 1996, Hull 1997).

Species concepts have been reviewed many times in the past (e.g. Mishler and Donoghue 1982, Luckow 1995, Mallet 1995, Hull 1997, Mayden 1997). In a review by Mayden (1997), 22 species concepts were listed from taxonomic literature. These concepts can be arranged in three broad classes: definitions that entail similarity between organisms (morphological and phenotypic); those that invoke evolutionary processes (biological species, evolutionary species, species mate recognition); and phylogenetic or lineage based concepts (Hull 1997; Perkins 2000). In the case of fungi, it has been suggested that species be defined based on a combination of at least one concept from each of the three main categories (phenotypic cohesiveness, reproductive isolation and common evolutionary descent) (Petersen and Hughes 1999).

Species concepts most eminent in fungal systematic literature are the morphological species concept, biological species concept and phylogenetic (diagnostic and genealogical) species concept. These concepts have contributed significantly to the current understanding of fungal diversity and resulted in the discovery of many previously undetected species. The conceptual basis, operational criteria and limitations of these concepts and their relation to general fungal taxonomy were extensively discussed in several recent reviews (Harrington and Rizzo 1999, Petersen and Hughes 1999, Taylor *et al.* 2000). In the current review, a broad theoretical background is presented of these species concepts with regard to holobasidiomycetes, after which the focus is narrowed to their history and use in *Armillaria* taxonomy.

## The Morphological Species Concept

Until the middle 20$^{th}$ century, the morphological species concept was the basis for fungal classification (Brasier 1997). Various definitions of a morphological species were proposed (e.g. Du Rietz 1930, Simpson 1943). One of these defines a species as "... a community, or a number of related communities, whose distinctive morphological characters are, in the opinion of a competent systematist, sufficiently definite to entitle it, or them, to a specific name." (Regan 1926). Thus, from a strictly morphological point of view, a species in basidiomycetes is a group of organisms congruent in the characteristics of their basidiocarp macro- and micro-morphology.

The application of basidiocarp morphology in species recognition presents various limitations. These are, however, resolved to some extent by employing additional phenotypic characters such

as vegetative mat characteristics, growth rate at different temperatures, secondary metabolite production, isozymes and immunology (Pantidou *et al.* 1983, Bruns *et al.* 1991, Kohn 1992, Guarro *et al.* 1999, Harrington and Rizzo 1999). Species are then defined as groups of organisms with a cluster of phenotypic characters more similar within groups than between groups (Sneath 1976). When overall phenotypic similarity is the primary criterion for defining species, without taking lineage with common descent into account, the concept is phenetic (Sneath 1976, Mayden 1997). The phenetic species concept is, however, considered to be synonymous with the morphological species concept (Mayden 1997).

The majority of fungal species are diagnosed by means of their morphological or phenotypic characters (Taylor *et al.* 2000). Currently, the morphological species concept also forms the basis for new fungal descriptions, as is required by the International Code of Botanical Nomenclature (St. Louis Code)[1]. The utility of the morphological species concept can partially be attributed to its long history and wide use. The fact that so many taxa have already been described in terms of their morphological characteristics allows for comparisons to be drawn between existing taxa as well as between new and existing and/ or described taxa (Taylor *et al.* 2000). However, taxa showing clear evidence of evolutionary divergence (e.g. having lost the ability to interbreed) are often morphologically indistinguishable (Taylor *et al.* 2000). Consequently these taxa, although potentially differentiated in terms of criteria derived from other species concepts, are regarded as conspecific from the perspective of the morphological species concept.

## MORPHOLOGICAL SPECIES CONCEPT IN *ARMILLARIA*

The morphological species concept has dominated *Armillaria* taxonomy since the recognition of species within the tribe, and later genus, by Fries (1821). Using the criteria set by this concept, any agaric with white spores, annulus and broadly attached gills were regarded as a species of *Armillaria* (Volk and Burdsall 1995). The acceptance of *A. mellea* Vahl: Fr. as type of the genus (Watling *et al.* 1982), however, narrowed *Armillaria* spp. to agarics with white spores, decurrent to adnate gills and diploid vegetative mycelium, that are wood inhabiting (parasitic or saprophytic) and produce black to reddish-brown rhizomorphs either in the field or in culture

[1] http://www.bgbm.fu-berlin.de/iapt/nomenclature/code/SaintLouis/0000St.Luistitle.htm

(Watling *et al.* 1991, Volk and Burdsall 1995). Adhering to this circumscription has meant that most of the species previously included in the genus have now been transferred to other genera (Volk and Burdsall 1995). Presently the genus includes at least 36 morphological species (Volk and Burdsall 1995) (Table 1), some which are depicted in Fig. 1.

***Recognition of morphological species***

Recognition of *Armillaria* spp. by means of basidiocarps requires analyses of qualitative and quantitative characteristics of both their macro- and micro-morphology. Although a large variety of characters are available from these structures, many of them are not useful for species recognition due their low interspecific variation. Morphological characters found to be important in species delineation include ornamentation and structure of the stipe and pileus, annulus characteristics, location of pigments, basidiospore size and ornamentation and presence or absence of clamp connections (Bérubé and Dessureault 1988, Watling *et al.* 1991). Data pertaining to the basidiocarp morphology for species currently accepted in *Armillaria* are given in Table 2.

***Practical and theoretical limitations of the morphological species concept***

As is the general case with basidiomycete taxonomy, the recognition of *Armillaria* spp. based on basidiocarp morphology is beset with practical and theoretical limitations. Some of these limitations are outlined below:

- Basidiocarps of *Armillaria* spp. are ephemeral and produced at irregular intervals (Fox *et al.* 1994); consequently they are not readily available during surveys.
- Qualitative and quantitative characteristics are not always linked to the genetic attributes of a specimen but may be influenced by environmental factors, for example the dimensions and colour of the basidiocarps of *A. luteobubalina* that vary depending on the meteorological conditions (Kile and Watling 1981). In some cases, such environmentally determined phenotypic variation may result from the genetic or physiological block of a single enzyme (Petersen 1977).
- Morphological and genetic changes are sometimes not symmetrically linked. Small changes in the genome may lead to enormous changes in morphology; conversely large

genomic changes may yield small morphological changes (Mishler 1985). Some species, for example *A. ostoyae* and *A. gemina*, produce basidiocarps with identical morphology (Bérubé and Dessureault 1989). Speciation may, therefore, have occurred, but with little or no selection pressure for morphological change; consequently pleisomorphic morphological or phenotypic characters may be retained in sibling or cryptic species (Miller *et al.* 1994, Mayden 1997, Taylor *et al.* 1999).

- Convergent or parallel evolution may result in species with similar morphology but without sharing a common ancestor (Brasier 1997, Petersen and Hughes 1999).

In view of these problems, a large repertoire of methods has been developed to delineate *Armillaria* spp., either in combination with or as an alternative to basidiocarp morphology (Table 3).

## The Biological Species Concept

The primary tenet of the biological species concept is reproductive isolation between groups of organisms (Mayr 1942, Dobzhansky 1970). Species are defined "as groups of actually or potentially interbreeding natural populations which are reproductively isolated from other such groups" (Mayr 1942). In this concept, phenotypical and ecological differences are subordinated to interbreeding. Consequently if two populations are interfertile, which implies that they share the same gene pool, they are regarded as representing the same biological species, irrespective of variation in other characteristics (Petersen and Hughes 1999).

Evidence for intersterility groups (biological species) is provided by sexual compatibility between isolates using mating tests. Intersterility is governed by genetic factors that have an epistatic effect to the sexual incompatibility genes between different species (Chase and Ullrich 1990a, b). Thus, intersterility factors provide a mechanism for restricting gene-flow between species by overriding the effect of mating compatibility (Chase and Ullrich 1990a). Consequently isolates from different intersterility groups will not mate, even though they belong to different sexual compatibility groups. Sexual compatibility and intersterility are expressed by clearly identifiable phenotypic attributes (dikaryon formation or diploidization), which renders mating studies an effective means to determine intersterility groups.

Application of the biological species concept in homobasidiomycete taxonomy has proven to be most enlightening, in many cases revealing taxa previously considered to be a single species or representing complexes of species (Clémençon 1977, Vilgalys and Miller 1983, Fries 1984, Hallenberg 1985, Stenlid and Karlsson 1991, Vilgalys 1991, Hallenberg *et al.* 1994, Petersen 1995, Gordon and Petersen 1997, Aanen and Kuyper 1999, Miller and Methven 2000). The biological species concept has also been extensively applied to basidiomycete taxonomy, where its success is attributed to the various characteristics of these fungi that make mating studies relatively easy to conduct (Boidin 1986). Traits considered to be most eminent are their strong outbreeding mating systems and development of absolute intrinsic sterility barriers that often accompany speciation (Petersen and Hughes 1999). By virtue of these properties, holobasidiomycetes are amenable to the biological species definition and interfertility tests have become standard practice in delineating species of these fungi.

## BIOLOGICAL SPECIES CONCEPT IN *ARMILLARIA*

The Biological Species Concept was introduced in *Armillaria* taxonomy only during the late 1970's with mating studies among putative isolates of *A. mellea* (*sensu lato*) (Hintikka 1973, Korhonen 1978, Ullrich and Anderson 1978). This species had been viewed in earlier literature as a single taxon with highly variable basidiocarp morphology, rhizomorph production and morphology, pathogenicity, a broad host range and world-wide distribution (Singer 1956, Gibson 1961, Raabe 1966, 1972). Mating tests and, therefore, species delineation based on the biological species concept were, however, possible only after the sexual system of *A. mellea sensu lato* had been elucidated.

Early researchers observed that mycelia from monospore cultures, basidiocarp tissue and vegetative material of *A. mellea* have single nuclei in their hyphal tips and lack clamp connections (Kniep 1911, Motta 1969, Korhonen and Hintikka 1974). In contrast, higher homobasidiomycetes generate dikaryotic vegetative mycelia after anastomosis between sexually compatible monokaryotic hyphae, and clamp connections are observed that retain the dikaryon. These unique features of *A. mellea* have influenced its taxonomy in two ways: 1) The presence of a single nucleus and absence of clamp connections led researchers to consider the sexual system of *A. mellea* (*sensu lato*) as homothallic, asexual or homomictic (Kniep 1911, Burnett 1956, Raper 1966). 2) In mating studies with other basidiomycetes, the formation of clamp

connections is used instead of fruiting as criterion for sexual compatibility between strains. The absence of clamp connections in *A. mellea* precludes the use of this criterion. It is probably because of these two factors that mating tests were not used in *Armillaria* until the work of Hintikka (1973) was published. This is despite the fact that they had been employed in various other basidiomycetes e.g. *Fomes pinicola* (Mounce and MacRae 1938) and *Auricularia auricula* (Duncan and MacDonald 1967) for many years.

Hintikka (1973) observed that monospore cultures made from a single basidiocarp of *A. mellea* had profuse white aerial mycelia. In contrast, cultures made from rhizomorphs, mycelial fans on wood and basidiocarps were crustose and dark brown with aerial mycelia usually lacking. In crosses made between the monospore isolates the culture morphology was transformed to those of the vegetative cultures in accordance with a tetrapolar (bifactorial) mating system. Hintikka (1973) also suggested that, because a single nucleus is present in monospore isolates, the single nucleus in the vegetative mycelium of *A. mellea* should be diploid. These observations were later confirmed (Ullrich and Anderson 1978, Anderson and Ullrich 1982) and paved the way for the use of mating tests in *A. mellea sensu lato*.

Mating tests were first conducted among isolates of *A. mellea* from Europe by Korhonen (1978) and North America by Ullrich and Anderson (1978) and later Anderson and Ullrich (1979). Results of these tests revealed the presence of five intersterility groups in the *A. mellea* complex in Europe (Korhonen 1978) and ten groups in North America (Anderson and Ullrich 1979). Both research groups concluded that reproductive isolation between the sympatric intersterility groups was complete. This characteristic meets the criteria of the biological species concept (Mayr 1942) and the intersterility groups in Europe and North America were, therefore, equated with biological species (Korhonen 1978, Anderson and Ullrich 1979).

The discovery of biological species within the *A. mellea* complex resulted in its extensive use in *Armillaria* taxonomy. Consequently, at least 31 biological species are currently known from different parts of the world, many of which correspond to morphological species (Table 4). Seven biological species occur in Europe, all equated with morphological species (Korhonen 1978, Guillaumin *et al.* 1985, Roll Hansen 1985, Termorshuizen and Arnolds 1987, Zolciak *et al.* 1997). In North America, ten biological species have been found, of which only one (NABS X) is not described in terms of basidiocarp morphology (Anderson and Ullrich 1979, Anderson 1982, Anderson 1986, Morrison *et al.* 1985a, Motta and Korhonen 1986, Bérubé and Dessureault

1988, Bérubé and Dessureault 1989, Volk *et al.* 1996). At least ten biological species occur in Asia, with all but one (NAG E) linked to morphological species (Terashita and Chuman 1989, Cha and Igarashi 1994, 1995b, 1996, Cha *et al.* 1994, 1995, Mohammed *et al.* 1994a, Ota *et al.* 1998b). Australasian isolates representing the morphological species *A. hinnulea, A. luteobubalina, A. limonea, A. novae-zealandiae* and *A. pallidula* are intersterile and these species consequently also represent different biological species (Kile and Watling 1988). Only four biological species have been reported from Africa, of which two represent morphological species (Mohammed and Guillaumin 1993, Mohammed *et al.* 1994b, Abomo-Ndongo and Guillaumin 1997).

***Recognition of biological species***

Identification of biological species in *Armillaria* is based on either sexual or interspecific somatic incompatibility tests depending on the sexual system of isolates being studied. Most species have a heterothallic bifactorial (tetrapolar) mating system (Korhonen 1978, Ullrich and Anderson 1978, Kile and Watling 1988); it is therefore possible to employ sexual compatibility tests for routine use in species recognition (e.g. Proffer *et al.* 1987, Dumas 1988, Blodgett and Worrall 1992, Harrington and Rizzo 1993). Homothallic sexual systems have, however, been reported for a few species including *A. ectypa, A. heimii, A. mellea* (from Africa) and *A. mellea* subsp. *nipponica* (Cha and Igarashi 1995b, Abomo *et al.* 1997, Zolciak *et al.* 1997). These species produce diploid mycelium from their basidiospores (Fig. 2), which render them unsuitable for mating tests. It was, therefore, suggested that interspecific somatic incompatibility tests be conducted as a means to delineate biological species (Abomo- Ndongo and Guillaumin 1997). In both tests, pre-zygotic reproductive isolation mechanisms allow for a visual evaluation based on the culture morphology (Brasier 1987).

Identification of biological species in *Armillaria* with a heterothallic bifactorial (tetrapolar) mating system (Fig. 2) is usually based on the haploid-haploid sexual compatibility interaction between reference and unknown strains. Sexual compatibility between strains belonging to the same species is dependent on allelic differences at two unlinked mating type loci (e.g. *A* and *B*). Crosses between such isolates may, therefore, display one of the following interactions (Korhonen 1978):

1) Compatible ($A \neq B \neq$) for example ($A_1B_1$ x $A_2B_2$): Border between the mating mycelia disappears. Anastomosis takes place, cells become heterokaryotic followed by diploidization. The culture morphology is transformed from the haploid (white, cottony) to the diploid (crustose, brown) type (Fig. 3). This reaction is taken as evidence for conspecificity between the reference strain and the unknown isolate.
2) Incompatible ($A=$, $B=$) for example ($A_1B_1$ x $A_1B_1$): The haploid culture morphology is maintained and mycelia grow side by side.
3) Hemicompatible common $A$ ($A=$, $B \neq$) for example ($A_1B_1$ x $A_1B_2$): A barrage zone between the confronting mycelia is observed; some of the submerged hyphae have partially disintegrated septa.
4) Hemicompatible common $B$ ($A \neq B=$) for example ($A_1B_1$ x $A_2B_1$): Similar to incompatible interaction.

Strains belonging to different biological species display the same interaction as incompatible strains of the same species. Thus, while compatible interactions generally provide conclusive evidence of conspecificity, the converse conclusion cannot be drawn from incompatible interactions. This raises the possibility that conspecific sympatric species might erroneously be regarded as different species due their shared alleles at the mating type loci.

Diploid-haploid mating tests are useful for species identification when monospore (haploid) cultures are not available for the unknown isolates (Korhonen 1978, Anderson and Ullrich 1982). These tests are functionally equivalent to the "Buller phenomenon" where a compatible dikaryotic mycelium donates nuclei to the monokaryotic counterpart during mating (Raper 1966, Anderson and Ullrich 1982). In a compatible mating between heterothallic *Armillaria* isolates the diploid nuclei are transferred to the haploid isolate and subsequently displace the haploid nuclei (Rizzo and Harrington 1992, Rizzo and May 1994, Carvalho *et al.* 1995) or occasionally recombine with the haploid nuclei (Guillaumin *et al.* 1991, Carvalho *et al.* 1995). A compatible mating interaction in this test is judged by the transformation of the haploid culture morphology to that of the diploid culture (Korhonen 1978). Although diploid-haploid mating tests are regularly used for species identification (e.g. Gregory 1989, Mohammed *et al.* 1994a, Tsopelas 1999), diploidization is slow (Korhonen 1978, 1983) and results are often ambiguous (Siepmann 1985, Shaw and Loopstra 1988).

An alternative to diploid-haploid pairings in sexual compatibility tests is to induce somatic segregation of diploids with the use of Benomyl (Anderson 1983, Anderson and Yacoob 1984).

The artificial haploids are then used in a similar fashion to haploid-haploid tests. This method has been used in some studies (e.g. Proffer *et al.* 1987, Mwangi *et al.* 1989) but its success is not guaranteed (Holdenrieder 1986).

Species recognition based on interspecific somatic incompatibility tests employs diploid-diploid crosses between reference and unknown isolates of *Armillaria*. This method should, however, not be confused with intraspecific somatic incompatibility tests that use crosses between diploid isolates of the same species to distinguish between genotypes in population studies (Korhonen 1978, Kile 1983, Harrington *et al.* 1992). In intraspecific somatic compatibility tests, isolates of different genomic entities produce a demarcation line of faint hyaline mycelium at the confrontation point (Korhonen 1978). Interspecific somatic incompatibility between isolates, on the other hand, is determined by the formation of a black pigmented demarcation line between the confronting mycelia of different biological species (Mallett and Hiratsuka 1986, Mallett *et al.* 1989). This black demarcation line is often not clear and may be enhanced with L-DOPA (L-β-3,4-dihydroxyphenylalanine) (Hopkin *et al.* 1989). Isolates that do not produce the demarcation line are regarded as conspecific.

***Practical and theoretical limitations of the biological species concept***

The biological species concept is mechanistic in the sense that species are conceived as participants in an evolutionary process and not the end-points of evolution (Luckow 1995). The mechanistic paradigm, of which the biological species concept is a representative, is hampered by theoretical flaws that are related to its dependence on the biology of a particular organism under investigation and dependence on observation of process rather than pattern (Luckow 1995). Its major theoretical shortcoming, however, is its *a priori* decision to focus on a specific causal agent of speciation with disregard for the potential contribution of other factors (Donoghue 1985, Luckow 1995). It ignores the fact that reproductive isolation is but a single node in a complex web of interrelated processes, many of which may be regarded as both the cause and the product of speciation (Cracraft 1989, Endler 1989, Turelli *et al.* 2001). In view of these problems many systematists have rejected the biological species concept (Donoghue 1985, Cracraft 1989, 1997).

Practical problems with the biological species concept arise when sympatrically defined biological species are considered in allopatric terms. The European species, *A. cepistipes* (= *A. bulbosa*, EBS B), is reproductively isolated from its European counterparts (Korhonen 1978). This species is fully interfertile with the North American NABS XI and is, therefore, conspecific with it (Morrison *et al.* 1985a, Banik and Burdsall 1998). It is, however, also partially interfertile with two North American biological species, *A. sinapina* (NABS V) and NABS X (Anderson *et al.* 1980, Anderson 1986, Bérubé *et al.* 1996). The reproductive barriers between these allopatric intersterility groups are, therefore, not complete. Partial interfertility between these intersterility groups may be associated with recent speciation or with taxa in the process of speciation through geographic isolation, host specialisation or adaptation to changing environmental conditions without development of genetic isolation mechanisms (Boidin 1986). The ability to interbreed could, therefore, be ascribed to a retained ancestral trait (plesiomorphy) (Rosen 1978, 1979, Bremer and Wanntorp 1979, Donoghue 1985, Davis 1997). The occurrence of such reactions during mating tests poses a serious problem in assigning anonymous isolates unequivocally to a biological species.

It is possible that species might remain fully interfertile despite their being morphologically, ecologically or phylogenetically distinct e.g. *Auricularia* (Duncan and MacDonald 1967, Duncan 1972) and *Lentinula* (Hibbett *et al.* 1995, Petersen 1995). Intersterility is governed by relatively simple genetic determinants (Hallenberg 1988, Chase and Ullrich 1990a, b, Hallenberg and Larsson 1992) and are not necessarily linked to morphological, phenotypic, genetic and ecological traits (Petersen and Bermudes 1992). Divergence in these traits may, therefore, precede the emergence of reproductive barriers. The genetic basis for intersterility between biological species is, however, not well understood in most basidiomycetes, including *Armillaria*.

A further practical problem is the fact that the relational nature of biological species in terms of diagnosable characters makes it difficult to assign anonymous isolates to species, without the aid of a battery of tester isolates. Live mating monokaryotic/ haploid reference strains representing a biological species must, therefore, be readily available from culture collections. Currently testers for the North American Biological Species (NABS) are available from the American Type Culture Collection (Anderson 1986). However, mating tests yield better results with fresh strains and some haploid strains may become dark and crustose with age and are, therefore, not suitable for mating tests (Harrington *et al.* 1992). An additional problem posed by the relational nature

of the biological species concept is the fact that some species (e.g. *A. gallica*, *A. cepistipes* and *A. calvescens*) produce rather crustose haploid cultures whereas other species (e.g. *A. mellea*) may generate cottonous diploid mycelium that complicates interpretation of mating tests (Guillaumin *et al.* 1991, Harrington *et al.* 1992).

In addition to the problems outlined above, concern exists about the ability of mating tests to provide evidence of true interfertility (i.e. the ability to produce viable monokaryotic progeny) since mating is only the first step towards reproduction (Mueller and Gardes 1991, Harrington and Rizzo 1999). However, stable dikaryon formation between two monokaryotic hyphae and subsequent repetitive coupled nuclear division are considered to indicate close genetic relationships (Boidin 1986). The recognition of species is also complicated by the fact that intersterility barriers between populations might not always be an indication of species boundaries, but in some cases may be regarded as a species' propagation strategy, in particular when genetic differences between intersterility groups are small (Hallenberg and Larsson 1992, Hallenberg *et al.* 1994, 1996).

## Phylogenetic Species Concepts

Phylogenetic species concepts represent a diverse set of species concepts, all of which have their historical roots in Hennig's (Hennig 1966) phylogenetic systematics and later work by Rosen (Rosen 1978, 1979). Phylogenetic systematics defines the boundary between species as the interface between reticulated (tokogenetically related) and hierarchic (phylogenetically) descendent systems (Fig. 4) (Hennig 1966). From this perspective, the main focus of a phylogenetic species concept should be to recognize the boundary between the two systems. This is accomplished by determining the hierarchical ancestry and descendent structures among organisms and then interpreting and incorporating these structures in terms of a classification system (Davis 1996, 1999).

Phylogenetic species concepts comprise at least four different versions. These include the diagnostic species concept (Eldredge and Cracraft 1980, Nelson and Platnick 1981, Cracraft 1983, Nixon and Wheeler 1990, Wheeler 1990), monophyletic (autapomorphic) species concept (Donoghue 1985, Mishler and Donoghue 1982, Mishler and Brandon 1987, de Queiroz and Donoghue 1988, 1990a), a combination of the first two concepts (McKitrick and Zink 1988), and

the genealogical concordance species concept (also known as the genealogical species concept) (Baum and Donoghue 1995, Baum and Shaw 1995). Concepts within the body of the phylogenetic species concept differ significantly in their assumptions, criteria used for species diagnoses and adherence to the Hennigian phylogenetic systematic principles.

Phylogenetic species concepts such as the diagnostic and genealogical concordance species concepts view species as biological entities at the end point of evolution and are, therefore, considered historical species concepts (Luckow 1995). History based concepts are "theory neutral" in terms of evolutionary process; what matters is pattern, not process. Species recognition is therefore solely based on character evidence of ancestry. Other versions such as the monophyletic species concept employ a combination of historical and mechanistic approaches (Luckow 1995). These concepts give primacy to monophyly (an historical attribute) as grouping criterion and then rank taxa based on a speciation mechanism (e.g. reproductive isolation) believed to give rise to and maintaining the lineage (Donoghue 1985, Mishler and Donoghue 1982, Mishler and Brandon 1987).

A major source of conflict between advocates of different phylogenetic species concepts is their disagreement on the conceptualisation of monophyly (see Davis 1999 for an in depth discussion on this issue). Hennig (1966) defined monophyletic groups as "... a group of species descended from a single ('stem') species, and which includes all species descended from this species." Hennig (1966) also gave a second definition that states that "A monophyletic group is a group of species in which every species is more closely related to every other species than to any species that is classified outside this group." Monophyly in Hennigian terms is thus applicable at the phylogenetic level and refers to a specific relationship between at least two species. Some authors have, however, extended monophyly to the level of individual organisms (Donoghue 1985, Baum 1992) or populations (Mishler 1985, de Queiroz and Donoghue 1988).

Phylogenetic species concepts most prominent in contemporary systematic literature include the diagnostic species concept and the genealogical concordance species concept (Baum 1992, Davis 1996). These concepts have been the subject of numerous discussions and critical comparisons in the past (e.g. Baum and Donoghue 1995, Luckow 1995, Davis 1996, 1997). Application and limitations of these concepts in fungal taxonomy were discussed in depth and advocated with examples from various genera in recent reviews by Harrington and Rizzo (1999) and Taylor *et al.* (2000). These concepts have not received, however, much attention in *Armillaria* taxonomy.

The current review will therefore be limited to a broad overview of the general principles underlying these two types of phylogenetic species concepts.

## DIAGNOSTIC SPECIES CONCEPT

The diagnostic species concept (*sensu* Hull 1997) was developed and promoted by authors that include Eldredge and Cracraft (1980), Nelson and Platnick (1981), Cracraft (1983), Nixon and Wheeler (1990), Wheeler and Nixon (1990), Davis and Nixon (1992). In terms of this concept, a species is "the smallest aggregation of populations (sexual) and lineages (asexual) diagnosable by a unique combination of character states in comparable individuals (semaphoronts)" (Nixon and Wheeler 1990). A phylogenetic species, within this context, is thus a group of organisms among which there is a reticulated ancestry and descent structure (tokogenetic relationship) and forms the basal diagnosable element among the hierarchy (phylogenetic relationship) of taxa within a classification system.

The diagnostic species concept is consistent with Hennig's (Hennig 1966) view that a single species is not monophyletic; a species can only be monophyletic with another species (Luckow 1995, Davis 1999). As mentioned above, species in this concept are minimal basal phylogenetic elements with reticulated structure within the species. If they were to be monophyletic, this would imply that phylogenetic structure (hierarchic) exist within a species. Consequently, monophyly in terms of this species concept is not applicable for delimiting species. Key to the diagnostic species concept is constant characters or character states as evidence for divergence between species and phylogenetic pattern (Davis and Nixon 1992).

### *Recognition of diagnostic species*

Proponents of the diagnostic species concept see species as the result of speciation; pattern and not process is of importance in this concept (Cracraft 1983). Pattern reflects common ancestry and evolutionary history and is observed by assessing the inherited attributes of organisms. Inherited attributes are considered to represent either traits or characters (Nixon and Wheeler 1990, Davis and Nixon 1992). Traits are properties that are not fixed in a population and are, therefore, not present in all comparative individuals (semaphoronts) among a terminal lineage.

Traits do not reliably reflect historical relationships among organisms (Davis and Nixon 1992). Characters, in contrast, are fixed properties within a population and are therefore present in all comparative individuals in a terminal lineage. Fixed characters provide evidence for hierarchic descent (Davis and Nixon 1992). These characters need not be monomorphic but can represent the original or transformed states of a character (Davis and Nixon 1992). The nature of characters is not taken into account and can be any unique combination of derived (apomorphic) or primitive (pleisomorphic) characters. Characters are obtained from any of the comparable intrinsic attributes of organisms (Cracraft 1983, 1989, Harrington and Rizzo 1999).

One method for discovering diagnostic species is through "population aggregation analysis" (Davis and Nixon 1992). This method distinguishes traits from attributes by means of pattern variation analyses within local populations. Populations with fixed characters are then aggregated and assigned to a diagnostic species. Davis and Nixon (1992) indicated several sources of error that include incorrect homology assessment, undersampling of attributes, individuals or populations, incorrect delimitation of populations and parallel fixation. Most of these can, however, be avoided through rigorous study of characters and populations (Harrington and Rizzo 1999).

### Genealogical Concordance Species Concept

The genealogical concordance species concept (GCSC) was derived from the monophyletic species concept (Mishler and Donoghue 1982, Donoghue 1985, de Queiroz and Donoghue 1988, 1990a) that gives primacy to shared historical relationships between organisms as the attribute that unites them in a species. The GCSC was first proposed by Avise and Ball (1990) and further developed and promoted as the genealogical species concept by Baum and Shaw (1995). This concept defines species as "basal, exclusive groups of organisms" (Baum and Shaw 1995)

The GCSC adopted a variation of the second definition of monophyly provided by Hennig (Davis 1999). Baum and Shaw (1995) follow earlier views (Donoghue 1985, de Queiroz and Donoghue 1988) extending the concept of monophyly to a level that relates to relationships between individual organisms and not only between species. Monophyly at this level is equated with the term exclusivity (de Queiroz and Donoghue 1990b) where "an exclusive group is one whose members are more closely related to each other than they are to any organism outside the

group" (Baum and Donoghue 1995). Davis (1999), however, pointed out that that the term "exclusivity" in the context of the GCSC refers to a group of organisms whose members have gene copies that are more closely related to each other than to any gene copies of organisms outside the group.

Exclusive genealogical relationships are determined by means of coalescence patterns of gene genealogies of individual organisms from different populations (Baum and Shaw 1995). This approach stems from ideas adopted from "coalescence theory" whereby the transmission pathway of gene lineages is traced back in time to the point where they coalesce with their most recent common ancestor (MRCA) (Hudson 1990, Maddison 1995). In the GCSC, individuals with gene lineages that coalesce to a single lineage, the MRCA of the genealogy, constitute an exclusive genealogical relationship (Baum and Shaw 1995). In the light of coalescence theory, Baum and Donoghue (1995) have redefined genealogical species as "a basal group of organisms all of whose genes coalesce more recently with each other than with those outside the group."

***Recognition of genealogical species***

The GCSC invokes phylogenetic analysis of gene sequence data to construct gene trees representing the gene genealogy of organisms. Gene sequences are obtained from individuals sampled from different populations and often only portions of the genes are used. Genes, or gene regions, to be employed in phylogenetic analyses are not specified but a prerequisite is that they should not be recombining within the species (Baum and Shaw 1995).

Gene trees generated from single loci and species trees often do not correspond in their topological patterns. Reasons for this phenomenon include ancient divergence among gene lineages in contrast to a more recent divergence among species, use of paralogous genomic regions, and recombination through horizontal transfer or hybridisation between species (Hudson 1983, 1992, Nei 1987, Takahata 1989, Wu 1991, Doyle 1992, Maddison 1995, 1997, Brower *et al.* 1996). It is, therefore, suggested that genealogical concordance among multiple loci from the same set of individuals be used to delimit species (Baum and Donoghue 1995, Baum and Shaw 1995). Species limits in this approach are determined at the point of transition from incongruity to congruence in a consensus gene tree (Taylor *et al.* 2000) (Fig. 5). Alternatively, multi-loci

sequence data are combined and the point of transition determined at the branching node in the combined gene tree with high statistical support (Kroken and Taylor 2001).

## PHYLOGENETIC RELATIONSHIPS AMONG *ARMILLARIA* SPP.

The phylogenetic relationships among the Northern Hemisphere *Armillaria* spp. have received much attention and are consequently well resolved. Collectively, a number of studies suggest that the Northern Hemisphere species reside in at least five major clusters. Based on overall similarity and differences among taxa in terms of morphological and ecological characteristics, Korhonen (1995) identified these as the *A. ostoyae*, *A. gallica*, *A. mellea*, *A. ectypa* and *A. tabescens* clusters (in this review the *A. ectypa* and *A. tabescens* clusters will be referred to as the "exannulated cluster"). Assessing the relationships between taxa within these clusters is, however, complicated by the fact that many researchers have concentrated only on those species that are of specific interest to them. In contrast to the Northern Hemisphere species, the phylogenetic relationships among the Southern Hemisphere species have not received much attention and virtually nothing is known about them in this regard. One of the reliable conclusions that can be drawn, however, is that the Southern Hemisphere species can be sorted into two clusters: an African cluster and an Australasian cluster. The four Northern Hemisphere and two Southern Hemisphere clusters are discussed in turn below.

### The "*Armillaria ostoyae* cluster"

The "*Armillaria ostoyae* cluster" (Fig. 6) includes three species: *A. ostoyae*, *A. gemina* and *A. borealis*. These species are morphologically related by their thick annulus, more or less equal shape of the stipe and distinct dark scales (Gregory and Watling 1985, Bérubé and Dessureault 1989, Korhonen 1995). Phylogenetically these species are more closely related to one another than to other Northern Hemisphere *Armillaria* spp. (Anderson *et al.* 1989, Anderson and Stasovski 1992).

The three species in this cluster are distinct in their ITS and IGS-1 sequence data (Anderson and Stasovski 1992, Chillali *et al.* 1998a) and were separated into three respective rDNA classes based on their rDNA RFLP profiles (Anderson *et al.* 1989). Furthermore, they show variation in

terms of their geographic distribution: *A. borealis* is confined to Europe, *A. gemina* to North America and *A. ostoyae* is transcontinentally distributed between Europe, Japan and North America (Kile *et al.* 1994, Ota *et al.* 1998a). Some authors have therefore suggested that *A. ostoyae* is ancestral to *A. gemina* by virtue of its broader distribution (Miller *et al.* 1994, Piercey-Normore *et al.* 1998) and it is for the same reason probably ancestral to *A. borealis*.

## The "*Armillaria gallica* cluster"

The "*Armillaria gallica* cluster" (Fig. 6) represents the largest group of Northern Hemisphere species and includes *A. calvescens*, *A. cepistipes*, *A. gallica*, *A. jezoensis*, *A. nabsnona*, *A. sinapina*, *A singula* and NABS X. Morphologically these species, with the exception of NABS X for which the basidiocarp morphology is not known, are related by virtue of their thin delicate annulus and more bulbous or clavate stipes (Korhonen 1995). A combination of various phylogenetic studies based on ITS (Chillali *et al.* 1998b), IGS-1 (Anderson and Stasovski 1992, Terashima *et al.* 1998a), DNA-DNA hybridisation (Miller *et al.* 1994) and amplification of sequences with arbitrary primer pairs (SWAPP) (Piercey-Normore *et al.* 1998) supported their grouping and the conclusion that they share a common ancestor. The relationships between the species within this cluster are, however, not well resolved.

Analysis of rDNA operon sequence data revealed that the European and North American biological species in this cluster are separated into two rDNA classes (Anderson *et al.* 1989). The one rDNA class included *A. gallica, A. cepistipes* and *A. calvescens,* based on their shared 0.4 Kbp (Kilobase pair) insertion at 5' end of rDNA operon, while the second class included *A. sinapina*, *A. nabsnona* and NABS X (Anderson *et al.* 1989). Subsequent DNA-DNA hybridisation and IGS-1 sequence analyses, however, could not resolve the relationships between taxa within the two classes and therefore did not support their dichotomy (Anderson and Stasovski 1992, Miller *et al.* 1994). Recently, Piercey-Normore *et al.* (1998) showed that the morphologically similar species *A. gallica* and *A. calvescens* are more closely related to each other than to the other species in this cluster. It was also suggested that *A. gallica* might be the ancestor to *A. calvescens* based on the broad distribution of the former species in Europe, North America and Japan in contrast to that of the latter species, which is restricted to North America. The two Asian species, *A. singula* and *A. jezoensis*, are closely related and form a monophyletic group with *A. sinapina* and *A. cepistipes* isolates from Japan (Terashima *et al.* 1998a).

## The "*Armillaria mellea* cluster"

*Armillaria mellea* is the only member of this cluster and is distinct from the rest of the annulated Northern Hemisphere *Armillaria* spp. based on morphological and molecular characteristics. Representatives of this species cluster are characterised by the complete lack of clamp connections at the base of their basidia, prominent annulus, honey coloured caps and robust appearance of their basidiocarps (Motta and Korhonen 1986, Bérubé and Dessureault 1989). At the molecular level, this species is differentiated from other *Armillaria* spp. by a shorter IGS-1 region (Harrington and Wingfield 1995, Terashima *et al.* 1998a) and a 2.5 Kbp insertion in their rDNA operon (Anderson *et al.* 1989).

Phylogenetic studies indicate that this species is distantly related to the rest of the annulated *Armillaria* spp. from the Northern Hemisphere (Anderson and Stasovski 1992, Miller *et al.* 1994, Chillali *et al.* 1998b, Piercey-Normore *et al.* 1998). Consequently, some authors suggested that *A. mellea* is a basal species to the annulated species from the Northern Hemisphere (Miller *et al.* 1994, Piercey-Normore *et al.* 1998). The relationships between *A. mellea* and the annulated *Armillaria* spp. from the Southern Hemisphere have, however, not been investigated and a final conclusion can thus not be drawn.

Members of the "*Armillaria mellea* cluster" display considerable intraspecific variation. Differences are observed in their sexual systems with homothallic forms occurring in Africa and Japan, and heterothallic forms in Europe and North America (Hintikka 1973, Ullrich and Anderson 1978, Abomo-Ndongo *et al.* 1997, Ota *et al.* 1998a). Isolates from Europe and North America were differentiated based on differences in RFLP (restriction fragment-length polymorphism) patterns of the rDNA operon (Anderson *et al.* 1989) and RAPD (randomly amplified polymorphic DNA) profiles (Ota *et al.* 2000). The African and Japanese *A. mellea* are divergent from the heterothallic forms but are genetically similar and it was suggested that they originated in Japan (Ota *et al.* 2000). Phylogenetic studies based on ITS and IGS-1 sequence data showed that members of this cluster can be separated into four distinct geographic lineages representing Europe, western and eastern North America and Asia (Coetzee *et al.* 2000b). In view of the high diversity in *A. mellea,* it was suggested that *A. mellea* is in the process of speciation as a result of genetic isolation due to geographic barriers (Coetzee *et al.* 2000b).

## The "Exannulated cluster"

The "Exannulated cluster" includes *A. tabescens* and *A. ectypa* (Fig. 6). Both species are characterised by their complete lack of an annulus. *Armillaria ectypa*, however, is homothallic and a rare species in Europe, growing specifically in peat bogs (Zolciak *et al.* 1997). In contrast, *A. tabescens* is heterothallic (Darmono *et al.* 1992) and more widely distributed, occurring in Europe, Japan and North America[2] (Volk and Burdsall 1995).

Phylogenetic studies have shown that *A. tabescens* and *A. ectypa* are distantly related to the annulated species of *Armillaria* (Anderson and Stasovski 1992, Miller *et al.* 1994, Chillali *et al.* 1998b). Miller *et al.* (1994) suggested that *A. tabescens* is the oldest species and that it gave rise to the genus. These authors did not, however, include *A. ectypa* in their study. In a more resent study, Chillali *et al.* (1998b) suggested that *A. tabescens* is more closely related to *A. mellea* and that *A. ectypa* is the basal species to *Armillaria*. The narrow distribution of *A. ectypa*, however, renders the conjecture that this species is ancestral to *Armillaria* highly improbable.

## The "African cluster"

The "African cluster" includes *A. fuscipes* and *A. heimii* (Fig. 6). A distinguishing feature of this cluster is the fact that their 5S gene is in an inverted orientation relative to that of other *Armillaria* spp. (Coetzee *et al.* 2000a). The two species residing in this cluster were considered synonymous by some authors and the name *A. heimii* was given preference (Mohammed and Guillaumin 1993). A recent study by Coetzee *et al.* (2000a), however, separated isolates thought represent *A. heimii* into two monophyletic lineages based on their IGS-1 sequence data. The authors subsequently suggested that the one lineage be named A. *fuscipes* and the second *A. heimii*. The phylogenetic relationship between these species and the rest of the *Armillaria* spp. is currently unknown.

[2] The name *A. monadelpha* (Morgan) was erroneously used for this fungus in North America where it was thought to be intersterile with *A. tabescens* from Europe (Volk and Burdsall 1995).

## The "Australasian cluster"

The Australasian cluster includes the more common species reported from Australia and New Zealand (Fig. 6). These species include *A. fumosa*, *A. hinnulea*, *A. pallidula*, *A. novae-zelandiae*, *A. limonea* and *A. luteobubalina* (Podger *et al.* 1978, Kile and Watling 1981, 1983, 1988, Pearce *et al.* 1986, Hood 1989). Information pertaining to the phylogenetic relationships of these species to one another and to those from the Northern Hemisphere is not currently available from the literature. Hypotheses regarding the relationships of some species can, however, be formulated based on their distribution and morphological characteristics.

*Armillaria novae-zelandiae* has been reported from Australia and New Zealand, while *A. limonea* has been reported from New Zealand. Both species were also found on *Nothofagus* trees in South America by Singer (Singer 1969). These trees formed a continuous forest from Australia and New Zealand through Antarctica to South America when these landmasses were part of Gondwanaland (Poole 1987). It is therefore likely that *A. novae-zelandiae* and *A. limonea* have a Gondwanean origin and that they represent the ancestors of the species in the Australasian clade.

*Armillaria luteobubalina* is broadly distributed in eastern and western Australia (Kile and Watling 1981, 1983, Pearce *et al.* 1986) and may be ancestral to the Australian species, *A. fumosa* and *A. pallidula*. *Armillaria pallidula* was reported from only one location in Queensland in Australia (Kile and Watling 1988) and may therefore have a relatively recent origin within the Australasian cluster. *Armillaria hinnulea* resembles the Northern Hemisphere *A. cepistipes* (synonym *A. bulbosa*) in basidiocarp morphology and is the only species with clamp connections in the sub-hymenial layer of its basidiocarps (Kile and Watling 1983). Hence, *A. hinnulea* is probably closely related to the Northern Hemisphere species.

## CONCLUSIONS

This review shows that *Armillaria* is a highly diverse genus comprising several biological and morphological species. Much information is available regarding their distribution and their relationships to one another. The following conclusions are drawn from the reviewed studies:

- Species identification is possible through a variety of morphological, biochemical and DNA-based methods.

- All three major categories of species concepts (the morphological, biological and phylogenetic species concepts) have been employed in fungal taxonomic literature. The morphological species concept and the biological concept have made a major contribution to the current understanding of species within the genus *Armillaria*. Both concepts are, however, subject to certain limitations and the use of a single concept makes unequivocal identification of species problematic. The phylogenetic species concept, although widely used in fungal taxonomy, has not received much attention in *Armillaria* taxonomy. It may provide a valuable means for species delineation and identification.

- The phylogenetic relationships among species from the Northern Hemisphere are well resolved. In contrast, nothing is known about the relationships among species from the Southern Hemisphere and their relationship with those from the Northern Hemisphere.

- The distribution of *Armillaria novae-zelandiae* and *A. limonea* suggest that the Southern Hemisphere species might have a Gondwanean origin. It is therefore postulated that the Southern Hemisphere *Armillaria* spp. might be very old and may have given rise to the Northern Hemisphere species.

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**TABLE 1**: Species currently accepted in the genus *Armillaria* (Fr.:Fr.) Staude and their distribution (adopted from Watling *et al.* 1991 and Volk and Burdsall 1995).

| Species | Species |
|---|---|
| 1. *A. affinis* (Singer) Volk & Burdsall. Central America, Carribbean | 20. *A. melleo-rubens* (Berk. & M.A.Curtis) Sacc. Central America. |
| 2. *A. borealis* Marxmüller & Korhonen. Europe | 21. *A. montagnei* (Singer) Herink. South America, Europe. |
| 3. *A. calvescens* Bérubé & Desurr. Eastern North America. | 22. *A. nabsnona* Volk & Burdsall. Western North America. |
| 4. *A. camerunensis* (Henn.) Volk & Burdsall. Africa. | 23. *A. novae-zelandiae* (G.Stev) Herink. Australia, New Zealand, New Guinea, South America. |
| 5. *A. cepistipes* Velen. Europe, North America, Japan. | 24. *A. omnituens* (Berk.) Sacc. India. |
| 6. *A. duplicate* (Berk.) Sacc. India. | 25. *A. ostoyae* (Romagn.) Herink. (= *A. obscura* (Shaeff.) Herink, *Armillariella polymyces* (Pers.) Singer & Clémençon). Europe, North America, Japan. |
| 7. *A. ectypa* (Berk.) Emel. Europe. | 26. *A. pallidula* Kile & Watling. Australia. |
| 8. *A. fellea* (Hongo) Kile & Watling. New Guinea. | 27. *A. pelliculata* Beeli. Africa. |
| 9. *A. fumosa* Kile & Watling. Australia. | 28. *A. procera* Speg. South America. |
| 10. *A. fuscipes* Petch. India, Africa† | 29. *A. puiggarii* Speg. South America. |
| 11. *A. gallica* Marxmüller & Romagn.(= *A. lutea* Gillet, *A. bulbosa* (Barla) Kile & Watling). Europe, Japan, North America. | 30. *A. sinapina* Bérubé & Dessur. Japan, North America. |
| 12. *A. gemina* Bérubé & Dessur. Eastern North America. | 31. *A. singula* Cha & Igarashi. Japan. |
| 13. *A. griseomellea* (Singer) Kile & Watling. South America. | 32. *A. sparrei* (Singer) Herink. South America. |
| 14. *A. heimii* Pegler. Africa† | 33. *A. tabescens* (Scop.) Emel. Europe, North America, Japan |
| 15. *A. hinnulea* Kile & Watling. Australia, New Zealand. | 34. *A. tigrensis* (Singer) Volk & Burdsall. South America |
| 16. *A. jezoensis* Cha & Igarashi. Japan. | 35. *A. viridiflava* (Singer) Volk & Burdsall. South America, Europe? |
| 17. *A. limonea* (G.Stev) Boesewinkel. New Zealand. | 36. *A. yungensis* (Singer) Herink. South America. |
| 18. *A. luteobubalina* Watling & Kile. Australia. | |
| 19. *A. mellea* (Vahl.:Fr.) P.Kumm. Asia, Africa, Europe, North America. | † Synonymy proposed by Kile and Watling (1988) and Chandra and Watling (1981) |

TABLE 2: Basidiocarp morphology of some *Armillaria* spp.

| Species | *A. affinis* | *A. borealis* | *A. calvescens* | *A. camerunensis* | *A. cepistipes* |
|---|---|---|---|---|---|
| References | Singer 1989 (in Latin) | Gregory and Watling 1985 | Bérubé and Dessureault 1989 | Hennings 1895 (in Latin) | Motta and Korhonen 1986 (as *A. bulbosa*) |
| **Pileus** | | | | | |
| Size (mm) | 29-31 | (18-)28-50 | 20-100 | 5-10 | 50-70(90) |
| Shape | convex, obtuse, soon applanate; *centre* sub-depressed | convex almost campanulate then plano-convex | globose, convex then plano-convex, sometimes mammilate | plano-convex | plano-convex |
| Color | brown | yellow-brown with honey-coloured tinge towards the disk; *centre* faintly bay or purplish | tan to brown | reddish-brown | tan to pinkish-brown; *centre* paler than rest of the pileus |
| Surface | almost nude, translucent; striate; smooth or subsulcate; subviscid; *centre* minute brown scales | black to dark brown rather ephemeral floccules; hygrophanous | finely fibrillose, almost denuded; dry | small dark squamules | black scales; dry; *centre* black scales more densely than rest |
| Margin | | incurved at first; smooth; minutely striate | straight; sometimes with striations | inrolled at first then plane; somewhat striate | inrolled then down-turned; entire; striate |
| Lamellae | decurrent; crowded; horizontal; pale-brown, then brown (pale deep-brown) | subdecurrent to adnate; white, slightly tinged pinkish at first but bruising pinkish cream or with age unevenly pink | subdecurrent to sometimes strongly decurrent; close; thick; sinuate; cream, light brown when old | sinuate-adnate, barely decurrent; close; pale | attached to slightly decurrent; distant; thick at point of attachment to stipe, narrower to the margin, broad; white to pale pinkish buff |
| **Stipe** | | | | | |
| Size (mm) | 42-43x+/-4 (at apex mostly 3 diam) | 55-65 x 6-7 | 40-90x5-20 | 10-20x2-3 | 70-100x15 (at apex) |
| Shape | cylindrical, rarely slightly attenuate at apex | cylindrical, slightly bulbous or clavate | clavate, often bulbous | - | clavate when young, later more or less equal |
| Context texture | - | fluffy fleshy | fibrous | - | fibrous |
| Flesh | - | hollow in over mature basidiocarps | - | stuffed | slightly stuffed |

TABLE 2 (continued)

| Species | *A. affinis* | *A. borealis* | *A. calvescens* | *A. camerunensis* | *A. cepistipes* |
|---|---|---|---|---|---|
| **Annulus** | slightly membranaceous (not arachnoid); white | thick; double; white to cream; floccose | thin; submembranaceous; white to cream | thick; membranaeous; floccose | cortinate; evanescent |
| **Basidiospores** | | | | | |
| Size (µm) | (6.5-)7-8(-9)x(4.5-)4.7-5.5(-6) | (6.4-)6.8-8(-9.2)x4.4-5.7 | 8.5-10x5-7 | 7-8 | 8.4-12x6-7.2 |
| Shape | ellipsoid, some ovoid | broadly ellipsoid to elongate-ellipsoid | broadly elliptical to ovate, apiculate | subglobose | broadly elliptical to ovate, distinct apiculus |
| Colour | white-cream in mass; non-amyloid, hyaline | white in mass; non-amyloid, hyaline | ivory in mass; non-amyloid | hyaline | ivory in mass; non-amyloid, hyaline |
| Ornamentation | smooth | smooth | smooth | smooth | smooth |
| Wall | up to 0.5um when matured | slightly thickened | - | - | - |
| **Basidia** | | | | | |
| Size (µm) | 24-26.8x5.5-7.2 | 24-30x6-7 | - | - | - |
| Shape | - | elongate clavate | clavate | clavate | clavate |
| Clamp-connections | absent | present | present | - | present |
| **Hymenophoral trama** | bilateral | bilateral | bilateral | - | bilateral |
| **Subhymenial tissue - nuclei** | - | - | binucleate | - | binucleate |
| **Pigments** | often inside cell walls | in cell walls and vacuoles | in cell walls | - | - |
| **Habit** | caespitose | loosely grouped | single or fasciculate groups | - | - |
| **Rhizomorphs *in vitro*** | - | - | cylindrical, monopodial branches | - | - |

TABLE 2 (continued)

| Species | *A. fumosa* | *A. fuscipes* | *A. gallica* | *A. gemina* | *A. heimii* |
|---|---|---|---|---|---|
| References | Kile and Watling 1983 | Petch 1909, Chandra and Watling 1981, Pegler 1986 | Marxmüller 1987 | Bérubé and Dessureault 1989 | Pegler 1977 |
| **Pileus** | | | | | |
| Size (mm) | 20-120 | 25-60 | 40-130(-170) | 20-100 | 10-25 |
| Shape | convex expanding to plano-convex | broadly convex to applanate; *center* slightly umbonate, rarely umbilicate | at first campanulate, then convex | broad, hemispherical-campanulate or obtusely-parabolic, then convex and finally plane, sometimes mammilate | convex, applanate to umbonate |
| Colour | grey to hazel | yellowish-brown to brown or whitish; *centre* pale brown or whitish | yellowish brown to pinkish brown | dark to very dark brown | cream to orange; *centre* darker brown |
| Surface | *centre* densely covered with brown to fuscous black fibrillose squamules | glabrescent; *centre* covered with minute brown squamules | indistinct squamules, deep brown, olivaceous fibrils | distinct black scales; dry; *centre* scales more dense | brown squamules; dry; *centre* squamules crowed |
| Margin | initially incurved | finally recurved; striate | inrolled then irregular, undulate or lobbed; subtranslucent, striate when matured | inrolled then down-turned; entire; striate | incurved |
| Lamellae | decurrent to subdecurrent; fairly crowded; pliable; ivory-pale cream, yellowish cream or pale cinnamon with age | subdecurrent; rather crowded; narrow, 3-4mm broad; white | subdecurrent to sometimes strongly decurrent; close; thick; sinuate; cream, light brown when old | adnate to slightly decurrent, sinutate when matured; rather close; thick; cream when young, later greyish orange to cinnamon | adnate, with decurrent tooth; subdistant; pale cream; two lengths |
| **Stipe** | | | | | |
| Size (mm) | 55-130x5-14 | 30-100x50-90 | 60-150 | 50-80x5-10 | 25-45x2-3 |
| Shape | usually elongated, enlarging downwards to more or less clavate base | slender, curved, cylindrical | clavate to cylindrical | clavate, later more or less equal | cylindrical |

TABLE 2 (continued)

| Species | *A. fumosa* | *A. fuscipes* | *A. gallica* | *A. gemina* | *A. heimii* |
|---|---|---|---|---|---|
| Context texture | cartilaginous, fibrous | - | fibrous | fibrous | |
| Flesh | stuffed | solid | - | - | hollow |
| **Annulus** | thin; membranaceous; white; generally evanescent; floccose below | thick; floccose below | cortinate; arachnoid; whitish; evanescent | thick, membranaceous, white and brown | membranaceous; whitish; evanescent; floccose below |
| **Basidiospores** | | | | | |
| Size (µm) | 6.5-8.5(-9.5)x(4-)4.5-6 (-6.5) | 6-8.3x4.5-6.5 | 7.5-8.5x4.5-5 | 8.2-10x5.2-7 | 7.2-9x(4.4)5-5.5 |
| Shape | elongated-ellipsoid; apiculated | broadly ellipsoid but somewhat angled in outline | obtuse ellipsoid | broadly elliptical to ovate, apiculated | obvoid to angular, apiculated |
| Colour | almost white in mass, non-amyloid | non-amyloid, hyaline | - | ivory in mass; non-amyloid | non-amyloid, hyaline to tinged slightly honey |
| Ornamentation | smooth | smooth (but can be very slightly roughened) | smooth | smooth | smooth to very faintly irregular |
| Wall | moderately thick | slightly thickened | thin | - | thin, thicken slightly with age |
| **Basidia** | | | | | |
| Size (µm) | 35-47.5x7.5-9 | 22-31x5-7.5 | (20)30-45(-55)x(5)6-8 | - | 20-30x7.5-8 |
| Shape | clavate | clavate | clavate | clavate | clavate |
| Clamp-connections | absent | absent | present | present | (not seen) |
| **Hymenophoral trama** | bilateral | slightly bilateral | - | bilateral | bilateral |
| **Subhymenial tissue** | - | - | binucleate | binucleate | - |
| **Pigments** | in vacuoles | - | in cell walls and vacuoles | in cell wall | - |
| **Habit** | caespitose (5-20) | caespitose ( 6-9) | solitory | single, commonly in large fasciculated groups | fasciculate |
| **Rhizomorphs *in vitro*** | cylindrical, dichotomous branches | - | cylindrical, monopodial branches | cylindrical, monopodial branches | cylindrical, monopodial branches |

TABLE 2 (continued)

| Species | *A. hinnulea* | *A. jezoensis* | *A. limonea* | *A. luteobubalina* | *A. mellea* |
|---|---|---|---|---|---|
| References | Kile and Watling 1983 | Cha *et al.* 1994 | Stevenson 1964, Podger *et al.* 1978, Hood 1992 | Podger *et al.* 1978, Bougher and Syme 1998 | Watling *et al.* 1982, Motta and Korhonen 1986 |
| **Pileus** | | | | | |
| Size (mm) | 20-80(-120) | 47-68 | 80-130 | 40-70(-100) | up to 90 |
| Shape | subumbonate to broadly convex becoming plano-concave or regularly depressed | hemispherical-convex to convex when young, then plano-convex to plane, sometimes slightly umbonate | convex at first, becoming almost plane, waved at edges | convex at first, becoming expanded and subumbonate to umbonate, sometimes concave | convex , becoming plano-convex or plane |
| Colour | various shades of brown | dark yellowish-brown or strong brown; *centre* sometimes reddish | lemon yellow | lemon-yellow to honey-brown; *center* at first dark brown | weak yellow to dark honey |
| Surface | brown to fuscous black squamules; at most subviscid with age; hygrophanous; *centre* particularly squamulose | reddish-brown to brownish yellow fine fibres; dry; *centre* fine fibres or small dark brown to dusky-red scales | dark brown tufted scales, more sparsely towards the margin; dry | dark brown squamules, dense at disk, sparse towards the margin | silky fibrils or minute darker scales |
| Margin | sometimes distinctly striate | inrolled at first, then acute or slightly incurved later; striate | strongly down-rolled | strongly inrolled; dentate, occasionally striate | entire; striate |
| Lamellae | sinuate to subdecurrent; subcrowded; fleshy; pliable | sinuate, subdecurrent; close; thick; white when young, then reddish brown to pink; crenate | sinuate to subdecurrent; moderately crowded; cream white becoming stained pinkish fawn | subdecurrent, less frequently distinctly decurrent; crowded; white to pallid, becoming brownish cream or pinkish brown | emarginate, slightly decurrent, slightly sinuate; white to ivory, spotted rust-colour with age; slightly marginate |
| **Stipe** | | | | | |
| Size (mm) | 30-70(-100)x4-9 | 39-61x7-11 | 100-150x10-15 | 40-100(-120)x7.5-12(-15) | 85-145, 4.5-8.0, 0.8-10 |
| Shape | cylindrical tapering towards a bulbous to sub-bulbous base | cylindrical, clavate to subclavate | slightly bulbous at base | slightly thickened towards the base, sometimes sub-bulbose | clavate |
| Context texture | cartilaginous | fibrous | tough | tough | fibrous |

TABLE 2 (continued)

| Species | *A. hinnulea* | *A. jezoensis* | *A. limonea* | *A. luteobubalina* | *A. mellea* |
|---|---|---|---|---|---|
| Flesh | stuffed | solid when young, stuffed when old | solid | solid | stuffed then hollow |
| **Annulus** | arachnoid; grey to brown; evanescent, forming annular zone | thin; submembrananceous; white; fibrillate | arachnoid; white above, dark brown below | moderately thick; membranaceous; yellow; persistent; floccose | thick; double; membranaceous; pale above, citron yellow below; persistent; flocci below |
| **Basidiospores** | | | | | |
| Size (µm) | 6-8.5(-9)x(3.5-)4-6(-6.5) | 6.3-10.3x4.8-6.3 | 6.5-9x3.5-5.0 | (5-)6.5-7.5(-8)x4.5-5.5(-6) | 6.0-70.0x8.4-12.0 |
| Shape | ellipsoid to ovoid | broadly elliptical to ovate, apicululated | | broadly ellipsoid, broad apiculus | broadly ellipsoid to ovate, apiculated |
| Colour | white in mass; non-amyloid, hyaline | white in mass; non-amyloid, hyaline | white in mass; non-amyloid, | ivory white in mass; non-amyloid | ivory in mass; non-amyloid, hyaline |
| Ornamentation | faintly and irregularly sculptured | smooth | finely roughened | smooth | smooth |
| Wall | relatively thick | | moderately thick | moderately to slightly thick | thin or slightly thickened |
| **Basidia** | | | | | |
| Size (µm) | 21-47x5-9 | 39.1-44.1x6-7.8 | - | 20-35(-40)x5-10 | 25.5-37.8x6.5-8.5 |
| Shape | clavate-cylindrical | clavate | - | - | clavate |
| Clamp-connections | absent | present | - | absent | absent |
| **Hymenophoral trama** | bilateral | bilateral | bilateral | subregular to slightly divergent | slightly bilateral |
| **Subhymenial tissue – nuclei** | - | binucleate | - | - | uninucleate |
| **Pigments** | - | - | - | - | in vacuoles |
| **Habit** | solitary or in small fasciculate groups | solitary to caespitose | caespitose | single to subcaespitose | caespitose |
| **Rhizomorphs *in vitro*** | cylindrical, monopodial branching | cylindrical, monopodial branching | - | cylindrial to flattened, sparsely-branching | belt shape, dichotomous branching |

TABLE 2 (continued)

| Species | *A. montagnei* | *A. nabsnona* | *A. novae-zelandiae* | *A. ostoyae* | *A. pallidula* |
|---|---|---|---|---|---|
| Reference | Singer 1956, 1970 | Volk *et al.* 1996 | Stevenson 1964, Kile and Watling 1983, Hood 1992 | Bérubé and Dessureault 1988 | Kile and Watling 1988 |
| **Pileus** | | | | | |
| Size (mm) | 40-81 | 40-70 | 30-100(-150) | 50-100 | 45-90 |
| Shape | convex; *center* umbonate | convex later plane | subumbonate to umbonate becoming plano-convex and later often depressed; *center* sumbumbonate to umbonate | hemispherical-campanulate or obtusely parabolic, later convex and finally plane | campanulate, then convex to subumbonate later plano-convex or slightly depressed |
| Color | olive melleous, later yellowish | orange brown, paler towards the margin | olive-buff to olive-brown | dark to very dark brown | yellowish buff to pale fulvous, darker towards the centre |
| Surface | ochre brown squamules | smooth; hygrophanous; *centre* sometimes short dark fibrils when young | small reddish brown squamules; viscid; hygrophanous | distinct dark scales all over, more dense at centre; dry | fulvous or tawny scales, irregularly and sparsely distributed at first, disappearing with age |
| Margin | declivous; glubrescent; later slightly striate, eventually sulcate | somewhat incurved; translucent striate to furrowed | initially incurved; striate | at first inrolled then down- turned; sometimes striate | inrolled |
| Lamellae | initially arcuate-decurrent, later adnate-decurrent; close; broad; whitish, eventually pale yellow | adnate to subdecurrent; subdistant; white to cream, pinkish-tan when aged, brownish patches may develop | sinuate, subdecurrent; subcrowded; white to ivory, becoming cream, yellowish or pinkish tints when age | adnate to slightly decurrent becoming sinuate when matured; rather close; thick where attached to stipe, thinner towards margin; white or cream when young, greyish orange, cinnamon later | subdecurrent, decurrent in large basidiocarps; fairly crowded; relatively thick; pliable; pale tawny, somewhat mottled |
| **Stipe** | | | | | |
| Size (mm) | 120-220x5-11 | 80-100x 4-5 | 50-120(-150)x4-9(-13) | 50-200 | 52-64x20-24 |

TABLE 2 (continued)

| Species | *A. montagnei* | *A. nabsnona* | *A. novae-zelandiae* | *A. ostoyae* | *A. pallidula* |
|---|---|---|---|---|---|
| Shape | subequal | - | elongate expanding from mid-point downwards to semi-bulbous or bulbous base | cylindrical | usually elongated, clavate or bulbous, more cylindrical in larger basidiocarps |
| Context texture | fibrous | fibrous | cartilaginous | fibrous | cartilaginous |
| Flesh | stuffed then hollow | - | stuffed | - | stuffed |
| **Annulus** | thick; double; membranaceous; white; persistent; flocci below | sometimes persist as an evanescent cortina, difficult to observe | thin; membranaceous; dark brown; evanescent | thick; membranaceous; white and brown | thin; cortinate; pale; persistent; darker floccules below |
| **Basidiospores** | | | | | |
| Size (µm) | 6.2-9.0x4.5-6.5 | (6-)8-10x5.5-6.5 | 7-8(-8.5)x4.5-5.0(-5.5) | 5.5-7x8-11 | 4.4-6.3x5.6-10 |
| Shape | ovoid-ellipsoid | ovoid to subglobose | ellipsoid to elongate-ellipsoid, broad apiculus | broadly elliptical to ovate, apiculate | elongate to broadly ellipsoid, broad prominent apiculus |
| Colour | pure white in mass; non-amyloid | white in mass; non-amyloid, hyaline | nearly white in mass | white in mass, non-amyloid | cream in mass; non-amyloid, hyaline |
| Ornamentation | smooth | smooth | smooth or very slightly roughened | smooth | smooth |
| Wall | thin to medium-thick | somewhat thick at maturity | moderately thick | - | moderately to distinctly thick |
| **Basidia** | | | | | |
| Size (µm) | - | 25-35x5.5-6.0 | 24-45x6-9 | | 42.5-55x4-5.5 |
| Shape | clavate | clavate | clavate | clavate | elongate-clavate |
| Clamp-connections | absent | present | absent | present | absent |
| **Hymenophoral trama** | regular to subbilateral | regular | bilateral | strongly bilateral | bilateral |
| **Subhymenial tissue- nuclei** | - | - | - | binucleate | - |
| **Pigments** | - | - | - | in cell walls | - |
| **Habit** | - | gregarious, but not caespitose | solitary or fasciculate | fasciculate | - |
| **Rhizomorphs *in vitro*** | - | - | belt shape, dichotomous branches | belt shaped, dichotomous branches | cylindrial to flattened, sparsely-branched |

TABLE 2 (continued)

| Species | *A. procera* | *A. puiggarii* | *A. sinapina* | *A. singula* | *A. sparrei* |
|---|---|---|---|---|---|
| References | Singer 1969, 1970 | Singer 1956, 1970 | Bérubé and Dessureault 1988, Cha *et al.* 1994 | Cha *et al.* 1994 | Singer 1956, 1969 |
| **Pileus** | | | | | |
| Size (mm) | 49-65(-85) | (11-)21-100(-175) | 20-60 | 24-38 | 18-66 |
| Shape | convex; *centre* depressed often with umbo in depression | semiglobose, then convex; *center* depressed but with subumbonate elevation, or more distinctly umbonate | conical-campanulate to campanulate, convex then plano-convex; *center* occasionally mammilate | convex to hemispherical when young, later plano-covex to plane; *centre* obtusely umbonate | convex-subcampanulate, then flatter-convex, often subumbonate |
| Colour | greyish; *centre* ochraceous | "indian buff" to honey colour; *centre* deeper brown | pale to dark brown with reddish tinges | yellow to brownish yellow; *centre* pale yellow to very pale brown | varying from pale coloured to deep olive |
| Surface | viscid; hygrophanous; *centre* spinulose-flocconous small scales | small concolorous scales, later darker brown squamules; dry; hygrophanous; *centre* dark brown squamules | brown scales; usually dry; sometimes hygrophanous | dark reddish-brown to very dark gray tufts of fine fibers; dry; *centre* fibers concentrated | smooth or rugose; viscid; |
| Margin | sulcate and transparently striate | uplifted when aged; transparently striate when matured | decurved; sometimes with striations | inrolled at first then acute later; translucent-striate | upturned; transparently striate |
| Lamellae | sinuate-decurrent or short-decurrent; close or subclose; rather broad; pure white, pallid with age | adnate, the adnato-decurrent or adnate with decurrent tooth; subclose; narrow to rather broad; varying from white to brown pallid, edge tends to be brown-spotted | sinuate, subdecurrent to sometimes strongly decurrent; close; thick; cream to cinnamon when old | subdecurrent; close; thick at apex, thin towards the margin; cream when young, light brown later | adnate, irregularly decurrent tooth, or subdecurrent; moderately close to close; relatively rather broad and often ventricose when aged; crisp or forked but not intervenose; ocher whitish to cream |
| **Stipe** | | | | | |
| Size (mm) | 37-58x4.5-9(-12) | 25-70(-170)x2-8 above, 2-18 below | 47-68x5-8 | 42-60x4-6 | as long or longer than size of pileus |
| Shape | equal or tapering downwards, or slightly tapering upwards | equal with bulbous base, later sometimes ebulbose or tapering downwards | clavate | cylindric, clavate | cylindrical or tapering upwards |

TABLE 2 (continued)

| Species | *A. procera* | *A. puiggarii* | *A. sinapina* | *A. singula* | *A. sparrei* |
|---|---|---|---|---|---|
| Context texture | fleshy | fragile | fibrous | fragile | fleshy |
| Flesh | solid | solid | solid when young, stuffed when old | solid when young, slightly hollow later | solid |
| **Annulus** | thick; membranaceous; persistent | subcortinoid to thin membranaceous; white; fibrils below | thin; sometimes membranaceous; whitish above, yellowish below; fibrous | thin; membranaceous; white to cream | thin; white; not persistent |
| **Basidiospores** | | | | | |
| Size (µm) | 6.5-11.7x4.5-7.3 | 6.5-11x6.5-7.3 | 5.9-8x8.2-10 | 6.2-10.6x3.6-6.2 | (7.3-)8-12x(4.5-)5.3-7.3 [2] |
| Shape | ellipsoid to ovoid; | subcylindrical to ovoid-ellipsoid | broadly elliptical to ovate, apiculated | broadly elliptical to ovate, apiculated | ellipsoid or cylindrical |
| Colour | pure white in mass; non-amyloid, hyaline | pure white in mass; non-amyloid, hyaline | ivory in mass; non-amyloid | cream in mass; non-amyloid, hyaline | pure white in mass |
| Ornamentation | smooth | smooth | smooth | smooth | rarely roughened |
| Wall | slightly thick | thin to slightly thickened | - | - | first thin, later gradually thickening |
| **Basidia** | | | | | |
| Size (µm) | 23-38x6.5-11.7 | 40-47x7.3-8.8 | 37.9-44.9x7.2-9.4 | 33-37.8-5.4-7.5 | 30-44x6.7-8 |
| Shape | - | clavate | clavate | clavate | clavate |
| Clamp-connections | present | present | present | present | absent |
| **Hymenophoral trama** | bilateral | bilateral | bilateral | bilateral | subparallel or very slightly interwoven |
| **Subhymenial tissue- nuclei** | - | - | binucleate | binucleate | - |
| **Pigments** | - | - | in cell walls | - | - |
| **Habit** | caespitose or densely fasciculate | fasciculate to caespitose | small fasciculate groups | solitary | fasciculate in large bunches |
| **Rhizomorphs *in vitro*** | - | - | cylindrical, monopodial branching | cylindrical, monopodial branching | - |

TABLE 2 (continued)

| Species | *A. tabescens* | *A. tigrensis* | *A. viridiflava* | *A. yungensis* |
|---|---|---|---|---|
| References | Singer 1970 | Singer 1970 | Singer 1989 (in Latin) | Singer 1970 |
| **Pileus** | | | | |
| Size (mm) | (25-)40-70(-100) | (11-)21-127(-175) | 30-64 | 34-64 |
| Shape | convex, sometimes slightly depressed in age around a slight umbo, or exumbonate, often sulcate | semiglobose or convex, later flattened, in larger basidiocarps subumbonate to umbonate | campanulate-convex the convex, later sometimes subapplanate; *centre* umbonate | semiglobate then applanate; *center* +/- depressed or subumbilicate to subumbonate |
| Colour | light brownish yellow; *centre* stramineous buff | pale ochraceous or yellow later dark honey; *centre* sometimes deeper brown | olive to olive-blackish | pale-brown to dark-brown |
| Surface | smooth; subhygrophanous | rugulose to subrugulose; somewhat subviscid, later dry; subhygrophanous or hygrophanous; *centre* concolorous scales, later darker brown | fibrillose; not viscid; hygrophanous; *center* generally rugulose, fibrillose | slightly fibrillose; not viscid; *center* blackish dotted squamulose |
| Margin | - | upturned with age | - | striate when matured |
| Lamellae | irregularly decurrent; subclose; broad; arcuate; whitish later dark cream, or flesh-pallid, sometimes brown-spotted | adnate, or sometimes adnexed, with subdecurrent to decurrent tooth, or adnato-decurrent to sinuate decurrent; close or subclose; narrow to rather broad; white to cinnamon-white, tending to become fulvous-brown spotted | decurrent; crowded; moderately broad; white then pale-yellow | decurrent; close or subclose; narrow; arcuate; beige |
| **Stipe** | | | | |
| Size (mm) | (35-)60-150x(3-)4-11 | 25-90(-170)x2-18 | 80-125x9-11.5 | 25-65x3.5-12 |
| Shape | tapering towards base or at least with thickened apex and tapering base | equal with bulbous base, later subequal or slightly ventricose with bulbous base, at times tapering down | subequal or tapering towards the base | equal or tapering upwards |

TABLE 2 (continued)

| Species | *A. tabescens* | *A. tigrensis* | *A. viridiflava* | *A. yungensis* |
|---|---|---|---|---|
| Context texture | flexous | - | - | - |
| Flesh | solid or stuffed, sometimes hollow when aged | solid, later stuffed or hollow | solid, later stuffed | solid |
| **Annulus** | absent | membranous or thin-membranous; white; persistent | thick; membranaceous; yellow; persistent | thick; cortinoid; whitish |
| **Basidiospores** | | | | |
| Size (µm) | 7.7-8.8x5.2-6 | 9.3-11x6.5-7.3 | 6.2-8.5x4.5-5.5(-6) | 7-9x4-5.3 |
| Shape | short-ellipsoid or somewhat ovoid | ellipsoid | ellipsoid | ellipsoid, ovoid, or short-cylindric |
| Colour | white in mass; non-amyloid | pure white; non-amyloid, hyaline | cream-yellowish in mass | pure white in mass; non-amyloid, hyaline |
| Ornamitation | smooth | smooth | smooth | smooth |
| Wall | - | somewhat thick | thickened | - |
| **Basidia** | | | | |
| Size (µm) | 30-40x8-9 | 40-47x7.3-8.8 | (16-)21.8-31.8x(6-)6.7-9(-10) | 20-32x5.3-8.7um |
| Shape | clavate, elongated when matured | clavate, strongly elongated when matured | - | - |
| Clamp-connections | - | not always present | present | sometimes |
| **Hymenophoral trama** | somewhat bilateral | subregular-subbilateral to more distinctly bilateral | subregular-bilateral | bilateral |
| **Subhymenial tissue** | - | - | - | - |
| **Pigments** | - | in vacuoles | - | - |
| **Habit** | fasciculate or caespitose | fasciculate to caespitose | - | - |
| **Rhizomorphs *in vitro*** | - | - | - | - |

**TABLE 3**: Phenotypic and genotypic characters used to differentiate *Armillaria* spp. in conjunction with or instead of basidiocarps (sexual compatibility studies are dealt with under the biological species concept and are therefore not included in this table).

| **Characters** | **Differentiate:** |
|---|---|
| *Phenotypic* | |
| 1. Morphology of mycelium and rhizomorphs (in many cases this is not unique for a specific species but can be used to differentiate between two species with similar basidiocarp morphologies). | • North America: *A. gemina* from *A. ostoyae*, *A. calvescens* and *A. sinapina* (Bérubé and Dessureault 1988, 1989)<br>• Europe: all species except *A. cepistipes* and *A. galllica* (Rishbeth 1982, 1986, Zolciak *et al.* 1997, Tsopelas 1999).<br>• Africa: *A. mellea*, *A. heimii*, interspecific somatic compatibility group (SIG) III and SIG IV (Mohammed *et al.* 1989, 1994b, Mwangi *et al.* 1989)<br>• Australia: *A. novae-zelandiae*, *A. hinnulea*. *A. fumosa* and *A. luteobubalina* are the same but different from the other species (Kile and Watling 1983).<br>• New Zealand: *A. limonea* and *A. novae-zelandiae* (Shaw *et al.* 1981) |
| 2. Response to temperature | • Europe: all species, especially *A. tabescens* and *A. mellea* (Rishbeth 1982, 1986)<br>• Africa: *A. mellea*, *A. heimii*, (SIG) III and IV (Mohammed *et al.* 1994b) |
| 3. Response to phenolic acids and terpens | • Europe: *A. mellea*, *A. ostoyae*, *A.cepistipes* and *A. tabescens* (Rishbeth 1986) |

TABLE 3 (continued)

| Characters | Differentiate: |
|---|---|
| 4. Isozyme and protein profiles | • North America: *A. ostoyae, A. calvescens, A. sinapina, A. nabsnona* and *A. gallica* (Morrison *et al.* 1985b, Lin *et al.* 1989)<br>• Europe: all species (Wahlström *et al.* 1991, Bragaloni *et al.* 1997)<br>• Africa: *A. mellea, A. heimii* and SIG III (Agustian *et al.* 1994, Mwenje and Ride 1997)<br>• Japan: *A. ostoyae, A. gallica, A. jezoensis, A. singula* and *A. sinapina* (Cha and Igarashi 1995a, Matsushita *et al.* 1996) |
| 5. Mono- and polyclonal antibodies | • Europe: all species (Lung-Escarmant and Dunez 1979, 1980, Lung-Escarmant *et al.* 1985, Fox and Hahne 1989). |
| *Genotypic* | |
| 6. DNA/DNA hybridization | • North America: *A. cepistipes, A. mellea* and *A. ostoyae* (Jahnke *et al.* 1987) |
| 7. DNA base composition (mol % G+C) | • North America: *A. mellea* and *A. cepistipes* (Motta *et al.* 1986) |
| 8. Restriction fragment length polymorphisms (RFLP's) | |
| 8.1 mitochondrial DNA (mtDNA) | • North America: all species (Anderson *et al.* 1987, Smith and Anderson 1989)<br>• Europe: *A. cepistipes, A.ostoyae* and *A. mellea* (Jahnke *et al.* 1987) |

TABLE 3 (continued)

| Characters | | Differentiate: |
|---|---|---|
| 8.2 | whole cell nuclear DNA (nDNA) | • North America: all species (Anderson *et al.* 1987) |
| 8.3 | complete ribosomal nDNA operon (rnDNA) | • North America: *A. mellea*, *A. ostoyae* and *A. gemina*. *Armillaria gallic* and *A. cepistipes* similar but distinct from other species. *Armillaria sinapina*, *A. nabsnona* and NABS X similar but distinct from other species (Anderson *et al.* 1989)<br>• Europe: *A. mellea*, *A. gallica*, *A. ostoyae*, *A. borealis*, *A. cepistipes* and *A. tabescens* (Anderson *et al.* 1989, Schulze *et al.* 1995) |
| 8.4 | PCR generated rnDNA intergenic spacer region (IGS-1) | • North America: all species except *A. gallica* and *A. calvescens* (Harrington and Wingfield 1995, Banik *et al.* 1996, Volk *et al.* 1996, White *et al.* 1998)<br>• Europe: all species (Harrington and Wingfield 1995, Pérez Sierra *et al.* 1999)<br>• Africa: *A. fuscipes* and *A. heimii* (Coetzee *et al.* 2000a)<br>• Japan: all species (Terashima *et al.* 1998b) |
| 8.5 | PCR generated rnDNA internal transcribed spacer (ITS) | • Europe: *A. mellea*, *A. tabescens* and *A. ectypa* (Chillali *et al.* 1998a)<br>• Africa: *A. mellea*, *A. heimii* and SIG III (Chillali *et al.* 1997) |

TABLE 3 (continued)

| Characters | | Differentiate: |
|---|---|---|
| 9. | Interspecific DNA sequence character differences | |
| | 9.1 IGS-1 | • North America: *A. ostoyae*, *A. gemina*, *A. borealis*, *A. mellea*, *A. tabescens* and *A. nabsnona*. Few differences between *A. sinapina*, *A. cepistipes*, *A. gallica*, *A. calvescens* and NABS X (Anderson and Stasovski 1992, Coetzee *et al.* 2000b)<br>• Europe: *A. borealis*, *A. mellea*, *A. tabescens* and *A. ostoyae*. Few differences between *A. gallica* and *A. cepistipes* (Anderson and Stasovski 1992, Coetzee *et al.* 2000b)<br>• Africa: *A. fuscipes* and *A. heimii* (Coetzee *et al.* 2000a)<br>• Japan: all species (Terashima *et al.* 1998a) |
| | 9.2 ITS | • North America: *A. mellea* and *A. tabescens* (Anderson and Stasovski 1992)<br>• Europe: *A. mellea*, *A. tabescens* and *A. ectypa*. Single nucleotide differences between *A. borealis*, *A. ostoyae*, *A. cepistipes* and *A. gallica* (Anderson and Stasovski 1992, Chillali *et al.* 1998b) |

TABLE 4: Biological species and corresponding morphological species of *Armillaria* in Europe, North America, Japan and Africa.

| Morphological species | Biological species | | | |
|---|---|---|---|---|
| | **Europe** | **North America** | **Japan** | **Africa** |
| *A. borealis* | A | | | |
| *A. calvescens* | | NABS[a] III | | |
| *A. cepistipes* | B | NABS XI | NAG[b] D | |
| *A. ectypa* | *[c] | | | |
| *A. gallica* | E | NABS VII | NAG A | |
| *A. gemina* | | NABS II | | |
| *A. heimii* | | | | SIG[d] II |
| *A. jezoensis* | | | H | |
| *A. mellea* | D | NABS VI | NAG Am | SIG I |
| *A. nabsnona* | | NABS IX | NAG B | |
| *A. ostoyae* | C | NABS I | NAG C | |
| *A. sinapina* | | NABS V | F | |
| *A. singula* | | | G | |
| *A. tabescens* | * | * | T[e] | |
| Undescribed | | NABS X | NAG E | SIG III<br>SIG IV |

[a] NABS: North American Biological Species

[b] NAG: Nagasawa

[c] Asterisk denotes intersterility groups without vernacular.

[d] SIG: Somatic Incompatibility Group

[e] Compatible with European strains of *A. tabescens* but not with North American strains (Ota *et al.* 1998b).

[3] http://www.uoguelph.ca/~gbarron/index.htm
[4] http://www.hiddenforest.co.nz
[5] http://morwellnp.pangaean.net

**Figure 1.** Basidiocarps of commonly found *Armillaria* spp. 1) *A. calvescens*, 2) *A. cepistipes*, 3) *A. fumosa*, 4) *A. fuscipes*, 5) *A. gallica*, 6) *A. gemina*, 7) *A. hinnulea*, 8) *A. jezoensis*, 8) *A. jezoensis*, 9) *A. limonea*, 10) *A. luteobubalina*, 11) *A. mellea*, 12) *A. nabsnona*, 13) *A. novae-zelandiae*, 14) *A. ostoyae*, 15) *A. pallidula*, 16) *A. sinapina*, 17) *A. singula*, 18) *A. tabescens*. (Photo credits. TJ Volk: 1, 2, 5, 6, 12, 14, 16, 18. GS Ridley: 7. JY Cha: 8, 17. G. Barron[3]: 9. C. Shirley[4]: 4. C. Harris[5]: 10. Armillaria Root Disease Handbook Figure 1.2: 3, 15.).

[3] http://www.uoguelph.ca/~gbarron/index.htm
[4] http://www.hiddenforest.co.nz
[5] http://morwellnp.pangaean.net

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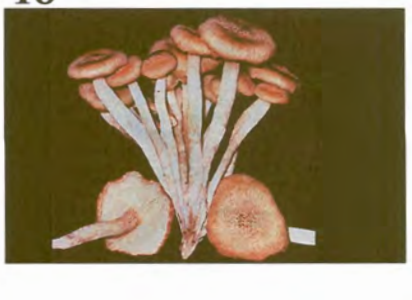


**Figure 2.** Life cycle of *Armillaria* spp. with different mating systems. A) Heterosexual bifactorial (tetrapolar) mating compatibility system (genotypes are arbitrarily chosen); B) Non-heterosexual mating system. ●: diploid nuclei, ○: haploid nuclei. (Redrawn and expanded from Fig. 6, Ota *et al.* 1998)

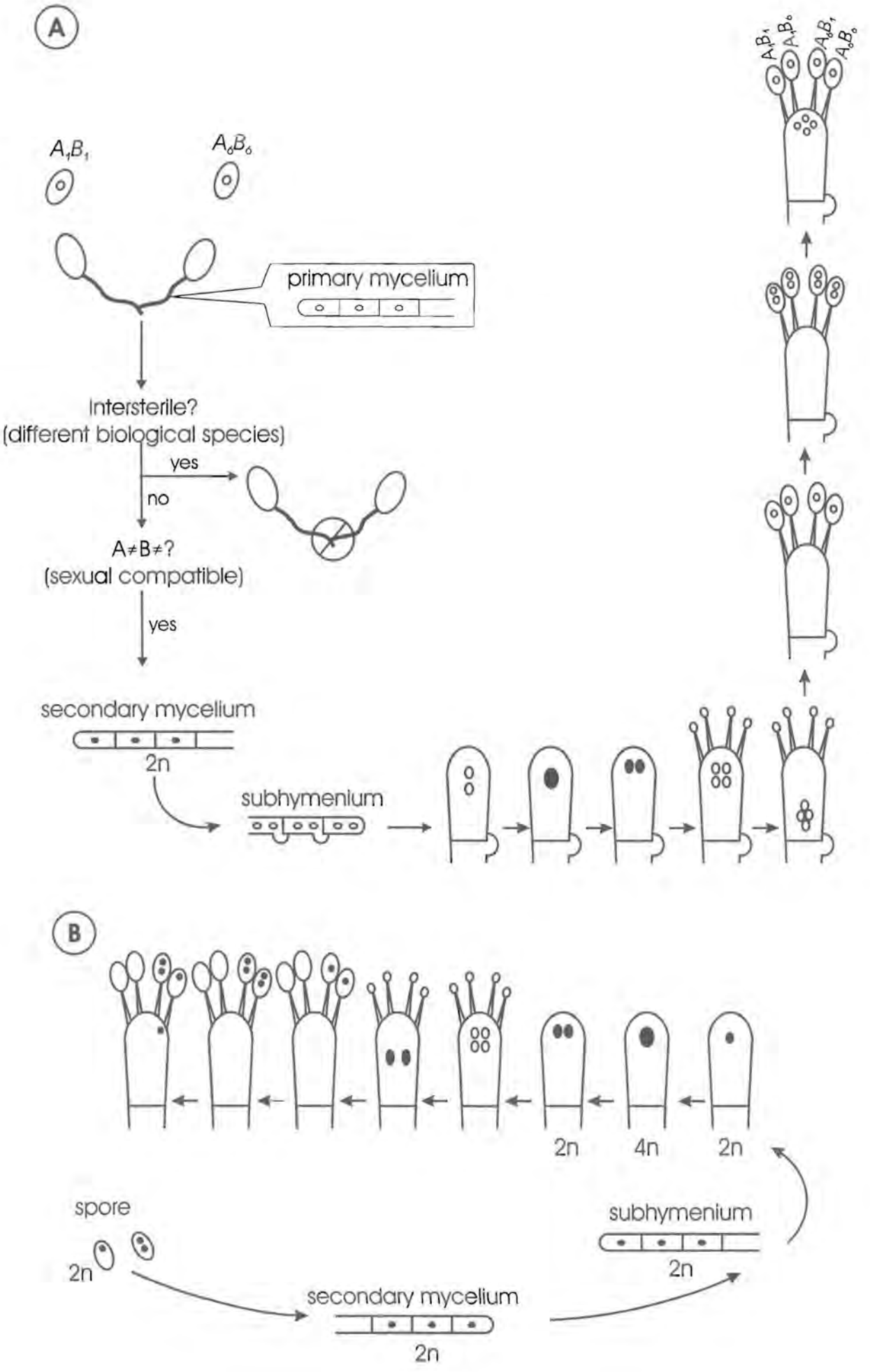


**Figure 3**. Haploid – haploid mating interaction between two sexually compatible isolates. The culture morphology of the haploid isolates is white with abundant aerial mycelium (left and right pictures). The culture morphology of the compatible isolates changes to brown and crustose after successful diploidization (middle picture).

**Isolate 1: haploid** **Isolate 1 and 2: diploid** **Isolate 2: haploid**

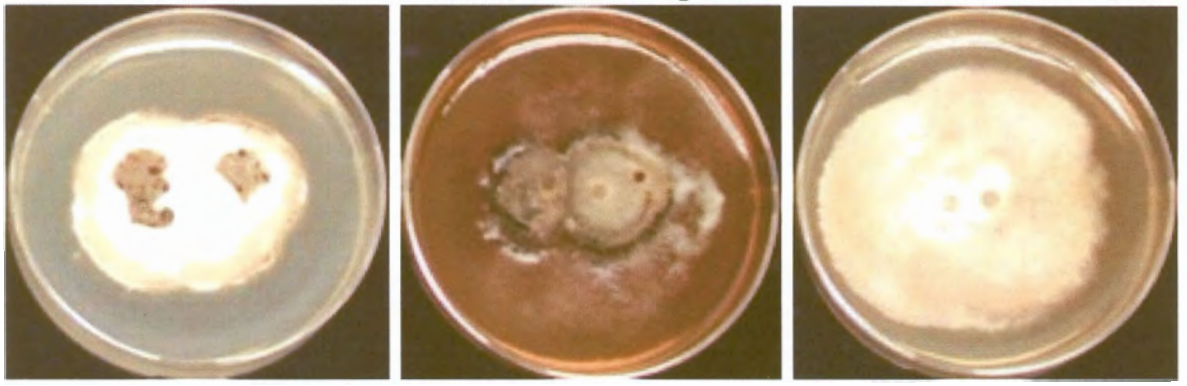


**Figure 4.** The relationship between reticulated (tokogenetically related) and hierarchic (phylogenetically related) descendent systems. (Redrawn from Fig. 6, Hennig 1966)

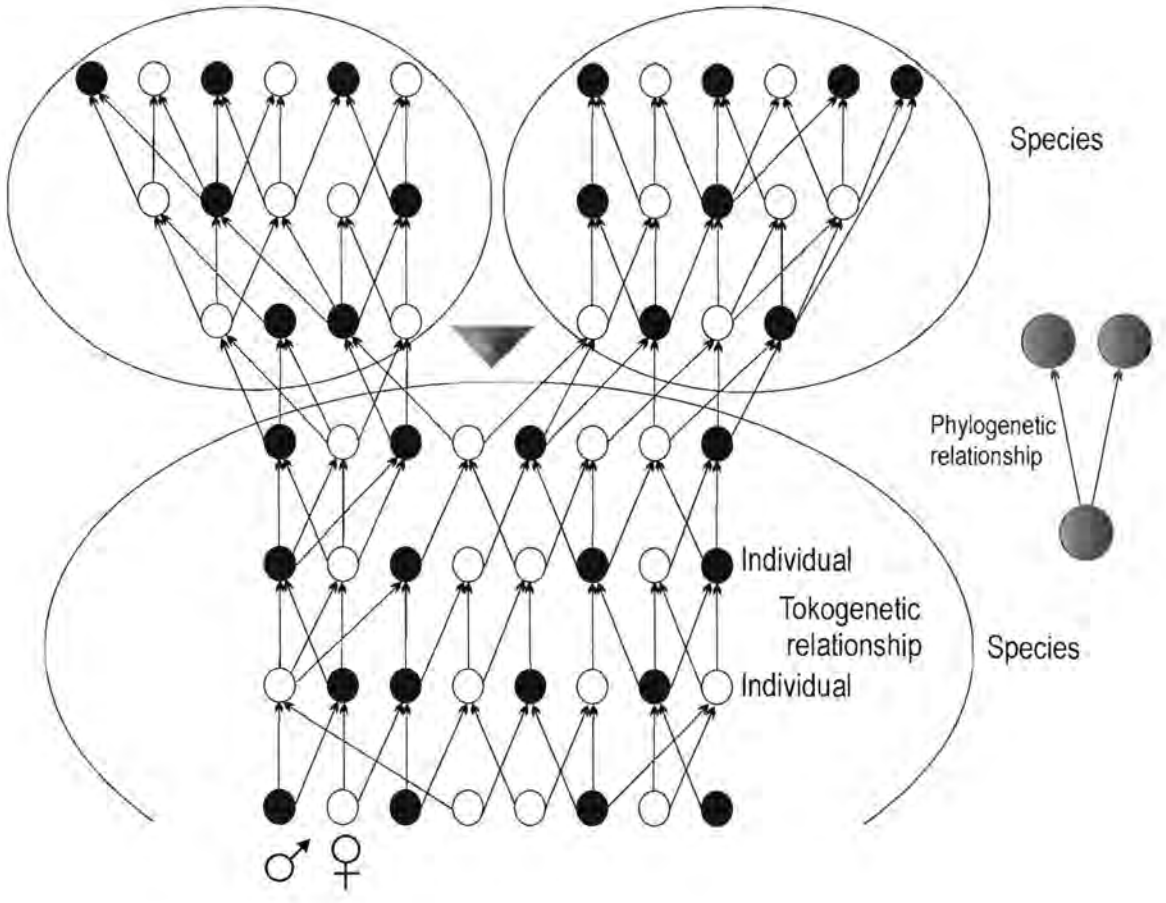
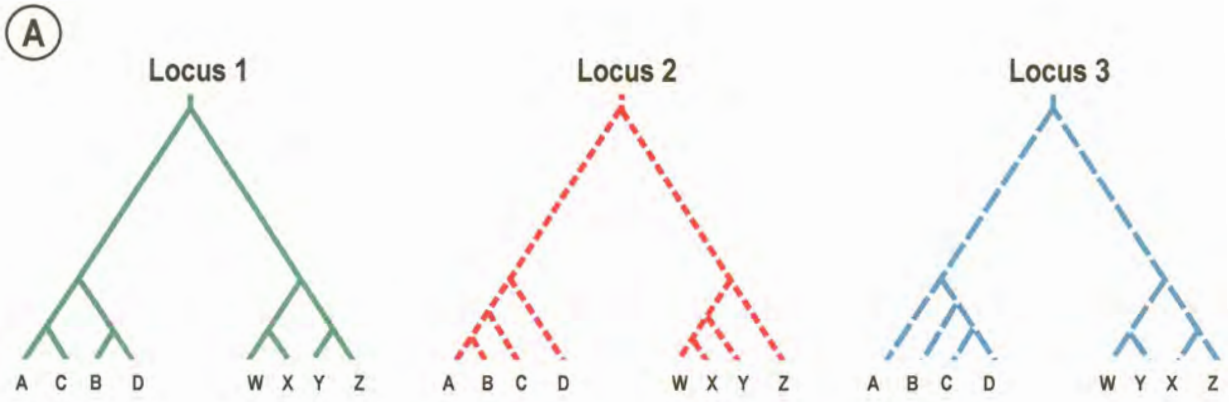


**Figure 5**. Genealogical concordance among multi-loci data sets. A) Cladograms depicting the genealogy of three individual loci for eight taxa. B) Consensus tree of the three cladograms shows the limit of species at the point of transition from incongruity to concordance among branches. (Redrawn from Fig. 2, Taylor *et al*. 2000)



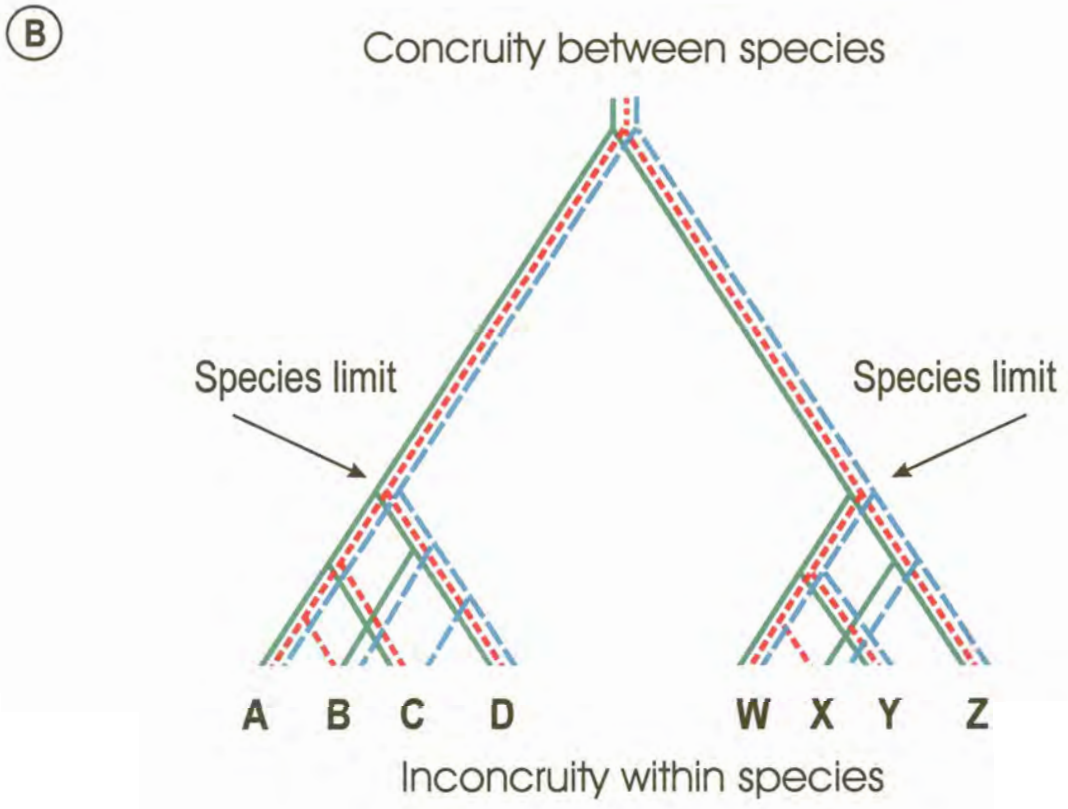
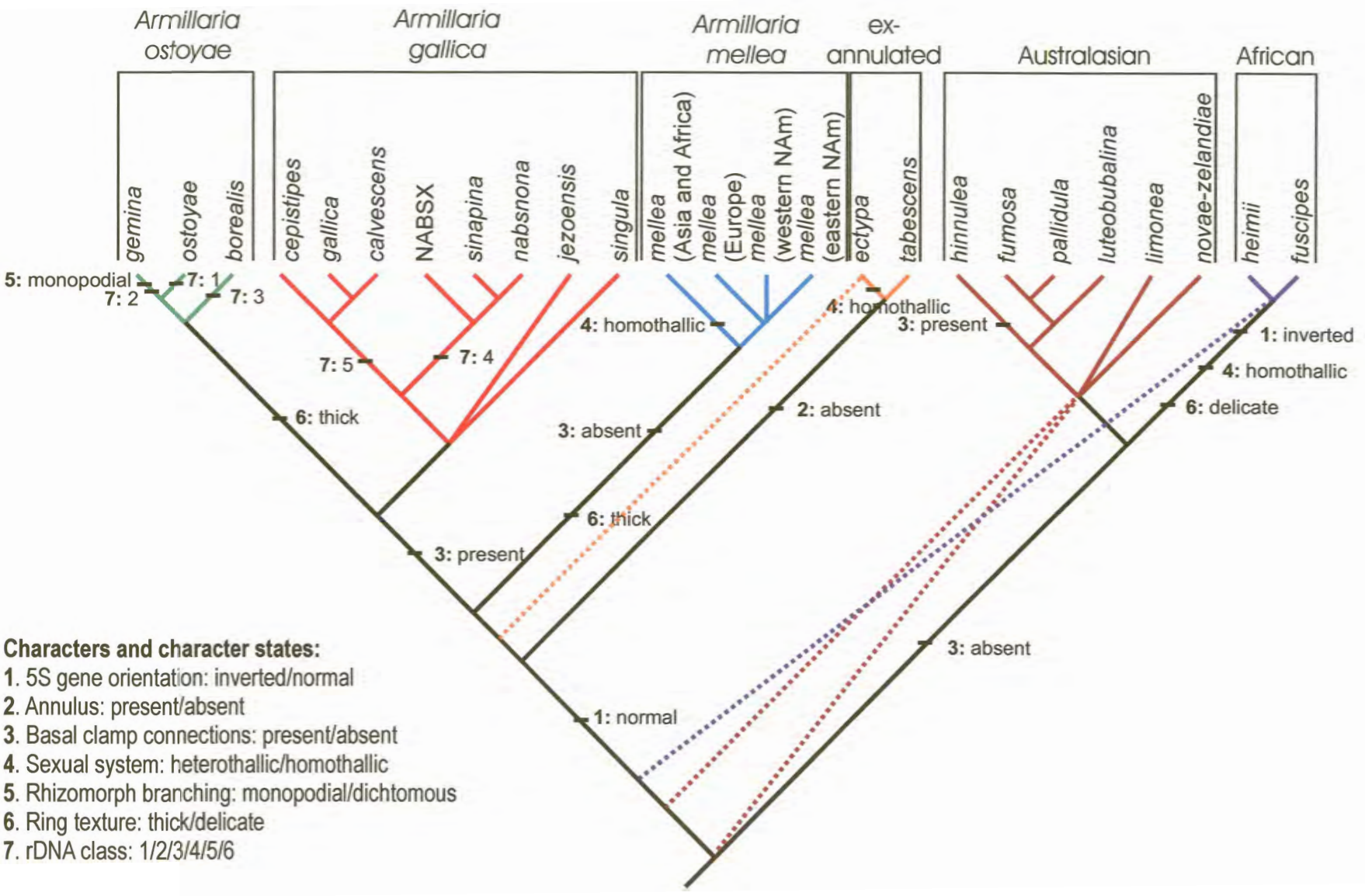



**Figure 6.** Cladogram showing the phylogenetic relationships among taxa within the species clusters and the relationships among clusters based on morphological and molecular data. Alternative relationships are indicated with a dashed line. Character states that differentiate between clusters or species within the clusters are indicated on the branches.




**Characters and character states:**

1. 5S gene orientation: inverted/normal
2. Annulus: present/absent
3. Basal clamp connections: present/absent
4. Sexual system: heterothallic/homothallic
5. Rhizomorph branching: monopodial/dichtomous
6. Ring texture: thick/delicate
7. rDNA class: 1/2/3/4/5/6

# CHAPTER TWO

# PHYLOGENETIC RELATIONSHIPS OF AUSTRALIAN AND NEW ZEALAND *ARMILLARIA* SPECIES

Published: *Mycologia* **93**: 887 – 896 (2001)

# PHYLOGENETIC RELATIONSHIPS OF AUSTRALIAN AND NEW ZEALAND *ARMILLARIA* SPECIES

## ABSTRACT

*Armillaria* species cause Armillaria root rot on a wide range of plant species throughout the world. Based on morphology and sexual compatibility, various species of *Armillaria* have been reported from Australia and New Zealand. These include *A. hinnulea*, *A. fumosa*, *A. pallidula*, *A. novae-zelandiae* and *A. luteobubalina* from Australia. In New Zealand, *A. limonea*, *A. novae-zelandiae*, *A. hinnulea* and a fourth undescribed but morphologically distinct species are recognized. To determine the phylogenetic relationships between *Armillaria* spp. from Australia and New Zealand, the ITS region (ITS1, 5.8S rRNA gene and ITS2) of the rRNA operon was amplified and the DNA sequences determined for a collection of isolates. The ITS sequences of *A. ostoyae* (from USA) and *A. sinapina* (from USA) were included for comparison. Phylogenetic trees were generated using parsimony analysis. *Armillaria hinnulea* was found to be more closely related to *Armillaria* spp. occurring in the Northern Hemisphere than it was to the other Australian and New Zealand species. The remainder of the Australian and New Zealand *Armillaria* spp. included in this study formed a monophyletic clade and confirmed separation of species based on morphology and sexual compatibility.

***Key words:*** *Armillaria*, ITS, phylogeny, evolution.

species using sequence data from the ITS1, ITS2 and the 5.8S gene regions of the rRNA operon.

## MATERIALS AND METHODS

### Fungal isolates

Haploid and diploid isolates of *Armillaria* spp. originating from different regions in Australia and New Zealand were obtained (Table 1). These isolates are maintained in the culture collection of the Tree Pathology Co-operative Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

### DNA extraction

Isolates were grown in liquid MY (2% malt extract and 0.3% yeast extract) at 22 °C in the dark for two weeks. Mycelium was harvested by centrifugation (15 300 *g*, 20 min), lyophilised and ground to a fine powder in liquid nitrogen. DNA was extracted according to the method described by Coetzee *et al.* (2000b). RNase A (0.01 mg/µL) (Roche Diagnostics) was added to the suspension at 37 °C to remove contaminating RNA.

### PCR

PCR fragments for the ITS1 and ITS 2 regions including the 5.8 S gene between the small subunit (SSU) and large subunit (LSU) were obtained using the primer set ITS1 and ITS4 (White *et al.* 1990). The IGS-1 region was amplified using the primer set P-1 (Hsiau 1996) and O-1 (Duchesne and Anderson 1990). The PCR conditions were the same as those described by Coetzee *et al.* (2000b).

### DNA sequencing

DNA sequences were determined using the ABI PRISM™377 DNA sequencer. The ITS region was sequenced in both directions with primers ITS1 and ITS4 and newly designed internal primers CS2B (5' caaggtgcgttcaaagactcg 3') and CS3B (5' cgagtctttgaacgcaccttg 3'). The sequence reactions were carried out using an ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase, FS (Perkin Elmer, Warrington, U.K.) according to the manufacturer's directions.

**Sequence analysis**

Multiple alignments of ITS DNA sequences were done using the Clustal W vers. 1.6 (Thompson *et al.* 1994) program and manually adjusted. Aligned ITS sequences for the Australian and New Zealand *Armillaria* isolates were deposited in TreeBase (accession number S569, matrix accession number M862). Phylogenetic analyses were conducted using searches based on maximum parsimony and maximum likelihood in PAUP* vers. 4 (Swofford 1998). Ambiguously aligned sequence regions were excluded from the data matrix before analysis. In the parsimony analysis, insertions and deletions (indels) of more than one base were treated in various ways to assess their influence on the topology of the trees obtained. However, with the exception of indels included without coding and gaps treated as newstate, the topology of the trees remained the same, irrespective of the indel treatment. Indels were, therefore, regarded as the result of a single evolutionary event and were coded with multistate characters (0 = deletion, >0 = insert). Phylogenetic trees were rooted to *A. ostoyae* (B481, GenBank accession number AF169645) and *A. sinapina* (B493, GenBank accession number AF169646) as the outgroup.

Most parsimonious (MP) trees were generated by heuristic searches with TBR (Tree Bisection Reconnection) branch swapping and MulTrees effective. Starting trees were obtained via stepwise addition with 100 random taxon addition sequences. Maxtrees was set to auto-increase. Zero length branches were collapsed. Parsimonious trees obtained according to the procedure described above were optimized by applying successive weighting according to the mean consistency of each parsimony informative character. This weighting scheme was applied until the number of MP trees obtained after heuristic searches had stabilized. The confidence levels of the branching points on the phylogenetic trees were determined by bootstrap (1000 replicates) (Felsenstein 1985). Heuristic searches were used in this analysis with MulTrees and TBR active. Starting trees were obtained via stepwise addition of taxa with *A. ostoyae* (B481) as the reference taxon. MaxTrees were set to auto-increase, zero length branches were set to collapse and topological constraints were not inforced. Bremer support / decay indexes (Bremer 1988, Donoghue *et al.* 1992) were calculated for monophyletic clades using AutoDecay v. 4.0 (Eriksson 1998).

The phylogenetic relationship between *A. hinnulea* and the Northern hemisphere *Armillaria* spp. was determined in preliminary analysis. ITS sequence data for various *Armillaria* spp., with the exception of *A. fuscipes* and *A. heimii*, were obtained from GenBank. Sequences were aligned using Clustal W vers. 1.6 (Thompson *et al.* 1994) and manually adjusted by inserting gaps. Most

parsimonious trees were obtained as described for the Australian and New Zealand *Armillaria* spp. Indels were, however, included without coding in this analysis.

Phylogenetic analysis based on maximum likelihood was done to estimate nucleotide frequencies, gamma distribution and the transition/ transversion (ti/tv) ratio. Search settings corresponded to the Hasegawa-Kishino-Yano (HKY) model (Hasegawa *et al.* 1985). Starting branch lengths were obtained using the Rogers-Swofford approximation method. Molecular clock was not enforced. Starting trees were obtained via stepwise addition and the addition of sequences followed the order of taxa in the data set. Heuristic searches were conducted with TBR and Multrees effective. Maxtrees was set to auto-increase. Branches were collapsed if branch lengths were less than, or equal to $10^{-8}$.

## RESULTS

### PCR

The IGS-1 region was successfully amplified using the primers P-1 and O-1. Double bands were observed for certain isolates within the same species. IGS-1 amplicon sizes varied between 400 bp (base pairs) and greater than 1500 bp for the various *Armillaria* spp. (Table 1). The ITS regions and 5.8S gene were successfully amplified using the primers ITS1 and ITS4. ITS amplicon sizes were the same within species but varied between 800 and 1000 bp among the different species.

### Phylogenetic analysis

Data for the ITS1 region included sequences starting 22 bp downstream from the 3' end of the SSU while sequences for the ITS2 region stopped approximately 3 bp upstream from the 5' end of the LSU. The total number of characters obtained after alignment by inserting gaps (without coding indels) was 867. The number of nucleotides sequenced, however, varied between 658 and 763 characters between the different isolates. The ITS1 and ITS2 regions were characterized by the presence of numerous indels. The largest indel was observed in *A. limonea* (CMW4991, CMW4992, CMW4678 and CMW4680) and was 127 bp in size. Indels were, with few exceptions, conserved within species.

Parsimony analysis of the ITS sequences in which indels were treated in various ways, generated MP trees that differed in length, number of trees retained, constancy index and retention index.

The MP trees generated using different indel treatments were similar in topology, with some variation of branches at the tips of trees. Analysis with indels excluded and gaps treated as newstate, however, produced MP trees that differed in the placement of *A. limonea* and *A. novae-zelandiae* clades relative to other clades.

The MP tree (Fig. 1) generated with indels coded with multistate characters and gaps treated as missing, grouped the isolates of *A. hinnulea*, *A. luteobubalina*, *A. pallidula*, *A. fumosa*, *A. novae-zelandiae*, *A. limonea* and the unknown New Zealand species into six strongly supported monophyletic lineages. *Armillaria pallidula* isolates and *A. fumosa* isolates grouped in a strongly supported (100%) clade and could not be differentiated from each other. Isolates representing *A. novae-zelandiae* formed a sister group with the *A. pallidula – A. fumosa* group. In this analysis we were not able to clearly differentiate between *A. novae-zelandiae* isolates from New Zealand and isolates representing the same species from Australia. *Armillaria limonea* formed a basal group to the *A. pallidula – A. fumosa* and *A. novae-zelandiae* sister group. *Armillaria luteobubalina* isolates were placed basal to *A. limonea* on the most parsimonious tree. The most parsimonious tree generated from the data set placed isolates representing the unknown species basal to *A. luteobubalina*. *Armillaria hinnulea* was placed basal to the rest of the Australian and New Zealand species. It was intriguing that, in our preliminary study, *A. hinnulea* grouped strongly within a clade representing the Northern hemisphere *Armillaria* spp. and not in the Australian - New Zealand *Armillaria* clade (Fig. 2).

## DISCUSSION

Molecular analysis of the IGS-1 of the rRNA operon of *Armillaria* spp. from Australia and New Zealand indicated that this is a highly divergent group of fungi. In this study the IGS-1 amplicon sizes varied significantly among the species. These size differences can only be attributed to the presence of large indels. This is in contrast to the Northern Hemisphere *Armillaria* species where the IGS-1 region was found to range between 845 bp and 920 bp among the different *Armillaria* species (Anderson and Stasovski 1992, Harrington and Wingfield 1995, Terashima *et al.* 1998). The large size variation observed in the IGS-1 region for the Australian and New Zealand species made it unsuitable for use in a robust phylogenetic study and it was, therefore, not included in this study.

The ITS regions (ITS1, 5.8S gene and ITS2) of the rDNA operon were used as an alternative to the IGS-1 region to determine the phylogenetic relationships between the Australian and New Zealand *Armillaria* species. Anderson and Stasovski (1992) found that the ITS regions for the majority of the Northern Hemisphere *Armillaria* spp. were excessively conserved for determining the phylogenetic relationships. ITS sequence data obtained in this study indicated a higher degree of DNA sequence similarity between the various lineages but with sufficient variation to be used in phylogenetic analysis of the Australian and New Zealand *Armillaria* spp.

Cladograms generated indicated that *A. hinnulea* is more closely related to the Northern Hemisphere *Armillaria* spp. than to the other Australian and New Zealand species. In a preliminary analysis of ITS sequences for *A. hinnulea* and Northern Hemisphere and African *Armillaria* spp. (Fig. 2), *A. hinnulea* grouped within the Northern Hemisphere clade. This is in agreement with the views of Kile and Watling (1983) who indicated that *A. hinnulea* resembles the European *A. bulbosa* Velen. (synonym: *A. cepistipes*). *Armillaria hinnulea* is further distinguishable from the other Australian and New Zealand *Armillaria* spp. in general basidiocarp morphology, and is the only Australian species with clamp connections in the subhymenial layer of the basidiocarp (Kile and Watling 1983). The New Zealand population of *A. hinnulea* differs from the Australian collections by having clamp connections in both the subhymenium and the hymenium (GS Ridley unpubl). Sexual compatibility studies (Kile and Watling 1988) confirmed the separation of *A. hinnulea* from the other *Armillaria* spp. based on morphology and indicated that this is a distinct species. Our grouping of the *A. hinnulea* isolates in a strongly supported monophyletic clade distant to the other Australian and New Zealand *Armillaria* spp. is thus in congruence with the differentiation of this species based on morphology and sexual compatibility tests.

Using interfertility tests, *A. hinnulea* isolates from Australia and putative *A. hinnulea* isolates from the central North Island of New Zealand were shown not to be conspecific by Kile and Watling (1988). Cladograms generated in the current study support this observation where the isolate of *A. hinnulea* (CMW4983) from Australia and the putative *A. hinnulea* isolates from the New Zealand North Island (CMW5597, CMW4994 and CMW4993) segregated in different clades. However, isolates derived from basidiomes collected in the South Island of New Zealand and identified as *A. hinnulea* based on micro-morphology, were grouped into the same clade as the Australian isolate of *A. hinnulea*. This indicates that *A. hinnulea* is present

in the South Island of New Zealand and is the same species as that occurring in Australia. It also indicates the presence of a new undescribed species in the central North Island of New Zealand.

*Armillaria fumosa* and *A. pallidula* could not be separated based on their ITS sequence differences resulting in one strongly supported monophyletic group. *Armillaria pallidula,* while sharing some morphological features with *A. fumosa*, was shown to be a distinct biological species (Kile and Watling 1988). *Armillaria pallidula* was described from one location in Queensland but possibly overlaps *A. fumosa* in geographic distribution (Kile and Watling 1988). Data presented in this study indicate that the two species are closely related and are probably sibling species. Differences in morphology and mating type were not congruent with the differentiation at ITS level.

Analysis of the ITS sequence data showed that the *A. novae-zelandiae* isolates from New Zealand and Australia belong to a single monophyletic clade and basal to the *A. pallidula – A. fumosa* group. *Armillaria novae-zelandiae* occurs in the temperate rainforests of south-eastern Australia and New Zealand (Hood 1989). Macro- and micro-morphology of the type material from New Zealand (Stevenson 1964) was similar to the morphology of basidiocarps found in Australia (Kile and Watling 1983). Kile and Watling (1983) also found that the vegetative morphology of the Australian and New Zealand isolates of *A. novae-zelandiae* is very similar. At the biological species level, it was shown that *A. novae-zelandiae* isolates from New Zealand, mainland Australia and Tasmania are sexually compatible (Kile and Watling 1983). Morphological descriptions, sexual compatibility tests and ITS sequence analyses presented here indicated that *A. novae-zelandiae* from Australia and New Zealand are very closely related.

The grouping of *A. limonea* isolates in a monophyletic clade basal to the *A. novae-zelandiae* clade supports the differentiation of these two species based on vegetative and basidiocarp morphology. The vegetative morphologies of *A. limonea* and *A. novae-zelandiae* are distinctly different and can be used to differentiate between isolates representing these species (Shaw *et al.* 1981). *Armillaria novae-zelandiae* and *A. limonea* can be separated on micro-morphology, particularly on the structure of the pileipellis (GS Ridley unpubl). Comparisons between the descriptions of the macro-morphology of *A. limonea* (Stevenson 1964) and *A. novae-zelandiae* (Stevenson 1964, Kile and Watling 1983) indicated that they are distinct species. The

grouping of *A. limonea* from New Zealand basal to *A. novae-zelandiae* and not to the Australian *A. pallidula – A. fumosa* clade is supported by their biogeography. It is apparent that *A. limonea* and *A. novae-zelandiae*, although divergent in morphology, are very similar in ITS sequences and, therefore, phylogenetically related.

*Armillaria luteobubalina* grouped basal to *A. limonea* on the cladogram generated in this study. *Armillaria luteobubalina* is the most prevalent *Armillaria* sp. in Australia and is widely distributed in New South Wales, Victoria, South Australia, Western Australia, Tasmania and parts of south-east Queensland (Kile and Watling 1981, 1983, Pearce *et al.* 1986, Shearer and Tippett 1988, Shearer 1994). The grouping of *A. luteobubalina* close to the New Zealand *A. limonea* and not the other *Armillaria* spp. from Australia is interesting since there are limited similarities in their basidiocarp morphology (Stevenson 1964, Podger *et al.* 1978). These two species, however, are similar in their yellow pigmentation of the pileus. Based on sexual compatibility tests, Kile and Watling (1988) showed that *A. limonea* and *A. luteobubalina* are distinct biological species.

Our results indicate that the unknown *Armillaria* sp. included in this study is different from the other Australian and New Zealand *Armillaria* spp. This species is only known in the central North Island of New Zealand (Hood 1992). Haploid cultures of this fungus were crossed with haploid tester strains of *A. novae-zelandiae* but failed to form dikaryons (Hood and Sandberg 1987). It was thus suggested that the unknown North Island of New Zealand fungus probably represented *A. hinnulea* (Kile and Watling 1983). Haploid isolates were, however, incompatible with *A. hinnulea* tester strains from Australia (Kile and Watling 1988) and the micromorphology of the basidiocarps of the two species also differed (Hood 1989). Based on our phylogenetic analysis and evidence from the reported sexual compatibility tests and morphology of the basdiocarps, we believe that this is a distinct taxon that needs to be formally described.

Large indels were present in both ITS1 and ITS2 regions obtained in this study. To reduce the effect of the indels we applied a multistate coding system, by which blocks of indels were replaced by numeric characters. This coding system resulted in an increase in resolution at the branch tips of the trees obtained after heuristic searches. Phylogenetic analysis based on ITS data in this study showed that the *Armillaria* spp. from Australia and New Zealand, with the

exception of *A. hinnulea*, formed a strongly supported monophyletic group and that they are separated from one another.

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TABLE 1: *Armillaria* isolates used in phylogenetic analysis.

| Species | IGS amplicon size (*ca.*) | Culture no. | Alternative number | Host | Origin | Collector | Genbank accession number |
|---|---|---|---|---|---|---|---|
| *Armillaria hinnulea* | 660 bp | CMW4990 | 3512/13 | Basidiocarp on *Nothofagus* sp. | South Island, New Zealand | GS Ridley | AF329905 |
| | | CMW4988 | 3511/15 | " | South Island, New Zealand | GS Ridley | AF329906 |
| | | CMW4987 | 3511/10 | " | South Island, New Zealand | GS Ridley | AF329907 |
| | | CMW4983 | Lot2(11) | " | Australia | - | AF329908 |
| *A. luteobubalina* | 610 bp | CMW4978 | MtCole1(18) | Unknown | Victoria, Australia | - | AF329909 |
| | | CMW4979 | MtCole1(1) | " | Victoria, Australia | - | AF329910 |
| | | CMW3942 | 659.85 | *Eucalyptus regnans* | Australia | GA Kile | AF329911 |
| | | CMW4977 | SA(6) | Unknown | South Australia | - | AF329912 |
| | | CMW5704 | WA31(5) | " | Western Australia | - | AF329913 |
| *A. pallidula* | 400 bp | CMW4972 | Qld5761 | " | Queensland, Australia | - | AF329914 |
| | | CMW4968 | 3626, ATCC 66124 | *Pinus caribaea* var. *hondurensis* | Australia | P. Gordon | AF329915 |

TABLE 1 (continued)

| Species | IGS amplicon size (*ca.*) | Culture no. | Alternative number | Host | Origin | Collector | Genbank accession number |
|---|---|---|---|---|---|---|---|
| *A. fumosa* | 400 bp | CMW4960 | Qld.Coll.9(4) | Unknown | Queensland, Australia | GA Kile | AF329916 |
| | | CMW4957 | 123 | Basidiocarp on *Eucalyptus* sp. | Tasmania, Australia | GA Kile | AF329917 |
| | | CMW4955 | 123.1 | Basidiocarp on *Eucalyptus* sp. | Tasmania, Australia | GA Kile | AF329918 |
| | | CMW4956 | 123.2 | Basidiocarp on *Eucalyptus* sp. | Tasmania, Australia | GA Kile | AF329919 |
| | | CMW4959 | Qld.Coll.8(1) | *P. radiata* | Queensland, Australia | GA Kile | AF329920 |
| | | CMW4967 | Qld.Coll.9(3) | " | Queensland, Australia | GA Kile | AF329921 |
| *A. novae-zelandiae* | 830 bp | CMW4963 | 121, ATCC 66127, DAR41512 | Basidiocarp on *Antherosperma moschatum* | Tasmania, Australia | GA Kile | AF329922 |
| | | CMW4966 | Lot4(4) | Unknown | Australia | - | AF329923 |
| | | CMW4964 | Qld.Coll.10(3) | Basidiocarps on *P. radiata* | Queensland, Australia | GA Kile | AF329924 |

TABLE 1 (continued)

| Species | IGS amplicon size (*ca.*) | Culture no. | Alternative number | Host | Origin | Collector | Genbank accession number |
|---|---|---|---|---|---|---|---|
| | | CMW4986 | 3505/15 | Basidiocarps from *Nothofagus fusca* and *N. solandri* forest | South Island, New Zealand | GS Ridley | AF329925 |
| | | CMW4722 | G3.0.34.4 | Rhizomorphs from *Beilschmiedia tawa* forest | North Island, New Zealand | IA Hood | AF329926 |
| *A. limonea* | 580 bp | CMW4991 | 3522/2 | *P. radiata* | North Island, New Zealand | GS Ridley | AF329927 |
| | | CMW4992 | 3522/13 | *P. radiata* | North Island, New Zealand | GS Ridley | AF329928 |
| | | CMW4678 | A3.4.26.3 | Rhizomorphs from *Beilschmiedia tawa* forest | North Island, New Zealand | IA Hood | AF329929 |
| | | CMW4680 | C3.28.0.1 | Rhizomorphs from *Beilschmiedia tawa* forest | North Island, New Zealand | IA Hood | AF329930 |
| *Armillaria* sp. | > 1500 bp | CMW5597 | A35.4 | *Nothofagus fusca* | North Island, New Zealand | IA Hood | AF329931 |

**TABLE** 1 (continued)

| Species | IGS amplicon size (*ca.*) | Culture no. | Alternative number | Host | Origin | Collector | Genbank accession number |
|---|---|---|---|---|---|---|---|
| | | CMW4994 | 4698/10 | *Nothofagus* sp. | North Island, New Zealand | GS Ridley & JF Gardener | AF329932 |
| | | CMW4993 | 4698/9 | *Nothofagus* sp. | North Island, New Zealand | GS Ridley & JF Gardener | AF329933 |

**Figure 1.** One of the most parsimonious trees generated after a heuristic search from the ITS sequence data with indels coded and gaps treated as missing. Bootstrap (1000 replicates) values and Bremer support indexes for the branching nodes are indicated above the tree branches. Values below the branches are the branch lengths. Number of parsimony informative characters = 113, length of tree = 202, CI = 0.880 and RI = 0.967.

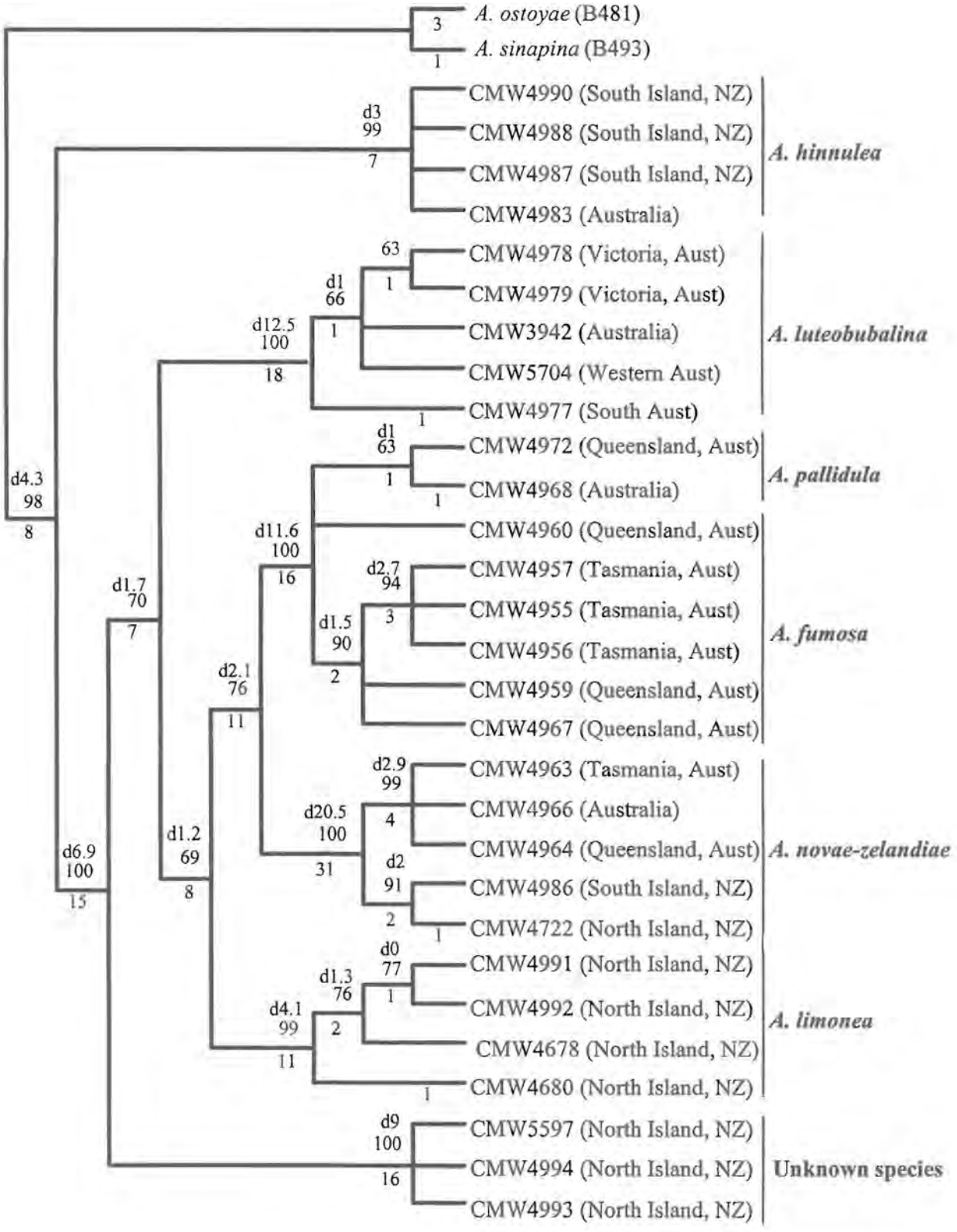
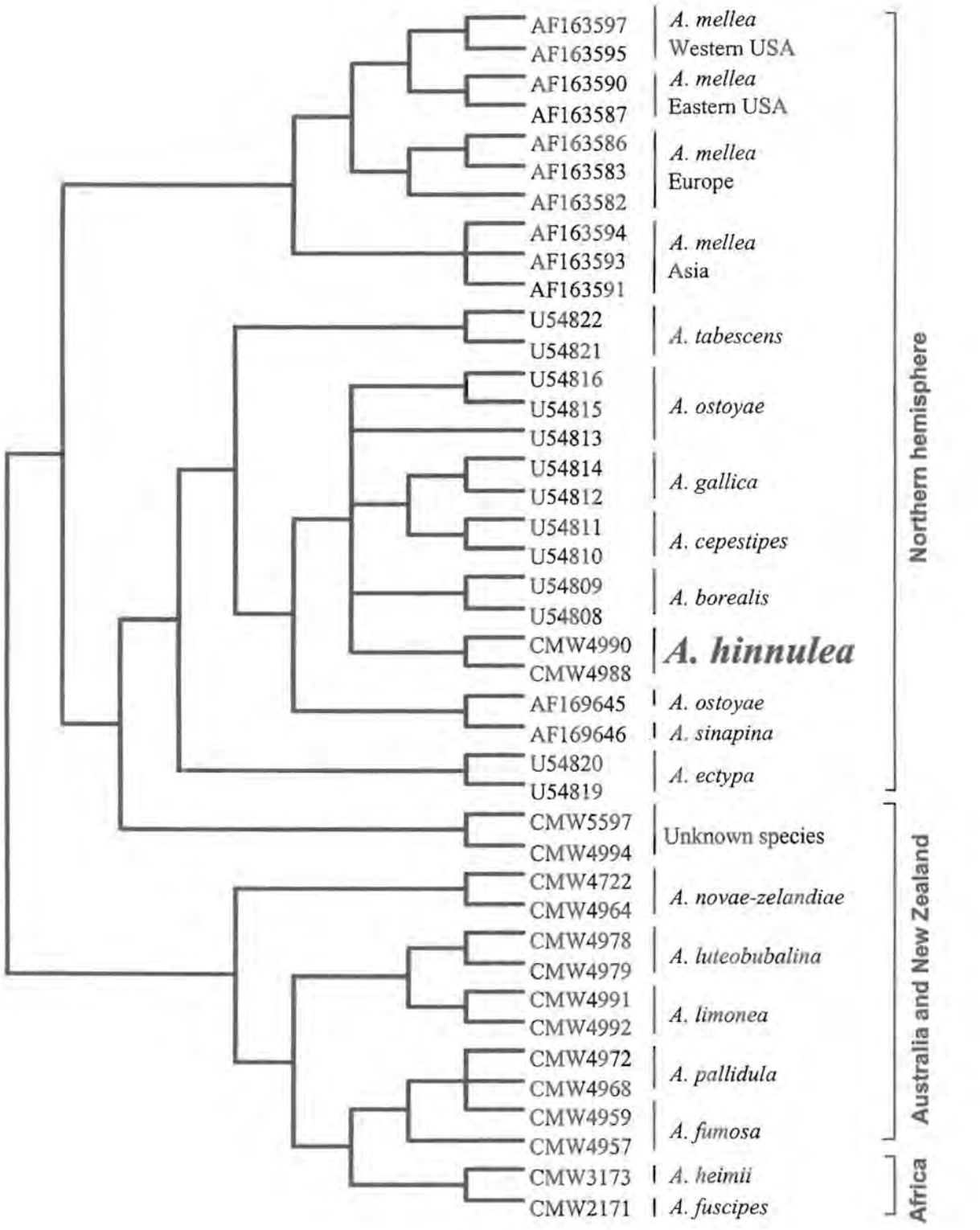


**Figure 2.** Strict consensus tree from 210 MP trees for *Armillaria* spp. from the Northern and the Southern Hemisphere. Number of parsimony informative characters = 339, length of tree = 485, CI = 0.786, RI =0.894. AF and U numbers refer to GenBank accession numbers.



# CHAPTER THREE

# MOLECULAR IDENTIFICATION AND PHYLOGENY OF *ARMILLARIA* ISOLATES FROM SOUTH AMERICA AND INDO-MALAYSIA

Published: *Mycologia* **95**: 285 – 293 (2003)

# MOLECULAR IDENTIFICATION AND PHYLOGENY OF *ARMILLARIA* ISOLATES FROM SOUTH AMERICA AND INDO-MALAYSIA

## ABSTRACT

Armillaria root rot is a serious disease, chiefly of woody plants, caused by many species of *Armillaria* that occur in the temperate, tropical and sub-tropical regions of the world. Very little is known about *Armillaria* in South America and Southeast Asia, although Armillaria root rot is well known in these areas. In this study, we consider previously unidentified isolates collected from trees with symptoms of Armillaria root rot in Chile, Indonesia and Malaysia. In addition, isolates from basidiocarps resembling *A. novae-zelandiae* and *A. limonea*, originating from Chile and Argentina, respectively, were included in this study because their true identity has been uncertain. All isolates in this study were compared based on their similarity in ITS sequences with previously sequenced *Armillaria* spp., and their phylogenetic relationship with species from the Southern Hemisphere was considered. ITS sequence data were also compared with those available on GenBank, for *Armillaria* species. Parsimony and distance analyses were conducted to determine the phylogenetic relationships between the unknown isolates and the species that showed high ITS sequence similarity. In addition, IGS-1 sequence data were obtained for some of the species to validate the trees obtained from the ITS data set. Results of this study showed that the ITS sequences of the isolates obtained from basidiocarps resembling *A. novae-zelandiae* are most similar to those for this species. ITS sequences for isolates from Indonesia and Malaysia had the highest similarity to *A. novae-zelandiae*, but were phylogenetically separated from this species. Isolates from Chile, for which basidiocarps were not found, were similar in their ITS and IGS-1 sequences to the isolate from Argentina that resembled *A. limonea*. These isolates, however, had the highest ITS and IGS-1 sequence similarity to authentic isolates of *A. luteobubalina* and were phylogenetically more closely related to this species than to *A. limonea*.

***Keywords:*** *Armillaria limonea*, *Armillaria novae-zelandiae*, *Armillaria luteobubalina*, ITS, IGS-1, phylogeny, systematics.

## INTRODUCTION

Armillaria root rot is a serious disease mainly of woody plants, caused by species of *Armillaria* (Fr.:Fr.) Staude. *Armillaria* spp. exist as pathogens, saprobes or necrotrophs on a wide range of host plants (Gregory *et al.* 1991, Hood *et al.* 1991, Kile *et al.* 1991, Fox 2000). They also tend not to show a species-specific interaction with their hosts, although some species have defined host ranges (Termorshuizen 2000).

*Armillaria* spp. are known in many parts of the world and can be found on infected plants in the temperate, sub-tropical and tropical regions (Hood *et al.* 1991). Species associated with root rot are best known in Northern Hemisphere countries where considerable effort has been made to identify them. Armillaria root rot has also been recorded on various planted and natural hosts in South America and Indo-Malaysia, although little is known about the species occurring in these areas (Hood *et al.* 1991). Many *Armillaria* spp. linked to outbreaks of the disease in South America are thought to be restricted to this area (Singer 1953, Kile *et al.* 1994). Two species, *A. novae-zelandiae* (G.Stev.) Herink and *A. limonea* (G.Stev.) Boesew., are the exception in that they have also been reported from Australia and New Zealand (Ivory 1987, Hood *et al.* 1991).

Little information is available regarding the identity of *Armillaria* in Indonesia and Malaysia (Hood *et al.* 1991, Kile *et al.* 1994). Reports of *Armillaria* in these regions are based mostly on the presence of the characteristic rhizomorphs or typical disease symptoms on infected trees (Kile *et al.* 1994). In most reports from Indo-Malaysia, Armillaria root rot has been attributed to *A. mellea sensu lato*, although this identity almost certainly does not include *A. mellea* (Vahl.:Fr.) P.Kumm. *sensu stricto*.

Conventional identification of *Armillaria* has been based on the morphology of the basidiocarps, but dependence on this character is beset with problems. Generally, these structures are produced only in the final stages of the disease and then only in some years and for a limited period of time (Fox *et al.* 1994). In some species, the morphology of the basidiocarps differs only slightly, making routine identification difficult (Bérubé and Dessureault 1989). In the past two decades, identification of unknown *Armillaria* isolates has depended strongly on the use of sexual compatibility tests with known haploid tester strains (Korhonen 1978, Ullrich and Anderson 1978). However, these tests are time consuming and often yield ambiguous results.

Furthermore, field isolates are usually diploid making their sexual interaction with haploid tester strains difficult to interpret (Guillaumin *et al.* 1991).

Problems surrounding the identification of *Armillaria* have led to important advances in developing robust but rapid DNA techniques. Such techniques have included DNA-base composition (Jahnke *et al.* 1987), DNA-DNA hybridization (Miller *et al.* 1994), sequence analyses of the first intergenic spacer region (IGS-1) (Anderson and Stasovski 1992) and internal transcribed spacer regions (ITS) (Coetzee *et al.* 2001a), restriction-fragment length polymorphisms (RFLPs) without PCR (Smith and Anderson 1989) and RFLPs of IGS-1 amplicons (Harrington and Wingfield 1995). Although several of these techniques might include some problems (Pérez-Sierra *et al.* 2000), by virtue of their relative simplicity they are gradually replacing traditional methods.

Sequence data for various *Armillaria* have increased substantially since the first publication on the phylogeny of *Armillaria* from the Northern Hemisphere (Anderson and Stasovski 1992). Understandably, the initial focus of such studies has concentrated on species in Europe and North America (Chillali *et al.* 1998, Coetzee *et al.* 2000b). More recently, however, substantial data sets for species in Africa, Australasia and Southeast Asia have become available (Terashima *et al.* 1998, Coetzee *et al.* 2000a, 2001a). At present ITS and IGS-1 sequences are available on GenBank for the best-known species of *Armillaria*. However, there are disjunctions in data sets and relatively little is known about species from Indo-Malaysia and South America.

The aim of this study was to identify a collection of isolates from dying trees, showing typical symptoms of Armillaria root rot in various parts of South America and Indo-Malaysia. These isolates had cultural characteristics typical of *Armillaria* but could not be identified based on morphology, due to the absence of basidiocarps in disease centres. In addition, isolates from a culture collection, of uncertain identity but thought to represent *A. novae-zelandiae* and *A. limonea* from Chile and Argentina, were included. Sequences from the IGS-1 and ITS regions of the rDNA operon were used to identify the unknown isolates and to determine their phylogenetic placement relative to other *Armillaria* spp. Evolutionary relationships between field isolates from Asia and South America and isolates representing the species that shared a high ITS sequence similarity with them, were determined in a phylogenetic study using distance and parsimony analyses.

## MATERIALS AND METHODS

### Fungal isolates

The majority of isolates used in this study originated from field investigations on dying *Eucalyptus* and *Pinus* species in Malaysia, Indonesia and Chile. Additional isolates from basidiocarps in Chile (CMW5448 and CMW5450) and Argentina (CMW5446), thought to represent *A. novae-zelandiae* and *A. limonea*, respectively, were included. All isolates used (Tables 1 and 2) are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

### DNA extraction

Isolates were grown in liquid MY (1.5% malt extract and 0.2% yeast extract) medium for two weeks at 22 °C in the dark. Mycelium was harvested by filtering through sterilized stainless-steel mesh, lyophilized and ground to a fine powder in liquid nitrogen. One mL preheated (60 °C) extraction buffer (O'Donnell *et al.* 1998) was added to approximately 0.5 g of the powdered mycelium, vortexed and incubated for 2h at 60 °C. Cell debris was precipitated by centrifugation (15 300 *g*, 15 min), followed by isoamyl alcohol: chloroform (1:24) extractions on the aqueous phase (0.5 v/v) until a clean interphase was obtained. A final chloroform (0.5 v/v) extraction was done to remove the remaining isoamyl alcohol. Nucleic acids were precipitated with ethanol (100%) overnight at -20 °C. The precipitate was collected by centrifugation (13 500 *g*, 30 min, room temperature), washed twice with ice-cold ethanol (70%), dried and dissolved in sterile distilled water. RNase A (0.01 mg/µL) was added to the suspension and incubated at 37 °C for 6 h to remove contaminating RNA.

### PCR and sequencing

Extracted DNA was used as template in the PCR reactions to amplify the ITS (including ITS1, 5.8S and ITS2 regions) and the IGS-1 regions for the unknown isolates from Asia and South America. The ITS region was amplified with primer set ITS1 and ITS4 (White *et al.* 1990) and the IGS-1 region with P-1 (Hsiau 1996) and O-1 (Duchesne and Anderson 1990). PCR reaction mixtures for amplification of the regions were the same. The mixture included dNTPs (0.25 mM of each), buffer with $MgCl_2$ supplied by the manufacturer, additional $MgCl_2$ (0.25 mM), 0.1 µM of each primer, Expand™ High Fidelity PCR System enzyme mix (1.75 U) (Roche Diagnostics)

and approximately 80 ng of template DNA. Reaction conditions were an initial denaturation at 96 °C (2 min), 35 cycles of primer annealing at 62 °C (30 s), elongation at 72 °C (1 min) and denaturation at 94 °C (30 s). A final elongation step was allowed at 72 C for 5 min. PCR products were purified before to sequencing with a QIAquick PCR Purification Kit (QIAGEN).

Sequences for both strands of the PCR products were obtained with an ABI PRISM™ 377 automated DNA sequencer. Sequence reactions were carried with an ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA polymerase FS (Perkin Elmer). The ITS region was sequenced with primers ITS1, ITS4, CS2B and CS3B (Coetzee *et al.* 2001a). IGS-1 sequences were obtained with primers P-1, O-1, MCO-2 and MCO-2R (Coetzee *et al.* 2000b).

### Identification of unknown isolates

Initial identification of the unknown isolates from Asia and South America was based on nucleotide similarity with sequences at GenBank, by using the BLAST search function of the database. In addition, ITS and IGS-1 DNA sequences for the unknown isolates were aligned with those from the same DNA regions, for the species that showed highest similarities to them. Sequence alignment was done with Clustal X version 1.8 software (Thompson *et al.* 1997). Regions poorly aligned due to indels were manually corrected with a text editor. Aligned ITS and IGS-1 sequences for the *Armillaria* isolates have been deposited in TreeBase (study accession number: S771, matrix accession numbers: M1219 and M1220). Sequence similarities among isolates were determined, based on uncorrected *p* distances converted to percentage similarity.

### Phylogenetic analyses

Relatedness of the unknown isolates and *Armillaria* showing high sequence homology to them was determined in a phylogenetic analysis based on distances and parsimony using PAUP* version 4 (Swofford 1998). Neighbour-Joining (NJ) trees (Saitou and Nei 1987) were generated with a Kimura 2-parameter substitution model (Kimura 1980) implemented in the analysis and random addition of taxa. Most-parsimonious (MP) trees were generated after a heuristic search, with starting trees obtained via stepwise addition with 100 random taxon additions, branch-swapping based on the tree-bisection-reconnection (TBR) algorithm, MulTrees effective and

topological constraints not enforced. MaxTrees was set to auto-increase and zero length branches were collapsed. The effect of indels on the tree topology was tested in separate analyses by exclusion of indels, inclusion of indels but with gapmode set as missing, and inclusion of indels but with gaps treated as a fifth character (newstate). Tree-length distribution of 100 randomly generated trees was determined for phylogenetic signal (g1) (Hillis and Huelsenbeck 1992). Confidence in branching points on the phylogenetic trees was determined with bootstrap (1000 replicates) (Felsenstein 1985).

## RESULTS

### Identification of unknown isolates

Unknown isolates CMW5448 and CMW5450 from Chile and CMW3951, CMW4143 and CMW4145 from Asia had ITS sequences most similar to sequences of *A. novae-zelandiae* at GenBank. ITS sequences of *A. limonea* had the next highest similarities to the unknown isolates, but the scores (bits) ranged from 436 to 442 in comparison with the 571 to 613 scores obtained for *A. novae-zelandiae*. Isolates CMW5448 and CMW5450 were identical in their ITS sequences. Isolates CMW4143 and CMW4145 from Indonesia were > 99% similar in their ITS sequences but showed a 5% difference from CMW3951 from Malaysia. Sequence similarity between the Chilean isolates and *A. novae-zelandiae* (CMW4722 and CMW4964) (Table 2) ranged between 94% and 97%. Similarity among the two Indonesian isolates and *A. novae-zelandiae* was lower than the Chilean isolates, ranging between 89% and 91%. Similarity between the Malaysian isolate (CMW3951) and *A. novae-zelandiae* sequences, CMW4722 and CMW4964, were 90% and 91%, respectively.

The unknown Chilean and the presumed *A. limonea* isolate from Argentina had ITS sequences that were most similar to ITS sequences for *A. luteobubalina* Watling & Kile at GenBank. ITS sequences for these isolates were also very similar to those for *A. limonea* in the database, but their scores were significantly lower, 737 - 745 in contrast to the 930 - 944 bits obtained for *A. luteobubalina*. Isolates CMW8876 and CMW8879 from Chile had identical ITS sequences but differed from isolate CMW5446 in Argentina (< 1%) due to a single 32bp indel. IGS-1 sequences for isolates CMW8876, CMW8877 and CMW8879 from Chile and CMW5446 from Argentina were identical. IGS-1 sequences for these isolates showed a 95% similarity with unpublished IGS-1 sequences of *A. luteobubalina* (CMW4977). *Armillaria limonea*

(CMW4991) had IGS-1 sequence similarity of 85% with the isolates from Chile and Argentina.

### Phylogenetic analyses

The choice of taxa in the ITS data set could be made only after initial identification of the unknown isolates. Thus ITS sequence data for *A. limonea* (CMW4678 and CMW4680), *A. luteobubalina* (CMW4977 and CMW5704), and *A. novae-zelandiae* (CMW4722 and CMW4964) (Table 2) were used to determine the phylogenetic relationships among the isolates. *Armillaria hinnulea* Kile & Watling (CMW4983 and CMW4990) (Table 2), a Southern Hemisphere species (Coetzee *et al.* 2001a) shown to be closely related to Northern Hemisphere species, was used as the outgroup.

The presence of large indels in the data set had a minimal effect on the parsimony analyses (Table 3). Most-parsimonious trees generated with indels treated in different ways were similar in overall topology (Fig. 1). Swapping between taxa on the terminal branches resulted in multiple MP trees when indels were excluded or included but gaps treated as missing. The placement of the unknown taxa within specific clades, however, was supported by bootstrap values, independent of the treatment of indels.

Neighbour-Joining and MP trees generated in this study (Fig. 1) placed isolates CMW5448 and CMW5450, resembling *A. novae-zelandiae* in Chile, within a well supported monophyletic group that included sequences from authentic isolates of species in Australia and New Zealand. Isolates, tentatively identified as *A. novae-zelandiae* in this study, from Malaysia (CMW3951) and Indonesia (CMW4145 and CMW4143), grouped together in a well-supported clade. The Indo-Malaysian clade formed a well-supported sister group with the *A. novae-zelandiae* clade that included isolates from Australia, Chile and New Zealand. Differences were observed among the Malaysian isolate (CMW3951) and Indonesian isolates (CMW4145 and CMW4143), with the Malaysian isolate separated from the Indonesian isolates by a long branch.

Isolate CMW5446 from Argentina, thought to represent *A. limonea,* grouped closely in a well-supported clade with the isolates from Chile (CMW8876 and CMW8879) in both NJ and MP trees generated (Fig. 1). These isolates, identified as *A. luteobubalina* based on ITS sequence similarity, resided in a highly supported group that included authentic isolates representing *A. luteobubalina* (CMW5704 and CMW4977). The South American group of isolates, however,

formed a sister group to the *A. luteobubalina* clade in distance and parsimony analyses with indels excluded or with indels included but gaps treated as missing.

The relationships among the unknown isolates from Chile and Argentina and those of *A. luteobubalina*, were further investigated based on their IGS-1 sequences. The number of characters included in the data set was 537 after exclusion of an ambiguously aligned CT rich region. Trees generated on distance and parsimony analysis had similar topologies and grouped the Chilean and Argentinean isolates in a strongly supported monophyletic group (Fig. 2). Isolates representing *A. luteobubalina* from Australia formed a well-supported monophyletic sister group with the South American isolates. Isolates representing *A. limonea* from New Zealand were placed basal to the South American *A. luteobubalina*.

## DISCUSSION

In this study, *Armillaria* isolates from Argentina, Chile, Indonesia and Malaysia of unknown or uncertain identity, were identified with ITS and IGS sequence data. We thus were able to confirm previous suggestions (Singer 1969) regarding the identity of species in South America. Our results also provide interesting new records pertaining to the geographic distribution of *Armillaria* spp. in the areas considered. Results from this study have confirmed the utility of sequence data for identifying *Armillaria* in the absence of basidiocarps. Moreover, they add substantial new information regarding phylogenetic relationships for this important group of root pathogens.

Two isolates from Chile, of uncertain identity but resembling *A. novae-zelandiae* based on basidiocarp morphology, were included in this study. Phylogenetic analyses confirmed their identity as *A. novae-zelandiae* by placing them in a strongly supported monophyletic group, with well recognized isolates of this species from Australia and New Zealand. There were, however, some differences in the ITS sequences between the Chilean isolates and those from Australia, due to indels and base substitutions. Differences between Australasian and South American collections of *A. novae-zelandiae* have been reported by Kile and Watling (1983), and our data support their observations.

Although *Armillaria* have been shown to be introduced into new areas (Coetzee *et al.* 2001b), it is unlikely that *A. novae-zelandiae* was introduced into Chile from Australia or New Zealand. The ITS sequences of the Australian, Chilean and New Zealand isolates, although highly similar, differed as a result of a number of indels. These differences suggest a long period of geographic separation between *A. novae-zelandiae* from Australasia and South America. Furthermore, isolates from Chile were collected from *Nothofagus*, a genus that occurs in Chile, Argentina, Australia, New Zealand, New Guinea and New Caledonia. *Nothofagus* species formed a continuous forest from New Guinea, through eastern Australia, west Antarctica, New Zealand and New Caledonia to southern South America when these landmasses were part of the super continent Gondwanaland (Poole 1987). Kile *et al.* (1994) noted that *A. novae-zelandiae* in Australia displays a particularly close association with *Nothofagus*. Likewise Singer (1953) and Horak (1983) noted relationships among fungi on *Nothofagus* in Australia, New Zealand and South America. The close phylogenetic relationship between the South American, Australian and New Zealand isolates of *A. novae-zelandiae* supports the notion that this fungus was associated with *Nothofagus* before the breakup of Gondwana and that it is native to South America.

Sequence-data comparisons lead us to tentatively identify isolates from Malaysia and Indonesia as *A. novae-zelandiae*. However, distance and parsimony analyses revealed that they form a strongly supported monophyletic group basal to the South American - Australia - New Zealand clade representing this species. Although these isolates are closely related to *A. novae-zelandiae*, it is possible that they represent a discrete taxon. This could be a species already known but for which sequence data are not available, or alternatively, it could represent an undescribed taxon.

At least eight biological species of *Armillaria* have been reported in Japan, and many of these are known or related, based on IGS-1 sequences, to those in other parts of the Northern Hemisphere (Terashima *et al.* 1998). Although IGS-1 sequence data were not obtained for the Malaysian and Indonesian isolates included in this study, it previously had been shown that the Southern Hemisphere *Armillaria* spp. differ significantly in their ITS sequences from those in the Northern Hemisphere (Coetzee *et al.* 2001a). It also was shown that *A. hinnulea* (used as outgroup in this study) is more closely related to the Northern Hemisphere species, than to the species in the Southern Hemisphere (Coetzee *et al.* 2001a). Thus the placement of the isolates from Indonesia and Malaysia within a strongly supported monophyletic clade, including the exclusively Southern Hemisphere *A. novae-zelandiae* and distant to *A. hinnulea,* makes it

unlikely that the isolates in Malaysia and Indonesia represent one of the known Japanese species.

It is unlikely that isolates from Indonesia and Malaysia in this study are related to species in India, despite the fact that India formed part of Gondwana. This view is supported by the findings of Kile and Watling (1988) who showed, based on morphology, that Indian species of *Armillaria* are most closely related to Northern Hemisphere species. Similarly Volk and Burdsall (1995) showed that Australian and New Zealand *Armillaria* spp. do not occur in India. The close phylogenetic relationship between isolates from Indonesia and Malaysia and *A. novae-zelandiae* from Australia and New Zealand, and the previously reported morphological differences between Australian and Indian *Armillaria* spp., reduces the likelihood that the Indonesian and Malaysian isolates in this study represent one of the Indian species.

A surprising discovery in this study was the fact that some isolates from Chile and one from Argentina were found to represent *A. luteobubalina*. This species has previously been known only in Australia, where it is a well-known pathogen of *Eucalyptus* (Kile *et al.* 1991, Volk and Burdsall 1995). The isolate from Argentina originated from a basidiocarp resembling *A. limonea* on *Nothofagus antarctica*. *Armillaria limonea* first was described from New Zealand as *Armillariella limonea* G.Stev. (Stevenson 1964) but was also found in a *Nothofagus* forest in South America by Singer (Singer 1969). The ITS sequence of the suspected *A. limonea* isolate was highly similar to the isolates from an exotic *Pinus radiata* plantation in Southern Chile and for which basidiocarps were not found. Although we expected the unknown Chilean isolates to represent *A. limonea*, their ITS sequences and that of the Argentinean isolate are closest to *A. luteobubalina*. Phylogenetic analyses based on parsimony and distances further supported the results based on sequence similarity, by placing the South American isolates within a strongly supported monophyletic group with *A. luteobubalina*. The Argentinean and Chilean isolates, however, were separated from the Australian group as a result of large indels.

The unexpected grouping of Chilean and Argentinean isolates with *A. luteobubalina* justified our further analysis based on sequences of the IGS-1 region. DNA sequences for this region have not previously been determined for any of the Australian and New Zealand species. Results unequivocally confirmed findings based on ITS sequences, that isolates from Argentina and Chile represent *A. luteobubalina*, forming a strongly supported monophyletic group with this species.

Although from different countries, the Chilean and Argentinean isolates of *A. luteobubalina* probably originated from areas relatively close to each other. The Chilean isolates were collected from dying *P. radiata* in the lower Andes and certainly originated in native vegetation, which predominantly includes *Nothofagus*. In Argentina, *Nothofagus* occurs in the Andes and the origin of the Argentinean isolate from this tree suggests a proximity of origin. The presence of *A. luteobubalina* in South America also suggests that this species has an early Gondwanan origin. This is the best-known species of *Armillaria* in Australia, where it occurs transcontinentally in natural wet and dry sclerophyll eucalypt forests as well as in horticultural plantings (Kile and Watling 1981, Shearer 1994). The wide distribution of this species in Australia, as well as its discovery in South America, support the view that it is an ancient species, with an origin preceding the separation of Gondwana. The fact that the South American isolates were separated from the Australian group in both ITS and IGS-1 trees, supports an extended period of geographical separation. Although available data support treating them as a single species, isolates clearly have existed independently for a long period and may later be regarded as independent taxa.

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**TABLE 1:** *Armillaria* isolates used in this study from Asia and South America.

| Culture number | Alternative number | Host | Origin | Collector | ITS GenBank accession no. | IGS GenBank accession no. |
|---|---|---|---|---|---|---|
| CMW3951 | O-1 | *Acacia mangium* | Malaysia | MJ Wingfield | AF448419 | - |
| CMW4143 | - | *Eucalyptus grandis* | Lake Toba, Sumatra, Indonesia | MJ Wingfield | AF448421 | - |
| CMW4145 | - | *E. grandis* | Lake Toba, Sumatra, Indonesia | MJ Wingfield | AF448420 | - |
| CMW5446 | 7348/10 | *Nothofagus* log | Neuquen Province, Argentina | RH Peterson | AF448422 | AF445068 |
| CMW5448 | 7365/2 | *Nothofagus* log | Grand Isla de Chiloe, Chile | RH Peterson | AF448417 | - |
| CMW5450 | 7365/4 | *Nothofagus* log | Grand Isla de Chiloe, Chile | RH Peterson | AF448418 | - |
| CMW8876 | Chile-1 | *Pinus radiata* | Temuco, Chile | MJ Wingfield | AF448423 | AF445065 |
| CMW8877 | Chile-2 | *P. radiata* | Temuco, Chile | MJ Wingfield | - | AF445066 |
| CMW8879 | Chile-3 | *P. radiata* | Temuco, Chile | MJ Wingfield | AF448424 | AF445067 |

TABLE 2: *Armillaria* isolates from Australia and New Zealand used in this study.

| Species | Culture no. | Alternative no. | Host | Origin | Collector | ITS GenBank accession no. | IGS Genbank accession no. |
|---|---|---|---|---|---|---|---|
| *Armillaria hinnulea* | CMW4980 | 119, CBS164.94 | Basidiocarp on *Eucalyptus obliqua.* | Hastings Caves, Tasmania | RH Peterson | - | AF445077 |
| | CMW4983 | Lot2(11) | Basidiocarp on *Nothofagus* sp. | Australia | - | AF329908 | - |
| | CMW4990 | 3512/13 | Basidiocarp on *Nothofagus* sp. | South Island, New Zealand | GS Ridley | AF329905 | AF445078 |
| *A. limonea* | CMW4680 | C3.28.0.1 | Rhizomorphs from *Beilschmiedia tawa* forest | North Island, New Zealand | IA Hood | AF329930 | AF445073 |
| | CMW4681 | 142B | *B. tawa* | North Island, New Zealand | M McKenzie | - | AF445074 |
| | CMW4678 | A3.4.26.3 | Rhizomorphs from *B. tawa* forest | North Island, New Zealand | IA Hood | AF329929 | - |
| | CMW4991 | 3522/2 | *Pinus radiata* | North Island, New Zealand | GS Ridley | - | AF445076 |

TABLE 2 (continued)

| Species | Culture no. | Alternative no. | Host | Origin | Collector | ITS GenBank accession no. | IGS Genbank accession no. |
|---|---|---|---|---|---|---|---|
| | CMW4992 | 3522/13 | *P. radiata* | North Island, New Zealand | GS Ridley | - | AF445075 |
| *A. luteobubalina* | CMW4974 | Runnymede | unknown | Australia | - | - | AF445071 |
| | CMW4976 | SA(1) | unknown | South Australia | - | - | AF445070 |
| | CMW4977 | SA(6) | unknown | South Australia | - | AF329912 | AF445069 |
| | CMW5704 | WA31(5) | unknown | Western Australia | - | AF329913 | AF445072 |
| *A. novae-zelandiae* | CMW4722 | G3.0.34.4 | Rhizomorphs from *B. tawa* forest | North Island, New Zealand | IA Hood | AF329926 | - |
| | CMW4964 | Qld.Coll. (10)3 | Basidiocarps on *P. radiata* | Queensland, Australia | GA Kile | AF329924 | - |

**TABLE 3:** Statistics for ITS data set with indels treated differently.

| Treatment | Nc[a] | Npic[b] | Nt[c] | Tl[d] | CI[e] | RI[f] | g1 |
|---|---|---|---|---|---|---|---|
| newstate | 1018 | 515 | 1 | 878 | 0.806 | 0.899 | -0.663 |
| missing | 1018 | 144 | 2 | 237 | 0.903 | 0.933 | -0.762 |
| complete deletion | 523 | 69 | 4 | 115 | 0.878 | 0.929 | -0.739 |

[a] Number of characters after alignment

[b] Number of parsimony informative characters

[c] Number of trees

[d] Tree length

[e] Consistency index

[f] Retention index

**Figure 1.** Phylogenetic tree generated after distance and parsimony analyses of the ITS sequence data. Bootstrap values (1000 replicates) are indicated above the branches for the Neighbour-Joining tree. Values below the branches are bootstrap support values for branching points obtained for trees generated after a heuristic search with indels included and gaps treated as missing. Values in italics are bootstrap-support values for branching nodes obtained after a heuristic search with indels included and gaps treated as a fifth character. Difference in tree topology when gaps were treated as a fifth character is depicted in the insert. Symbols indicate the connection between the tree and the branches in the inserts. (Abbreviations: NZ = New Zealand and Aust = Australia). Scale bar: 0.01 substitutions per site as determined in Neighbour-Joining analysis.

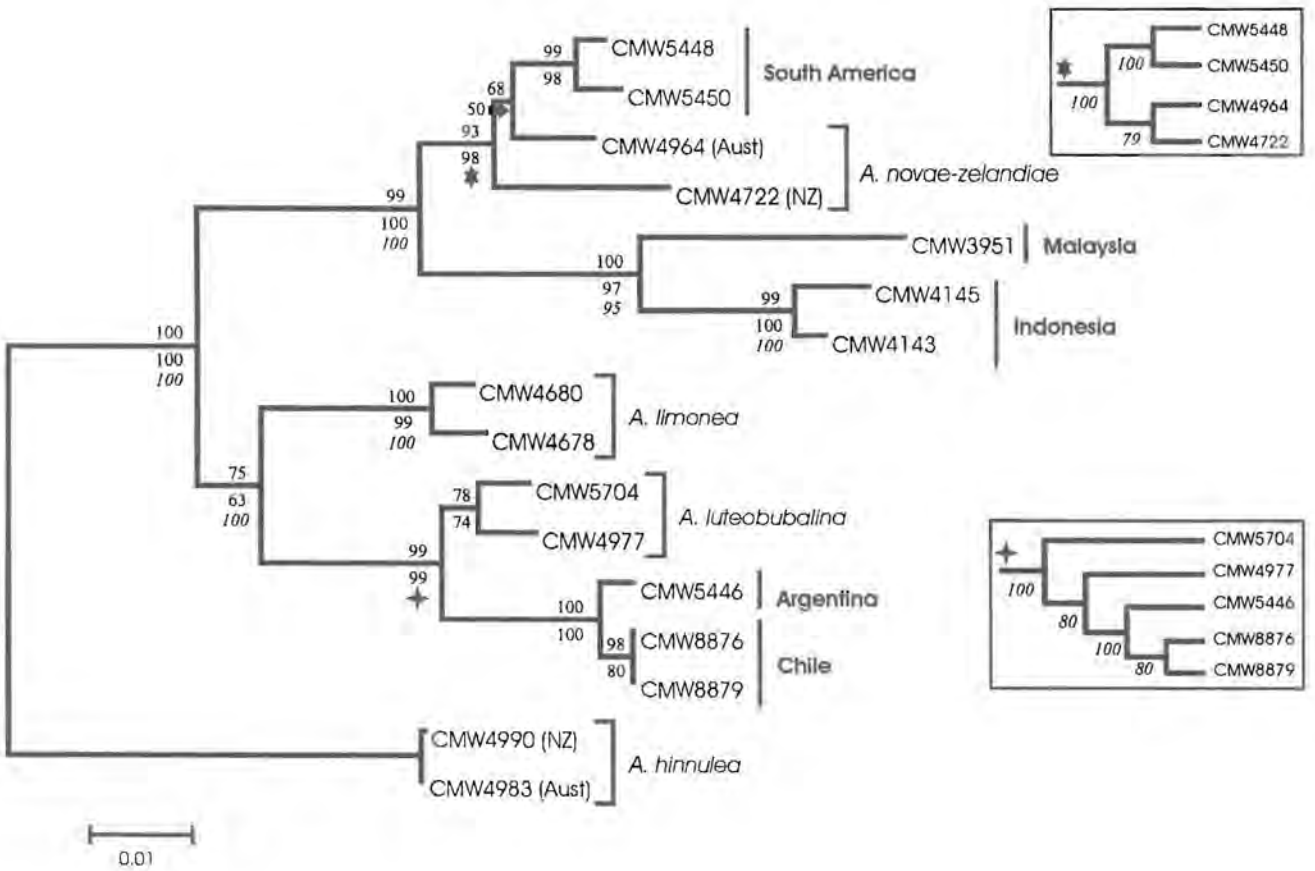
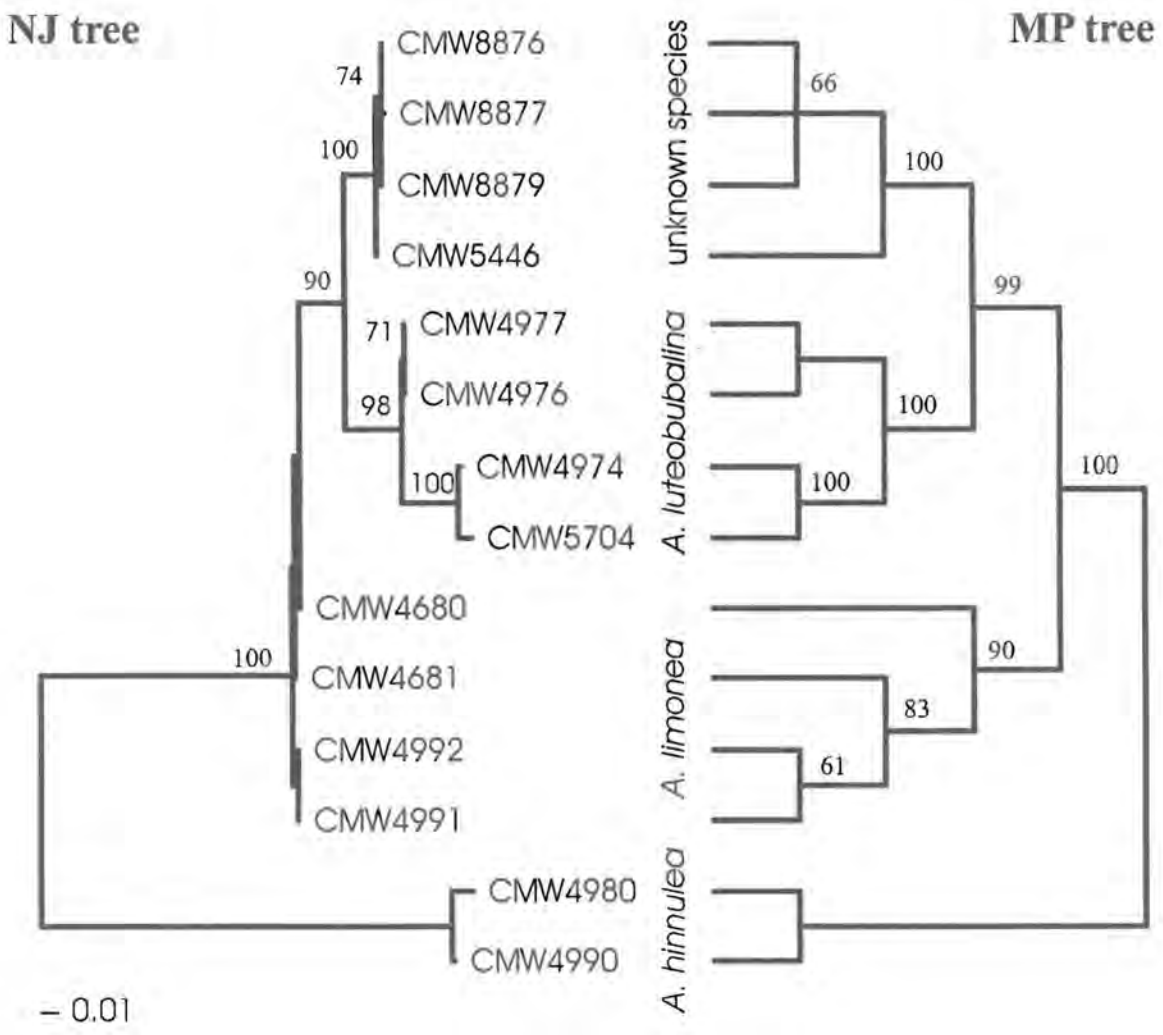

CMW5448
CMW5450
South America
CMW4964 (Aust)
CMW4722 (NZ)
A. novae-zelandiae
CMW3951
Malaysia
CMW4145
CMW4143
Indonesia
CMW4680
CMW4678
A. limonea
CMW5704
CMW4977
A. luteobubalina
CMW5446
Argentina
CMW8876
CMW8879
Chile
CMW4990 (NZ)
CMW4983 (Aust)
A. hinnulea
0.01
CMW5448
CMW5450
CMW4964
CMW4722
CMW5704
CMW4977
CMW5446
CMW8876
CMW8879

**Figure 2.** Neighbour-Joining and one of three MP trees generated from IGS-1 sequences with indels included and gaps treated as missing. Values above the branches are bootstrap-support values (1000 replicates) for the branching nodes. Number of parsimony-informative characters = 176, length of tree = 213, CI = 0.972 and RI = 0.979. Scale bar: 0.01 substitutions per site as determined in Neighbour-Joining analysis.


NJ tree
MP tree
CMW8876
CMW8877
CMW8879
CMW5446
CMW4977
CMW4976
CMW4974
CMW5704
CMW4680
CMW4681
CMW4992
CMW4991
CMW4980
CMW4990
74
100
90
71
98
100
100
– 0.01
unknown species
A. lufeobubalina
A. limonea
A. hinnulea
66
100
99
100
100
90
83
61

# CHAPTER FOUR

# A GLOBAL VIEW OF THE PHYLOGENY AND ORIGIN OF THE ROOT ROT PATHOGEN *ARMILLARIA*

# A GLOBAL VIEW OF THE PHYLOGENY AND ORIGIN OF THE ROOT ROT PATHOGEN *ARMILLARIA*

## ABSTRACT

*Armillaria* (Fr.:Fr.) Staude is a cosmopolitan plant pathogenic fungus that causes root rot in a large variety of primarily woody hosts. The phylogenetic relationships between *Armillaria* spp. from the Holarctic floral kingdom have been well studied. In contrast, very little is known regarding the relationships between species from the non-Holarctic (African, Australian, Indo-pacific and South American) floral kingdoms. The aim of this study was to determine the phylogenetic relationships among *Armillaria* spp. from the non-Holarctic and between these fungi and species from the Holarctic. An additional aim was to consider a previously presented hypothesis that *Armillaria* has a Gondwanan origin by estimating the time of divergence between the non-Holarctic and Holarctic *Armillaria* spp. Isolates included in this study originated from Africa, Asia, Australia, Europe, New Zealand, North America and South America. Analyses were based on DNA sequences from the large subunit (LSU) gene of the ribosomal RNA operon. Phylogenetic trees separated the species from the different floral kingdoms into two strongly supported clades representing the Holarctic and the non-Holarctic, respectively. Species in the non-Holarctic clade had a higher interspecific diversity than those from the Holarctic. Results suggest that the non-Holarctic *Armillaria* group is much older than the Holarctic and that the non-Holarctic species could have originated in Gondwana.

***Keywords:*** LSU, basidiomycetes, phylogeny, evolution.

## INTRODUCTION

Species of *Armillaria* (Fr.:Fr.) Staude (Basidiomycotina, Agaricales, Tricholomataceae) are well known plant pathogens that cause Armillaria root rot. *Armillaria* spp. are widely distributed, occurring in tropical, sub-tropical and temperate regions (Hood *et al.* 1991). They are also highly efficient at colonizing new areas owing to their ability to survive as pathogens, saprobes or necrotrophs on a wide variety of woody plants (Gregory *et al.* 1991, Hood *et al.* 1991, Kile *et al.* 1991, Fox 2000).

*Armillaria* has had a confused and controversial taxonomic history. Much of this confusion has since been resolved by integrating interfertility tests and DNA based identification techniques with conventional morphological classification systems. At present, at least 36 *Armillaria* spp. are known from tropical as well as temperate regions of the world (Volk and Burdsall 1995).

The first study using DNA sequences to compare species of *Armillaria* was published relatively recently by Anderson and Stasovski (1992). Subsequent phylogenetic studies based on ITS (Internal Transcribed Spacer) (Chillali *et al.* 1998b, Coetzee *et al.* 2001) and IGS-1 (Inter Genic Spacer) (Anderson and Stasovski 1992, Terashima *et al.* 1998, Coetzee *et al.* 2000b) sequence data as well as arbitrary primed primers (SWAPP) PCR (Piercey-Normore *et al.* 1998) on species from the Northern Hemisphere have resulted in a considerably enhanced understanding of the relatedness of these fungi. Much less is known regarding species in the Southern Hemisphere although recent phylogenetic studies dealing with species from Africa (Coetzee *et al.* 2000a), Australia (Coetzee *et al.* 2001, Dunne *et al.* 2002), New Zealand (Coetzee *et al.* 2001), South America (Coetzee *et al.* 2003), Indonesia and Malaysia (Coetzee *et al.* 2003) have been published. These studies deal with groups of species and individual areas, but a global analysis of the phylogeny of *Armillaria* spp. has never been attempted.

Nucleotide sequences from the variable spacer regions (IGS-1 and ITS) of the ribosomal RNA (rRNA) operon have provided an important source of data for *Armillaria* phylogenetics. However, these regions present difficulties for analyses that attempt to span the full diversity in *Armillaria*. The IGS-1 region, although extensively used in the past, was found to be inordinately variable for a robust phylogenetic analysis of *Armillaria* spp. from Australia and New Zealand (Coetzee *et al.* 2001). Moreover, the 5S gene of the African *Armillaria* spp. is

inverted in relation to the same gene in non-African species (Coetzee *et al.* 2000a), making it impossible to include sequence data from the IGS-1 in phylogenetic studies of species representing the entire genus. In comparison to the IGS-1 region, the ITS regions (ITS1 and ITS2) are more conserved but contain large indels (insertions / deletions) complicating phylogenetic analyses based on these regions (Coetzee *et al.* 2001). These difficulties have prompted the use of the more conserved large subunit (LSU) gene of the rRNA operon for a phylogenetic study of *Armillaria* spp. originating from both the Southern and Northern Hemispheres.

Recent studies using ITS and IGS-1 sequence data have shown that there is a higher level of inter- and intraspecific variation in the non-Holarctic *Armillaria* spp. than in the Holarctic species (Coetzee *et al.* 2001, Dunne *et al.* 2002, Coetzee *et al.* 2003). This correlates with the greater diversity of species that have been recorded from the non-Holarctic floral kingdoms when compared to the Holarctic. It has, therefore, been suggested that the non-Holarctic *Armillaria* group is older than that from the Holarctic and that *Armillaria* could have a Gondwanan origin (Dunne *et al.* 2002, Coetzee *et al.* 2003).

The primary aim of this study was to use DNA sequence data from the conserved LSU gene to determine relationships among a global collection of *Armillaria* spp. Thus phylogenetic relationships among known *Armillaria* spp. from the African, Australian, South American and Indo-Pacific Floral Kingdoms as well as between these species and those from the Holarctic Floral Kingdom were considered. In addition, the hypothesis that *Armillaria* might have had a Gondwanan origin was reconsidered by estimating the time of divergence between the non-Holarctic and Holarctic *Armillaria* spp.

## MATERIALS AND METHODS

### Taxon sampling

Taxa from the Holarctic were chosen to represent each of the species "clusters" described by Korhonen (1995). Species belonging to *A. ectypa* ("*A. ectypa* cluster") were exempted because cultures are not available for this species. Taxon sampling for species from the non-Holarctic Floral Kingdoms was complicated by a lack of cultures for some species reported from South

America. The southern floral kingdoms in this study are, therefore, represented mainly but not exclusively by species from Africa, Australia and New Zealand.

Isolates used in this study (Table 1) originated from a wide variety of hosts and continents and were collected by ourselves and by colleagues in various parts of the world. These isolates are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa. Duplicate cultures will be deposited with the Centraalbureau voor Schimmelcultures (CBS), Utrecht, Netherlands.

**Molecular techniques**

Isolates were grown in liquid MY (1.5% malt extract and 0.2% yeast extract) medium for two weeks at 22 °C in the dark. Mycelium was harvested by filtering through sterilized stainless steel mesh, lyophilized and ground to a fine powder in liquid nitrogen. DNA was extracted following to the method described by Coetzee *et al.* (2000b).

The LSU region was amplified with primers LR0R (Moncalvo *et al.* 2000) and LR11 (Hopple and Vilgalys 1999). The PCR reaction mixture included dNTPs (0.25 mM of each), buffer with $MgCl_2$ supplied by the manufacturer, additional $MgCl_2$ (0.25 mM), 0.1 µM of each primer, Expand™ High Fidelity PCR System enzyme mix (1.75 U) (Roche Diagnostics) and approximately 80 ng of template DNA. Reaction conditions were an initial denaturation at 96 °C (2 min), 35 cycles of primer annealing at 62 °C (30 s), elongation at 72 °C (1 min) and denaturation at 94 °C (30 s). A final elongation step was allowed at 72 °C for 5 min. PCR products were purified prior to sequencing with a QIAquick PCR Purification Kit (QIAGEN).

Sequences for both strands of the PCR products were obtained using an ABI PRISM™ 377 automated DNA sequencer. Sequence reactions were carried out using an ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA polymerase, FS (Perkin Elmer Applied Biosystems). Large subunit sequences were determined with primers LR0R, LR3R, LR5, LR6, LR7, LR8, LR9, LR11, LR14 and LR17R (Hopple and Vilgalys 1999, Moncalvo *et al.* 2000).

**Phylogenetic analyses with complete LSU gene data set**

The complete LSU sequence data set included divergent domains D1 to D8. Sequences from the LSU were aligned using Clustal X Version 1.8 (Thompson *et al.* 1997) and manually adjusted. Positions of the divergent domains in the LSU gene were determined by mapping their positions in relation to *Xenopus laevis, Saccharomyces carlsbergensis, Physarum polycephalum* and *Mus musculus* LSU sequences (Hassouna *et al.* 1984).

A limited number of D1 to D8 LSU sequences are available for the homobasidiomycetes, complicating the inclusion of various outgroup taxa from the Tricholomataceae. At present *Tricholoma matsutake* is the only member of the Tricholomataceae for which complete LSU sequence data (GenBank accession number U62964) is available (Hwang and Kim 2000). This species was, therefore, used as outgroup taxon in the analyses based on the D1 to D8 regions of the LSU.

The relationships among the non-Holarctic species and between these fungi and Holarctic species were determined based on parsimony and distance analysis within PAUP* version 4 (Swofford 1998). The first analysis incorporated the complete LSU data set. Maximum parsimony (MP) trees were obtained following a heuristic search with TBR (Tree Bisection Reconnection) branch swapping and MulTrees effective. Starting trees were obtained via stepwise addition with random addition of taxa (100 replicates). MaxTrees were set to auto-increase and zero length branches were collapsed. Successive weighting of characters according to their mean consistency index (Farris 1969) was applied to optimize the MP trees obtained after heuristic searches. This was done until the number of MP trees obtained after heuristic searches had stabilized. Gaps were treated as a fifth character (newstate). A Neighbour-Joining tree building algorithm (Saitou and Nei 1987) with a Kimura 2-parameter (Kimura 1980) substitution model was used to obtain trees in distance analysis. Support for tree nodes was determined using bootstrap analysis (1000 replicates) (Felsenstein 1985) using a heuristic search with TBR branch swapping, MulTrees effective, starting trees obtained via stepwise addition with simple addition of taxa and MaxTrees set to auto-increase and zero length branches collapsed.

### Phylogenetic analyses with D1 to D3 sequence data and outgroups from Tricholomacetaceae

Species of *Tricholoma* have been shown to be distantly related to *A. tabescens* (Moncalvo *et al.* 2000). Consequently, choosing *T. matsutake* as the outgroup taxon for the large part of the LSU could lead to the loss of phylogenetically informative characters. Thus a second analysis was performed based on sequences from the D1 to D3 regions that included the *Armillaria* spp. as well as species from clades D (*Marasmius pyrrocephalus*, AF042605 and *Rhodotus palmatus*, AF042565), E (*Baeospora myriadophylla*, AF042634 and *Hydropus scabripes*, AF042635), F (*Entoloma strictius*, AF042620 and *Macrocybe giganteum*, AF042625) (Moncalvo *et al.* 2000) and *T. matsutake*. Species in clade F and *T. matsutake* were used as outgroup taxa based on their phylogenetic relationship with species from clades D and E (Moncalvo *et al.* 2000). Phylogenetic trees were generated in this analysis using the same methods described above.

### Estimation of divergence times

Divergence times between the Holarctic and non-Holarctic *Armillaria* spp. were determined in a separate analysis. DNA sequences from the LSU gene of single isolates of *A. borealis* (EBS A), *A. ostoyae* (NABS I, EBS C), *A. nabsnona* (NABS IX), *A. gallica* (NABS VII), *A. gemina* (NABS II), *A. tabescens* and *A. mellea* (NABS VI) all of which are from the Holarctic region were included. The non-Holarctic group of isolates included a single isolate for each of the *Armillaria* spp. identified from Australia, New Zealand, South America and Africa. *Armillaria hinnulea* and the unknown *Armillaria* species from New Zealand (Hood 1992, Kile and Watling 1983) were, however, excluded from the data set due to their association with *Armillaria* spp. from the Holarctic (Coetzee *et al.* 2001). *Marasmius pyrrocephalus* and the distantly related *Entoloma strictiu* (Tricholomataceae) were included in the analysis. These taxa, together with *Armillaria,* formed the Euagaric clade, which also includes the 90 MY old mid-Cretaceous fossil *Archaeomarasmius* (Hibbett *et al.* 1997).

Binder and Hibbett (2002) suggested that the Boletales represent a sister group to the Euagarics clade. Sequences for *Boletus satanas* and *Scleroderma citrina* were, therefore, included as representatives of the boletes. The Hymenochaetoid fungi formed a clade distant to the euagarics-bolete sister group (Binder and Hibbett 2002). *Phelinus igniarius* from the Hymenochaetoid clade (Binder and Hibbett 2002) was, therefore, included as an outgroup taxon to the euagarics-bolete sister group in the present study. Trees were rooted to the outgroup *Thelephora* sp. that resides in the Thelephoroid clade, a basal group within the

homobasidiomycetes (Binder and Hibbett 2002). Phylogenetic trees were generated through an equally weighted parsimony analysis using the branch-and-bound algorithm in PAUP*. Missing data and ambiguously aligned regions were excluded prior to the analysis. Gaps were treated as missing.

One of the MP trees obtained from the above analysis was used to construct a user defined tree (see results section) in MacClade 3.08 (Maddison and Maddison 1992). Constrained branch-and-bound searches were performed as described above. The unconstrained and constrained trees were compared using the Shimodaira-Hasegawa test (Shimodaira and Hasegawa 1999) in PAUP*.

Evolutionary rate heterogeneity among lineages was tested for the LSU data set by using a maximum likelihood ratio test (LRT). Branch lengths were estimated for the tree obtained in the parsimony analysis using a HKY-Ggr substitution model with transistion/transversion ratio set to two, empirical nucleotide frequencies and an among site rate variation model with a discrete gamma distribution with four rate classes. Branch lengths and likelihood scores for trees with and without enforcement of the molecular clock were determined in the maximum likelihood analysis. The likelihood ratio between the two trees was determined with the test statistic being equal to twice the difference between likelihood scores which is $\chi^2$ distributed with $n - 2$ ($n$ equals the number of terminal taxa) degrees of freedom (Felsenstein 1981, Yang *et al.* 1995).

The LRT test indicated that there was rate heterogeneity amongst the lineages and the molecular clock was rejected. The tree obtained in the branch-and-bound search was, therefore, converted to an ultrametric tree by applying nonparametric rate smoothing (NPRS) (Sanderson 1997) using TreeEdit version 1. The node that split the euacarics from the boletes was calibrated at 90 MYA, based on *Archaeomarasmius*. A second calibration point was based on migration through the North Atlantic land bridge and final separation between North America and Europe during the Eocene, ~ 40 MYA (Graham 1999). The third calibration point was based on the latest connection between western Africa and eastern South America, ~ 120 MYA during the early Cretaceous, before the final opening of the South Atlantic (Filho *et al.* 2000). Confidence intervals for divergence dates were determined using the parsimony tree as a topologically constrained tree in a bootstrap analysis (100 replicates). Divergence times were then calculated for each of the 100 bootstrap trees within TreeEdit and the standard deviations were determined for the nodes.

# RESULTS

## PCR and sequencing

PCR products from the LSU gene that included the D1 to D8 regions were successfully obtained for all *Armillaria* isolates using primers LR0R and LR11. Single bands were observed on the agarose gel prior to cleanup and these were the same size for all isolates. Cleaning of the amplified PCR products often yielded multiple bands as a result of denaturation. High quality DNA sequence data was, however, obtained by direct sequencing of the cleaned PCR products.

## Phylogenetic analyses with complete LSU gene data set

The total number of characters after alignment and inclusion of gaps in this data set was 2508. Eighteen missing or ambiguously aligned characters were excluded from the data matrix. A total of 330 variable characters were obtained with 164 being parsimony informative.

Parsimony analysis of the complete data set yielded four MP (equally most parsimonious) trees before successive character weighting. The number of trees was reduced to two MP trees with similar overall topology after weighting characters. The length of these trees was 389 steps. The *CI* (consistency index) and *RI* (retention index) were 0.802 and 0.758, respectively.

The MP tree (Fig. 1a) obtained in this analysis placed *A. mellea* (from Europe and North America) basal to all the other *Armillaria* spp. (73% bootstrap support). The remainder of the *Armillaria* spp. grouped into two major clades. The Holarctic *Armillaria* spp. as well as *A. hinnulea* and the unidentified *Armillaria* sp. from New Zealand resided in one clade (77% bootstrap support). The non-Holarctic species resided in a second clade, but with lower bootstrap support (67%).

In this analysis the Australian, New Zealand, Indo-Malaysian and South American taxa formed a monophyletic group with a 76% bootstrap support. *Armillaria pallidula* and *A. fumosa* formed a strongly supported monophyletic clade (100% bootstrap support). Isolates representing *A. luteobubalina* from Chile and Argentina grouped together and formed a sister clade with an isolate of the same species from Australia. The relationship between *A. limonea*, *A. luteobubalina* and the *A. pallidula* - *A. fumosa* group was not clear as a consequence of low bootstrap support at the nodes of the MP tree. Isolates representing *A. novae-zelandiae* from

New Zealand and Chile grouped together and formed a sister group with isolates from Malaysia and Indonesia (100% bootstrap support). *Armillaria fuscipes* and *A. heimii*, from Africa, formed sister taxa (100% bootstrap support) and together they were placed basal to the non-Holarctic *Armillaria* spp. *Armillaria hinnulea* and the unknown species from New Zealand from the non-Holarctic floral kingdoms were placed paraphyletically within the Holarctic clade.

Two sister sub-clades, although not well supported by bootstrap, were observed in the Holarctic clade. The first sub-clade included *A. borealis, A. gemina* and *A. ostoyae* as well as the unknown species from New Zealand (51% bootstrap support). *Armillaria gallica, A. nabsnona* and *A. cepistipes* grouped together in the second sub-clade (65% bootstrap support). *Armillaria tabescens* was placed basal to the two sister clades (72% bootstrap support) and *A. hinnulea* basal to *A. tabescens* (62% bootstrap support).

The NJ (Neighbour-Joining) tree (Fig. 1b) generated from the complete LSU data set resembled the MP trees obtained from the same data set. Thus, the Holarctic *Armillaria* spp. were clearly separated from the non-Holarctic species. Branches connecting the terminal nodes of the non-Holarctic *Armillaria* spp., as well those for *A. mellea*, were long and branches connecting the deeper nodes were very short. In contrast, short branches connected the terminal and deeper nodes of the Holarctic *Armillaria* spp., *A. hinnulea* and the *Armillaria* sp. from New Zealand.

The relationships among *Armillaria* spp. from the non-Holarctic Floral Kingdom were difficult to determine as a result of low bootstrap support at the nodes that separate species in the NJ tree (Fig. 1b). *Armillaria pallidula* and *A. fumosa* formed highly supported sister taxa (100% bootstrap support) and grouped in a cluster together with *A. luteobubalina* and *A. limonea* (62% bootstrap support). *Armillaria novae-zelandiae* and the *Armillaria* sp. from Malaysia and Indonesia formed two highly supported sister groups; their association with the non-Holarctic species, however, did not have bootstrap support. The two African species, *A. fuscipes* and *A. heimii*, as well as *A. mellea* (from Europe and USA) formed monophyletic groups (100% bootstrap support), respectively but their phylogenetic relationships with the non-Holarctic species were not resolved.

The NJ tree generated in this analysis (Fig. 1b) was consistent with the MP tree (Fig 1a), grouping the Holarctic species, together with *A. hinnulea* and the unknown species from New Zealand, in a strongly supported cluster (84% bootstrap support). Two sister sub-clusters were

obtained within the Holarctic clade; the first included *A. borealis*, *A. gemina* and *A. ostoyae* (89% bootstrap support) and the second *A. gallica*, *A. nabsnona* and *A. cepistipes* (77% bootstrap support). In contrast to the MP tree, the unknown *Armillaria* sp. from New Zealand clustered outside the group accommodating the Holarctic *Armillaria* spp.

**Phylogenetic analyses with D1 to D3 sequence data and outgroups from Tricholomacetaceae**

The data matrix for analysis of the relationships between *Armillaria* spp. from the Holarctic and the non-Holarctic included 913 characters after alignment and inclusion of gaps. Missing and ambiguously aligned characters were excluded, resulting in a total of 234 variable characters. There were thus 157 parsimony informative characters.

A heuristic search yielded 16 MP trees before successive character weighting. Four MP trees were obtained with tree lengths of 310 steps after re-weighting of characters. The *CI* and *RI* for these trees were 0.740 and 0.762, respectively. Topological differences among the trees were observed with regard to the placement of the unknown species from New Zealand relative to the rest of the Holarctic species.

The *Armillaria* spp. included in this analysis formed a strongly supported monophyletic group (98% bootstrap support) with *A. mellea* from Europe and North America as the basal taxon (69% bootstrap support) (Fig. 2a). The MP tree grouped the remaining *Armillaria* spp. into two well-supported clades separating the Holarctic and the non-Holarctic species. In this smaller data set, the non-Holarctic *A. hinnulea* remained in the Holarctic clade and was basal to the rest of the taxa in this clade (64% bootstrap support). *Armillaria tabescens* had a position basal to the remaining Holarctic *Armillaria* spp. with a 56% bootstrap support. The unknown *Armillaria* sp. from New Zealand remained in the Holarctic clade but the relationship between this taxon and the remaining taxa in this clade could not be resolved.

*Armillaria heimii* and *A. fuscipes* formed a basal clade to the non-Holarctic group (74% bootstrap support). In this analysis *A. luteobubalina* from Chile and Argentina formed a basal group to *A. limonea*, with this taxon basal to *A. luteobubalina* from Australia. This relationship, however, did not have strong bootstrap support. *Armillaria novae-zelandiae* from Chile and New Zealand formed a strongly supported sister group with unidentified *Armillaria* isolates from

Indonesia and Malaysia. *Armillaria luteobubalina* was placed basal to these sister groups (79% bootstrap support).

The NJ trees (Fig. 2b) generated using DNA sequence data that included only the D1 to D3 regions, resembled the NJ tree in the previous analysis in terms of the lengths of branches connecting the terminal nodes and deeper nodes. The topology of the NJ tree (Fig. 2b) generated in this analysis was also congruent with the MP tree obtained with the whole LSU data set. Low bootstrap support was obtained for many of the branching nodes, making the phylogenetic relationships between taxa uncertain. In this analysis, the Holarctic species together with *A. hinnulea* and the unidentified *Armillaria* sp. from New Zealand grouped within a strongly supported monophyletic cluster (83% bootstrap support). The phylogenetic relationships between the non-Holarctic species reflected relationships shown in the MP tree with the whole LSU. In this analysis, the non-Holarctic *Armillaria* spp., with the exception of *A. fuscipes* and *A. heimii*, clustered in a monophyletic group (61% bootstrap support). The relationship between *A. mellea* and the two African species with the remaining non-Holarcic species could not be resolved due to low bootstrap support at the branching nodes.

### Estimation of divergence times

The LSU data set for molecular clock analysis was 875 characters of which 147 were parsimony informative. A branch-and-bound search generated three MP trees of 374 steps, $CI = 0.548$ and $RI = 0.626$. *Armillaria mellea* was placed as basal group to the non-Holarctic species of *Armillaria* in all three trees (Fig. 3) resulting from this search. The position of this species is in contrast to its phylogenetic relationships illustrated in other parts of this study. A constraint tree was, therefore, created where *A. mellea* was placed basal to the rest of the *Armillaria* spp. Parsimony analysis, with this tree topology enforced in a branch-and-bound search of the data set, resulted in a MP tree with a length of 376 steps, $CI = 0.545$ and $RI = 0.626$. Comparison between the constrained and unconstrained trees using the Shimodaira-Hasegawa ($P = 0.197$) test indicated that the differences in topology between the constrained and unconstrained trees were not significant. The constraint tree could thus not be rejected and it was used in further analyses. Maximum likelihood estimation of branch lengths was conducted on the tree depicted in Fig. 4. The likelihood score without the molecular clock enforced was –log 1734.98790 and with the molecular clock enforced, –log 1764.29152. The difference between the two trees was significant according to the LR test ($P < 0.05$) and the molecular clock was, therefore, rejected.

Absolute ages of divergence for nodes in Fig. 4 are given in Table 2. Bootstrap analysis on the tree with branches calibrated with the split between the Boletes and the Euagarics (90 MYA, node A, Fig. 4) and the separation between Africa and South America (120 MYA, node G, Fig. 4) gave smaller standard deviations than those calibrated with the latest connection between North America and Europe (40 MYA, node J, Fig. 4). The bootstrap trees obtained with the 40 MYA calibration point exhibited a high standard deviation; the resulting times of divergence were therefore considered unreliable, and they were rejected.

## DISCUSSION

This is the first study in which a large group of *Armillaria* spp. from different continents and hemispheres was subjected to phylogenetic analysis. Past phylogenetic studies of *Armillaria* have focused on the relationships among species from the Holarctic (Anderson and Stasovski 1992, Chillali *et al.* 1998b, Piercey-Normore *et al.* 1998, Terashima *et al.* 1998, Coetzee *et al.* 2000b), the African (Coetzee *et al.* 2000a), the Australian (Coetzee *et al.* 2001, Dunne *et al.* 2002) and South American (Coetzee *et al.* 2003) Floral Kingdoms. Coetzee *et al.* (2001), however, published preliminary results based on ITS sequence data for species from the Holarctic, Australian and African Floral Kingdoms and suggested that the Australian and African species form a group basal to those from the Holarctic Floral Kingdom. Results of the present study are the first to incorporate taxa from the world's six floral kingdoms (Cox 2001) and they confirm some of the findings of Coetzee *et al.* (2001).

### *Armillaria mellea* as basal taxon within *Armillaria*

Results from this study suggest that *A. mellea* (NABS VI / EBS D) is a basal taxon within *Armillaria*. This species is widely distributed and common to all areas in the Holarctic Floral Kingdom (Kile *et al.* 1994). At the interspecific level, *A. mellea* differs greatly from the rest of the Holarctic *Armillaria* spp. These differences pertain to macro-morphology (Bérubé and Dessureault 1988, Watling *et al.* 1982), absence of clamp connections in the suprapellis at the base of the basidia (Watling *et al.* 1982), large insertion (2.5 kb) in the rDNA repeat unit (Anderson *et al.* 1989), a larger number of shared base substitutions in anonymous nucleotide sequences (Piercey-Normore *et al.* 1998) and a shorter IGS-1 region (Harrington and Wingfield

1995). At the intraspecific level, this species varies in its mating strategy in having either a heterothallic or a homothallic life cycle (Korhonen 1978, Abomo-Ndongo *et al.* 1997, Ota *et al.* 1998). Furthermore, high levels of intraspecific variation have been observed in IGS-1 and ITS sequences for isolates from Europe, western and eastern North America and Asia and these can be separated according to their geographical origin (Coetzee *et al.* 2000b). The fact that *A. mellea* is widely distributed and shows a high degree of inter- and intra-specific variation, together with the grouping of this species basal to the rest of the *Armillaria* spp. included in this study, suggests that *A. mellea* represents a species ancestral to others from the Holarctic.

The fact that this study's results designate *A. mellea* as a basal taxon to species from the non-Holarctic Floral Kingdoms is interesting. *Armillaria mellea* is not commonly found in the non-Holarctic Floral Kingdoms, although it has been reported from Africa (Saô Thomé, Ethiopia, Tanzania and Kenya) (Mohammed *et al.* 1988, Ota *et al.* 2000). However, this collection closely resembles *Armillaria* spp. from the non-Holarctic in sharing the absence of basal clamp connections (Fig. 5a). With the exception of *A. procera* from South America, these structures are absent in *Armillaria* spp. described from Australia, New Zealand, Africa and South America, and they are rare in *A. fellea* from Australia and New Guinea, (Singer 1969, Kile and Watling 1983, 1988).

Unusually high variation was observed in the complete LSU sequence at the intra-specific level for isolates of *A. mellea* from California and Britain. Variation among these isolates was at least five times greater than that among isolates of *A. novae-zelandiae* from Australia and Chile and 1.5 times greater than among isolates of *A. luteobubalina* from these countries (data not shown). At the intra-specific level, variation within *A. mellea* was 2.75 times greater than within the Holarctic clade and 2.09 times less than the non-Holarctic clade (data not shown). The fact that there is more intra-specific variation between *A. mellea* from North America and Europe than between the two non-Holarctic species from distant continents suggests strongly that this is a very old species. The similarity between *A. mellea* and the non-Holarctic species in terms of absence of basal clamp connections, together with the high level of genetic variation within this species, is also consistent with the grouping of *A. mellea* isolates basal to the *Armillaria* spp. from the non-Holarctic Floral Kingdoms.

**Phylogenetic relationships among non-Holarctic *Armillaria* species**

Trees generated based on parsimony analysis of the complete sequence data set for the non-Holarctic *Armillaria* spp. yielded the highest bootstrap support values at nodes and high *CI* and *RI* values. The topology of these trees was furthermore similar to a previously published parsimony tree based on ITS sequence data (Dunne *et al.* 2002). The most parsimonious tree for the complete data set was, therefore, considered the best tree for inferring phylogenetic relationships amongst the non-Holarctic *Armillaria* spp.

*Armillaria hinnulea* and the undescribed species from New Zealand (Coetzee *et al.* 2001), both of which occur only in the non-Holarctic Floral Kingdoms, grouped within the Holarctic clade. *Armillaria hinnulea* occurs in Australia (including Tasmania) and New Zealand, and the undescribed species is from central North Island in New Zealand (Hood 1992, Kile and Watling 1983). The position of these two species in relation to the *Armillaria* spp. in the Holarctic clade is not entirely clear, but parsimony analyses on both data matrices show that *A. hinnulea* is basal to species from the Holarctic Floral Kingdom. The grouping of *A. hinnulea* and the undescribed species based on LSU sequence data is consistent with previous phylogenetic studies based on ITS sequence data (Coetzee *et al.* 2001, Dunne *et al.* 2002). Dunne *et al.* (2002) suggested that *A. hinnulea* is either related to a Holarctic *Armillaria* sp. that evolved in Gondwana or that it evolved from a common ancestor. Results of the parsimony analysis in the present study support the radiation of *A. hinnulea* and the Holarctic species from a common ancestor.

The inter-specific genetic diversity for the conserved LSU gene within the African group (*A. fuscipes* and *A. heimii*) was approximately the same as that amongst the rest of the species from the non-Holarctic Floral Kingdom. *Armillaria fuscipes* and *A. heimii* were previously thought to represent the same species (Kile and Watling 1988, Pegler 1986). Coetzee *et al.* (2000a) later showed that there is a clear sequence divergence in the IGS-1 region between isolates thought to be *A. heimii* and that they represent two distinct groups. The very high level of genetic diversity between *A. heimii* and *A. fuscipes* observed in this study further confirms the findings of Coetzee *et al.* (2000a) that these species represent discrete taxa.

All phylogenetic trees in this study suggest that the African taxa (*A. fuscipes* and *A. heimii)* are basal to the rest of the non-Holarctic species. *Armillaria heimii* and *A. fuscipes* are widespread over the African continent but also in Madagascar (reported as *Clitocybe elegans*) (Heim 1963), Sri Lanka (Petch 1909) and New Guinea (Heim 1967). At the micro-morphological level, these

taxa share features with the non-Holarctic species, such as the absence of clamp connections (Fig. 5a). They differ, however, from the *Armillaria* spp. from Australia, New Zealand and South America in being either homothallic or heterothallic with a bipolar unifactorial sexual incompatibility system (Fig. 5b). By contrast, the remainder of non-Holarctic species are heterothallic but with a tetrapolar bifactorial sexual incompatibility system (Kile and Watling 1988, Abomo-Ndongo *et al.* 1997). At the molecular level, *A. heimii* and *A. fuscipes* are the only *Armillaria* species with a 5S gene that is in the inverted orientation relative to the other genes in the rDNA operon (Coetzee *et al.* 2000a) (Fig. 5c). *Armillaria fuscipes* and *A. heimii* are clearly unique in many respects. This gives us confidence that their placement in phylogenetic trees as basal to the other species from the non-Holarctic floral kingdoms is a true reflection of their relationship with other species.

In this study, we have shown that the *Armillaria* isolates from Indonesia and Malaysia and those representing *A. novae-zelandiae* from Chile and New Zealand are closely related. Isolates from Chile and New Zealand as well as those from Indo-Malaysia, however, grouped in two strongly supported sister groups. This relationship is consistent with the findings of Coetzee *et al.* (2003) who, based on ITS sequence data, tentatively identified the Indo-Malaysian isolates as *A. novae-zelandiae*. LSU sequence variation between the Chile-New Zealand and Indo-Malaysian groups was also greater than that between *A. novae-zelandiae* from Chile and New Zealand. There are two possible explanations for this observation. One possibility is that the *Armillaria* sp. from Indonesia and Malaysia is in the process of speciation. This idea gains credibility from the fact that they have been geographically separated from the New Zealand group for an extended period of time. Another possibility is that they represent a species closely related to, but different from, *A. novae-zelandiae*. This question will only be resolved when basidiocarps representing the isolates from Indonesia and Malaysia are found and when an identification based on basidiocarp morphology and mating compatibility can be made.

Parsimony analysis of the complete LSU data set placed *A. novae-zelandiae* and the *Armillaria* sp. from Indo-Malaysia as a sister group to other *Armillaria* spp. from Australia, South America and New Zealand. This relationship was reflected in the topology of the Neighbour-Joining trees generated from both data sets, although it had low bootstrap support. Dunne *et al.* (2002) presented similar results based on parsimony analysis of ITS sequence data, and our results lend credence to the existence of this relationship.

*Armillaria novae-zelandiae* displays a number of characteristics that support a very old origin and the basal position of this species to *A. luteobubalina*, *A. limonea*, *A. fumosa* and *A. pallidula*. This species is the most widely distributed non-Holarctic species, occurring in eastern Australia, New Zealand, Papua New Guinea and in South America (Stevenson 1964, Singer 1969, Kile and Watling 1983, Guillaumin *et al.* 1992). It is pathogenic on various hosts, including *Nothofagus* spp., a genus that was widely distributed in the southern continents prior to the fragmentation of Gondwana (MacKenzie and Shaw 1977, Shaw and Calderon 1977, Kile 1980, 1983). There are a number of indels in the ITS sequences of the isolates of this species from Australasia and South America, suggesting that they have been geographically separated for a long time (Coetzee *et al.* 2003). In the present study, six nucleotide substitutions were also observed in the much more conserved LSU sequences between *A. novae-zelandiae* isolates from South America and New Zealand. This lends further support to the view that isolates of this species have been geographically separated for an extended period of time.

*Armillaria luteobubalina* was thought to be restricted to Australia, where it occurs transcontinentally. Based on comparisons of ITS and IGS-1 sequence data, however, Coetzee *et al.* (2003) identified isolates from Argentina and Chile as *A. luteobubalina* In the present study, *A. luteobubalina* from South America and Australia grouped in a monophyletic group providing additional evidence that the isolates from South America and the Australia are conspecific. A high level of intra-specific LSU sequence variation was, however, observed between the South American and the Australian isolates of this species This provides cogent evidence for the view of Coetzee *et al.* (2003), that this is an ancient species with a Gondwanan origin.

The relationships between *A. luteobubalina*, *A. limonea*, *A. pallidula* and *A. fumosa* are not well-defined, based on LSU sequence data, due to low or no bootstrap support at nodes. Although these relationships are not clear, both distance and parsimony analyses on the complete LSU data set generated phylogenetic trees suggesting that *A. luteobubalina* forms a sister group to *A. limonea*, *A. pallidula* and *A. fumosa*. Furthermore, *A. fumosa* and *A. pallidula* formed a sister group and are very closely related. These two species were previously shown to be phylogenetically closely related and could not be separated based on ITS sequence data (Coetzee *et al.* 2001). The topology of the sub-clade, including *A. luteobubalina*, *A. limonea*, *A. pallidula* and *A. fumosa*, is generally isomorphic to that of Coetzee *et al.* (2001) with the exception that these authors found *A. novae-zelandiae* to form a sister group to the *A. pallidula* – *A. fumosa* group.

**Phylogenetic relationship among the Holarctic *Armillaria* species**

Phylogenetic analyses indicated that *A. tabescens* is distantly related to the rest of the *Armillaria* spp. from the Holarctic floral kingdom. This species is restricted to Europe, North America and Japan, where it is a saprophyte or weak pathogen on trees and other plants (Kile *et al.* 1994). Amongst the Northern Hemisphere species, *A. tabescens* is very characteristic in having an exannulated stipe (Pegler 2000). The only other species having this characteristic is *A. ectypa*, but this species is homothallic, and found only in peat-bogs (Zolciak *et al.* 1997). The placement of *A. tabescens* distant and basal to the rest of the Holarctic species, in the present study, is also consistent with previous reports based on IGS-1 (Anderson and Stasovski 1992) and ITS (Chillali *et al.* 1998b) sequence data.

Chillali *et al.* (1998b) suggested that there is a close association between *A. mellea* and *A. tabescens* and that both species form basal taxa to the species from the Holarctic. Their study, however, was based only on species from Europe and did not include species from other regions. It is possible that the inclusion of non-Holarctic species in the present study resulted in an increase in synapomorphic characters between *A. tabescens* and the Holarctic species. Differences in morphology of the species as well as IGS-1 and ITS sequences, lead us to believe that phylogenetic trees generated from parsimony analyses in this study, reflect a true relationship between *A. tabescens* and the strictly Holarctic species and that it is an ancestral species. Isolates of *A. ectypa* were not available for this study, but it would be interesting to determine the relationship of this species to the Holarctic *Armillaria* spp. based on LSU sequence data.

*Armillaria* spp. from the Holarctic were characterized by low overall inter-specific DNA sequence divergence, which resulted in low resolution between tree nodes that separated the species. The Holarctic species, however, clustered in two groups based on distance and parsimony analyses of the complete LSU sequence data set. The first group included *A. borealis*, *A. gemina* and *A. ostoyae* while the remaining species, *A. gallica, A. nabsnona* and *A. cepistipes*, resided in the second group. This bipartition of Holarctic *Armillaria* spp. is in agreement with previous phylogenetic studies using the ITS and IGS-1 rDNA regions, have indicated that *A. borealis*, *A. ostoyae* and *A. gemina* are closely related and separate from the rest of the *Armillaria* spp. from Europe and North America (Anderson and Stasovski 1992, Chillali *et al.* 1998a, b).

### Phylogenetic relationship between the Holarctic and non-Holarctic *Armillaria* species

Phylogenetic trees obtained in this study showed that species of *Armillaria* could be separated into two clades that represent the Holarctic and the non-Holarctic Kingdoms. This dichotomy is supported by the presence of basal clamp connections in the basidia of the species from the Holarctic and the absence of these structures in species from the non-Holarctic Floral Kingdoms (Fig. 5a). An interesting observation emerging from this study was that species from the Holarctic, although widely distributed and morphologically variable, had a very low inter-specific genetic diversity. In contrast, species from the more southern Floral Kingdoms showed great inter-specific genetic diversity, suggesting that the *Armillaria* spp. from the non-Holarctic Floral Kingdoms are much older than those from the Holarctic.

### Dating the divergence between Holarctic and non-Holarctic *Armillaria* species

Divergence dates obtained for species from the different floral kingdoms, using three different calibration points, confirmed that the species from the non-Holarctic are much older than those from the Holarctic. Radiation from the most recent common ancestor (MRCA) and diversification of *Armillaria* spp. from the non-Holarctic must, therefore, have occurred earlier than between species from the Holarctic, resulting in greater numbers of taxa in the non-Holarctic. This is reflected in the greater variety of extant *Armillaria* spp. reported from the non-Holarctic Kingdoms when compared with the Holarctic Floral Kingdom (Volk and Burdsall 1995).

Calibration with *Archaeomarasmius* yielded very recent divergence dates between the *Armillaria* spp. from the various Floral Kingdoms. With this calibration point, the MRCA of *Armillaria* was dated at 39 (±3) MYA, placing it between the late Eocene (54 – 38 MYA) and early Oligocene (26 – 38 MYA). Furthermore, the divergence between species such as *A. novae-zelandiae* that occur on more than one continent, were found to be younger than 23 MY. During these times, the position of modern continents was already established as a result of continental drift. If ages based on the fossil record are taken as correct, distribution of extant non-Holarctic *Armillaria* spp. that are shared between continents must have been the result of long distance dispersal by basidiospores. This mechanism of population distribution is rare for fungi but has been shown to occur in some species (Nagaraja and Singh 1990, Et-touil *et al.* 1999, Fisher *et al.* 2001, Hibbett 2001, Brown and Hovmøller 2002). Long distance dispersal, as an explanation for the spread of *Armillaria* spp. from the non-Holarctic, is highly improbable due to the great

distance between countries such as New Zealand and South America. We do not believe that it would account for the number of polymorphic sites observed between isolates of the same species from these continents.

The geographic radiation of *Armillaria* spp. is perhaps best explained by vicariance events such as continental drift. If this is the case, the MRCA of *Armillaria* must have a Gondwanan origin. To test this hypothesis nodes were calibrated against the split between Africa and South America (~120 MYA). Using this calibration point, the radiation between the Euagarics and the Boletes was estimated to have occurred at 330 (±24) MYA in the Carboniferous period. This time of divergence is approximately 3.5 times older than that of the fossil *Archaeomarasmius leggettii* (Hibbet *et al.* 1995) which, like *Armillaria*, is a member of the Tricholomataceae. It also predates the oldest holobasidiomycetous fossils, *Palaeancistrus martinii* and *Palaeosclerotium pusillum*, from Pennsylvanian (~300 MYA) (Dennis 1970, 1976). Berbee and Taylor (2001) indicated that the radiation between the basidiomycetes and the ascomycetes occurred in the Paleozoic (~ 500 MYA), but it was later shown to have occurred between 1400 to 1200 MYA in the Precambrian by (Heckman *et al.* 2001). It is thus possible that the divergence between the boletes and the euagarics is older than reflected in the fossil record, if the earlier dates presented by Heckman *et al.* (2001) are taken as correct.

Nodes calibrated based on the separation of South America and Africa dated the MCRA of *Armillaria* at 142 (±9) MYA and the radiation of the Holarctic and non-Holarctic species at 132 (±6) MYA during the early Cretaceous (144 - 112 MYA). At this time, the fragmentation of Gondwanaland had already begun with rifting between the east (South America and Africa) and west (Antarctica, Australia, India and New Zealand) of this southern landmass (~ 150 MYA) (Wilford and Brown 1994). This calibration point is, however, very conservative since it is based on the latest connection between Africa and South America and gives the latest possible divergence dates at nodes. It is thus reasonable to assume that the radiation from the MRCA and divergence between the Holarctic and non-Holarctic species must have occurred earlier than the estimated date, possibly in the late Jurassic period (157 – 152 MYA).

Placing the radiation from the MRCA back to the late Jurassic period, or even earlier, concurs with the Gondwanan origin hypothesis for *Armillaria* spp. Furthermore, it correlates with results obtained from distance analyses in this study. The deeper nodes of taxa from the non-Holarctic

Floral Kingdom are connected by very short branches in the NJ trees, suggesting a rapid ancient radiation from a common ancestor. Long branches connecting internal nodes with terminal nodes of the extant non-Holarctic taxa suggest that they have evolved separately over a long period of time. In contrast, the Holarctic species have short branches connecting the terminal nodes with internal nodes in the NJ tree, suggesting that they evolved more recently from their common ancestor. These observations, together with the dating and biogeography of the non-Holarctic *Armillaria* spp., suggest that they or their progenitors evolved in Gondwana and spread to modern continents before these drifted apart. Data further suggests that the occurrence of non-Holarctic species such as *A. novae-zelandiae* and *A. luteobubalina* on different continents has resulted from continental drift.

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**TABLE 1:** *Armillaria* isolates for which LSU sequences were determined

| Species | Isolates nr | Alternative nr | Host | Country | Collected by |
|---|---|---|---|---|---|
| *A. borealis* | CMW3172 | B370 | Unknown | Finland | K Korhonen |
| *A. cepistipes* | CMW6909 | 33 | Unknown | USA (or Canada) | Morrison |
| *Armillaria* sp. | CMW4993 | 4698/9 | *Nothofagus* sp. | New Zealand | GS Ridley & JF Gardener |
| *A. fumosa* | CMW4955 | 123.1 | Basidiocarp on *Eucalyptus* sp. | Australia | GA Kile |
| *A. fuscipes* | CMW4953 | LR2 | *Pelargonium asperum* | La Reunion | C Fabregue |
| *A. gallica* | CMW6902 | 22 | Black elm | USA | M Banik |
| *A. gemina* | CMW6888 | 5 | Jack pine | USA | M Banik |
| *A. heimii* | CMW4873 | Z1 | *Brachystegia utilis* | Zimbabwe | M Ivory |
| *A. hinnulea* | CMW4980 | 119 | Basidiocarp on *Eucalyptus obliqua* | Australia | RH Peterson |
| *A. limonea* | CMW4680 | C3.28.0.1 | Rhizomorphs from *Beilschmiedia tawa* forest | New Zealand | IA Hood |
| *A. luteobubalin* | CMW4977 | SA6 | Unknown | Australia | Unknown |
| *A. luteobubalina* | CMW5448 | 7348/10 | *Nothofagus* log | Argentina | RH Peterson |
| *A. luteobubalina* | CMW8876 | Chile-1 | *Pinus radiata* | Chile | MJ Wingfield |
| *A. mellea* | CMW4603 | B253, KJS-6PS | Unknown | UK | S. Gregory |
| *A. mellea* | CMW4609 | B623 | Unknown | USA | PJ Zambino |

TABLE 1 (continued)

| Species | Isolates nr | Alternative nr | Host | Country | Collected by |
|---|---|---|---|---|---|
| *A. nabsnona* | CMW6905 | 28 | Unknown | USA | M Banik |
| *A. novae-zelandiae* | CMW4722 | G3.0.34.4 | Rhizomorphs from *B. tawa* forest | New Zealand | IA Hood |
| *A. novae-zelandiae* | CMW5448 | 7365/2 | *Nothofagus* log | Chile | RH Peterson |
| *A. novae-zelandiae* | CMW4143 | | *E. grandis* | Indonesia | MJ Wingfield |
| *A. novae-zelandiae* | CMW3951 | O-1 | *Acacia mangium* | Malaysia | MJ Wingfield |
| *A. ostoyae* | CMW3162 | B481 | *Abies balsamea* | USA | J Anderson |
| *A. pallidula* | CMW4968 | 3626, ATCC66124 | *Pinus caribaea* var. *hondurensis* | Australia | P Gordon |
| *A. tabescens* | CMW3165 | B531, TAB2 | Unknown | France | JJ Guillaumin |

TABLE 2: Estimated dates of divergence of nodes.

| Node | Branch length | Date[1] | Std[2] | Date | Std | Date | Std |
|---|---|---|---|---|---|---|---|
| A | 102.7612 | ***90*** | na | 330 | 24 | 181 | 74 |
| B | 57.0206 | 50 | 3 | 183 | 11 | 100 | 41 |
| C | 44.1884 | 39 | 3 | 142 | 9 | 78 | 31 |
| D | 41.1365 | 36 | 3 | 132 | 6 | 72 | 29 |
| E | 14.2997 | 13 | 8 | 46 | 28 | 25 | 24 |
| F | 4.3064 | 4 | 4 | 14 | 15 | 8 | 10 |
| G | 37.3158 | 33 | 3 | ***120*** | na | 66 | 27 |
| H | 26.6132 | 23 | 3 | 86 | 7 | 47 | 20 |
| I | 16.5891 | 15 | 3 | 53 | 9 | 29 | 13 |
| J | 22.7386 | 20 | 7 | 73 | 22 | ***40*** | na |

[1]Figures in bold and italics are calibration points for branches.

[2]Standard deviation.

**Figure 1.** Phylogenetic trees generated from D1 to D8 LSU gene sequence data. a) Cladogram, b) Neighbor-Joining tree. Numbers above tree branches in the cladogram indicate branch-length. Asterisks denote branches that collapsed in a strict consensus tree obtained from MP trees before character weighting. Bootstrap values are indicated below the branches in the MP tree and above the branches in the NJ tree.

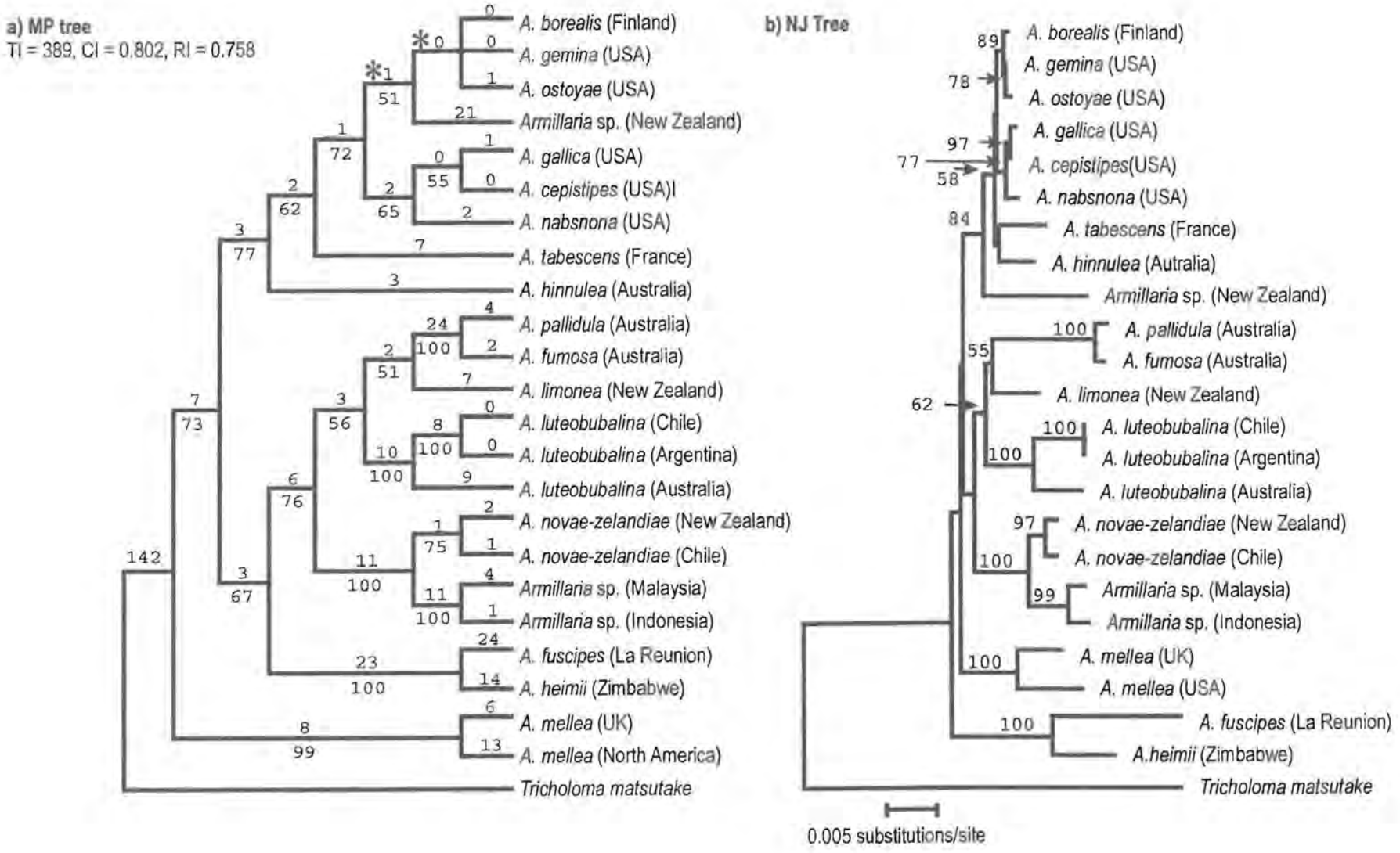


**Figure 2.** Phylogenetic trees obtained form D1 to D3 sequence data. a) Cladogram (MP), b) Neighbor-Joining tree. Numbers above tree branches in the cladogram indicate branch-length. Bootstrap values are indicated below the branches on the MP tree and above the branches on the NJ tree. Branches that collapsed in a strict consensus tree generated from MP trees before character weighting are indicated by an asterisk.

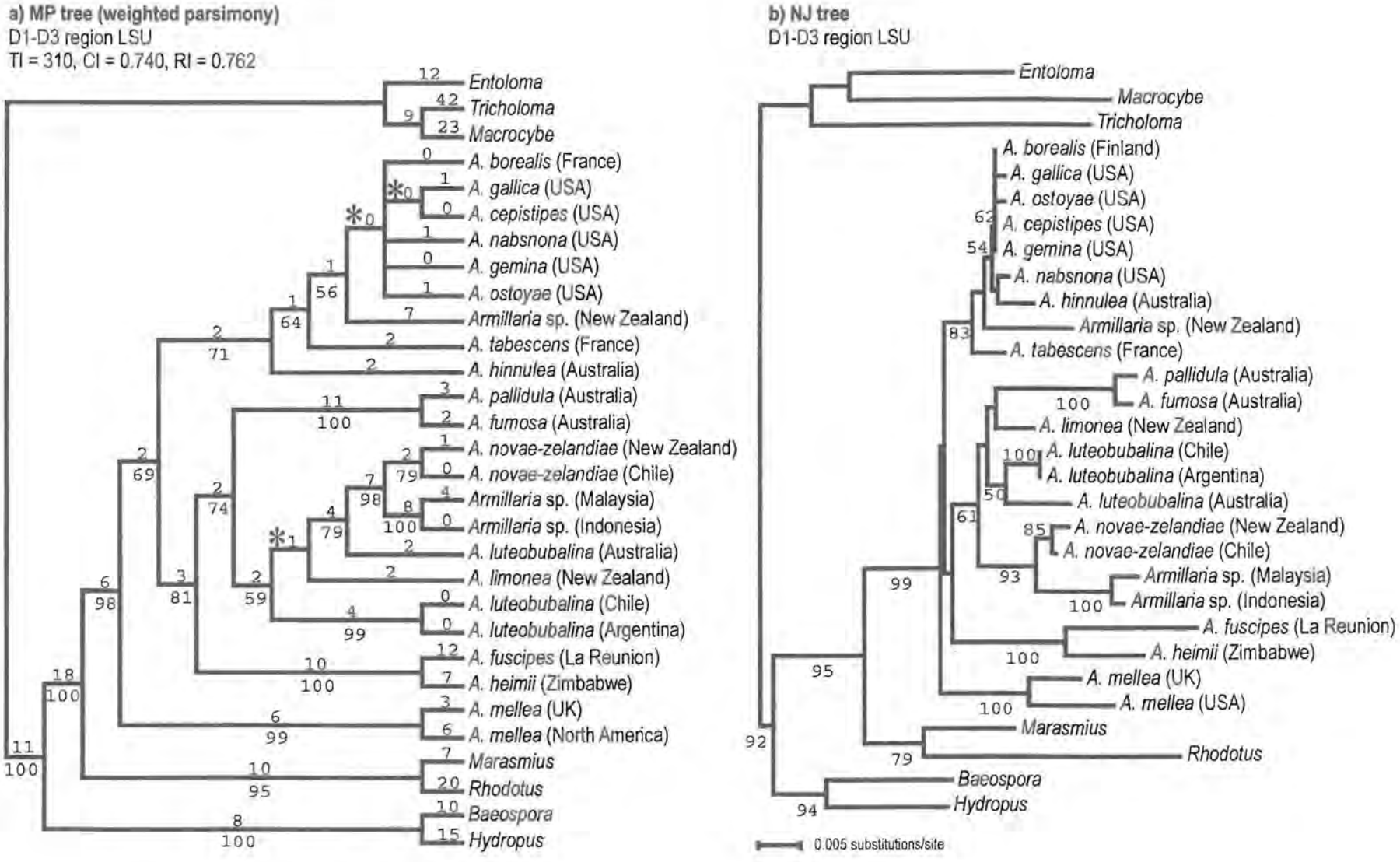


**Figure 3.** Branch-and-bound phylogenetic tree generated from D1 to D3 sequence data. Dashed lines indicate the position of *A .mellea* in the unconstrained branch-and-bound tree.

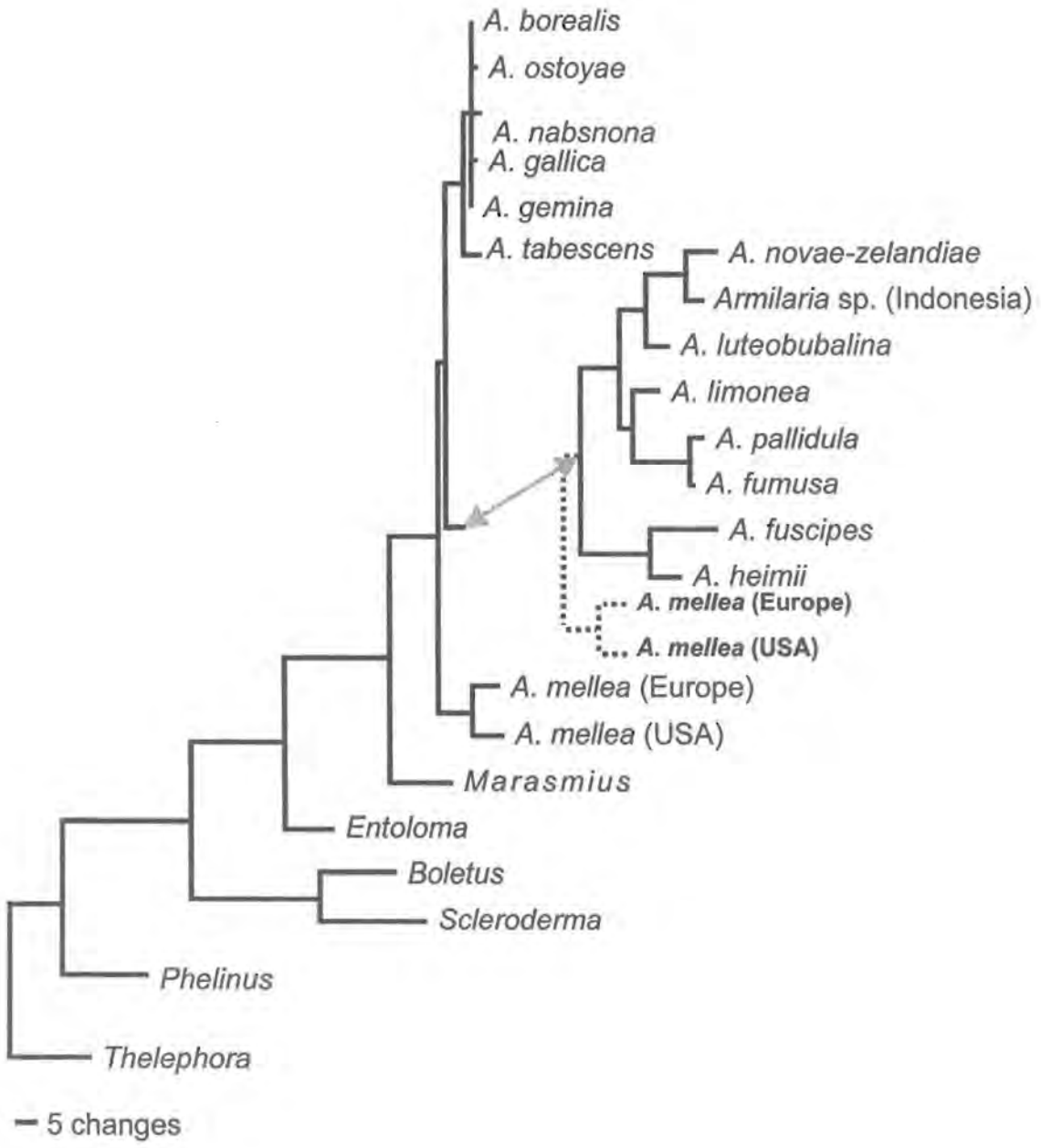

A. borealis
A. ostoyae
A. nabsnona
A. gallica
A. gemina
A. tabescens
A. novae-zelandiae
Armilaria sp. (Indonesia)
A. luteobubalina
A. limonea
A. pallidula
A. fumusa
A. fuscipes
A. heimii
A. mellea (Europe)
A. mellea (USA)
A. mellea (Europe)
A. mellea (USA)
Marasmius
Entoloma
Boletus
Scleroderma
Phelinus
Thelephora
5 changes

**Figure 4.** Ultrametric tree obtained after NPRS on the branch-and-bound tree in Fig. 3. Letters at the nodes: A; split between Boletes and Euagarics, B; hypothetical ancestor (HA) of *Marasmius* and *Armillaria*, C; split between *A. mellea* and the remainder of *Armillaria* spp., D; split between Holarctic and non-Holarctic *Armillaria* spp., E; HA of Holarctic *Armillaria* spp., F; HA of "*A. ostoyae* and *A. gallica* clusters" (Korhonen 1995), G; separation between Africa and South America, H; HA of Australian, New Zealand, South American and Indo-Malaysian taxa, I; HA of African *Armillaria* spp. and J; latest connection between North America and Europe.

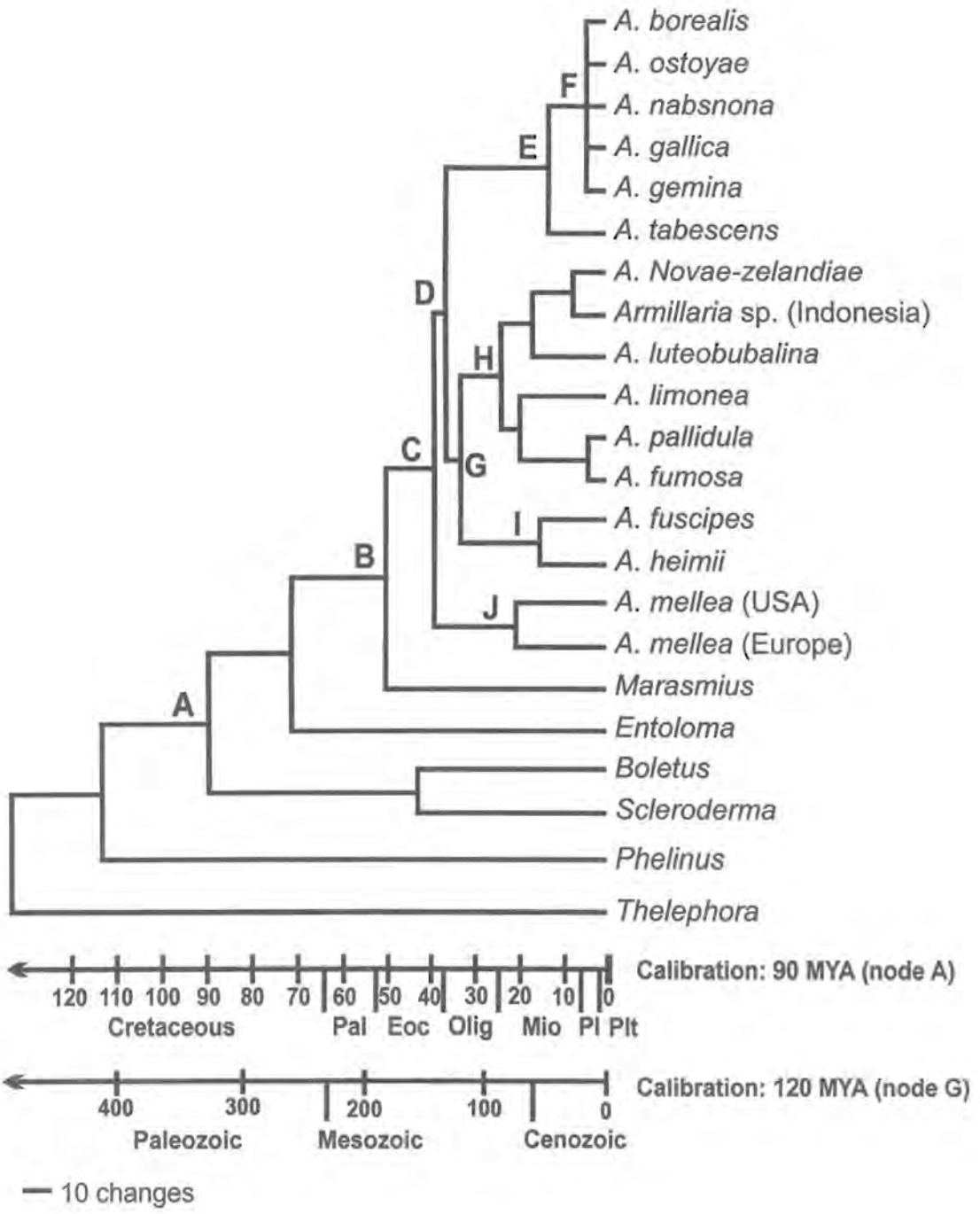
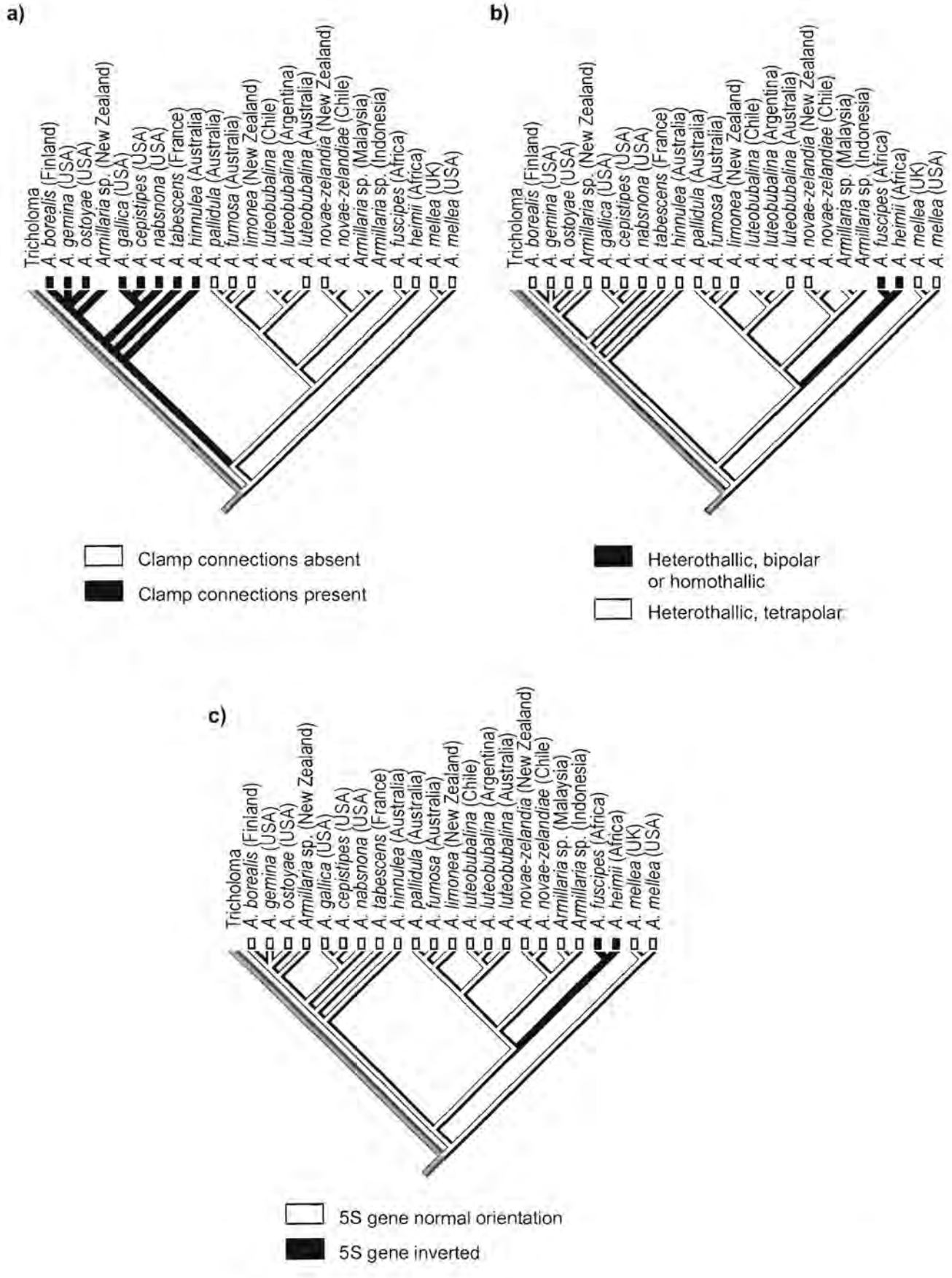

A. borealis
A. ostoyae
A. nabsnona
A. gallica
A. gemina
A. tabescens
A. Novae-zelandiae
Armillaria sp. (Indonesia)
A. luteobubalina
A. limonea
A. pallidula
A. fumosa
A. fuscipes
A. heimii
A. mellea (USA)
A. mellea (Europe)
Marasmius
Entoloma
Boletus
Scleroderma
Phelinus
Thelephora
A
B
C
D
E
F
G
H
I
J
120 110 100 90 80 70 60 50 40 30 20 10 0
Cretaceous
Pal Eoc Olig Mio Pl Plt
Calibration: 90 MYA (node A)
400 300 200 100 0
Paleozoic
Mesozoic
Cenozoic
Calibration: 120 MYA (node G)
10 changes

**Figure 5.** Cladograms showing character differences between *Armillaria* spp. a) Presence or absence of clamp connections at the base of basidia. b) Sexual system. c) Orientation of the 5S gene.



# CHAPTER FIVE

# PHYLOGENETIC RELATIONSHIPS BETWEEN AFRICAN *ARMILLARIA* SPECIES

# PHYLOGENETIC RELATIONSHIPS BETWEEN AFRICAN *ARMILLARIA* SPECIES

## ABSTRACT

Armillaria root rot is an important disease resulting in the loss of indigenous and exotic woody plant species in natural forests, plantations, orchards and gardens. *Armillaria* spp. are basidiomycetes that have a wide distribution and are found across the temperate and tropical regions of the world. Armillaria root rot has been described from various parts of Africa on many different hosts. However, very little is known regarding the evolutionary relationships among *Amillaria* species in Africa. The aim of this study was to determine the phylogenetic relationships between isolates originating from different regions in Africa using nDNA sequences from two non-coding gene regions. The ITS and the IGS-1 regions of the ribosomal DNA operon were sequenced and analysed using different phylogenetic tree searching methods. Phylogenetic trees grouped the African taxa in two strongly supported clades. One of these represented *A. fuscipes* and the other an undescribed but distinct species.

***Keywords***: *Armillaria heimii*, *Armillaria fuscipes*, Basidiomycetes, Armillaria root rot, IGS-1, ITS, phylogeny.

## INTRODUCTION

Fungi residing in the genus *Armillaria* (Basidiomycotina, Agaricales, Tricholomataceae) are phytopathogenic hymenomycetes that cause the disease known as Armillaria root rot. These fungi generally do not show strong host specificity and occur worldwide in natural forests and on planted woody crops (Hood *et al.* 1991, Kile *et al.* 1991, Termorshuizen 2001). The impact of the disease is exacerbated by the ability of *Armillaria* spp. to survive either as parasites, saprophytes or perthophytes (Gregory *et al.* 1991), depending on the available substrate in a particular niche. Consequently, Armillaria root rot poses a significant problem for forestry and agricultural industries worldwide and the species causing disease syndromes warrant identification.

The manner in which species are delineated in *Armillaria* depends heavily upon the species concept that is employed. Where emphasis is placed on morphology (Regan 1926), specimens with similar basidiocarp form are considered the same species. When the biological species concept (Mayr 1942) is applied, however, species are viewed as a group of organisms that are sexually or interspecificaly somatically compatible and reproductively isolated from those outside the group. A third perspective is the phylogenetic species concept (*sensu* Baum and Mishler 1995), according to which a species represents a group of organisms with a shared exclusive genealogical history. Disparities that arise through the application of these different species concepts have led to considerable lack of consensus regarding the identification of African *Armillaria* species.

Numerous taxonomic studies have been undertaken on African *Armillaria* spp. (Mohammed *et al.* 1989, 1994, Mohammed and Guillaumin 1993, Mwenje and Ride 1993, 1996, 1997, Agustian *et al.* 1994, Abomo-Ndongo and Guillaumin 1997, Chillali *et al.* 1997). These have included species identifications based on *in vitro* cultural characteristics, basidiocarp morphology, biochemical properties and sexual or interspecific somatic incompatibility tests. Because basidiocarps are short lived and seldom encountered in *Armillaria* spp., especially those in tropical Africa (Swift 1972), basidiocarp morphology has been of limited use in mapping the species populating the continent. Taxonomic studies based on interspecific somatic incompatibility tests, on the other hand, have been more successful. These studies suggested that

isolates from Africa can be separated into at least four somatic incompatibility groups (SIGs) (Mohammed *et al.* 1989, 1994, Abomo-Ndongo and Guillaumin 1997). The first of these (SIG I) includes isolates from Kenya, Tanzania and São Tomé that represent the homothallic African form of *A. mellea* (Vahl.:Fr.) P.Kumm. Isolates considered to represent *A. heimii* Pegler from East, Central, West and South Africa were designated as SIG II. Isolates residing in the third group (SIG III) originated from Kenya, but were not assigned to a morphological species. One isolate from Kenya was incompatible with all other isolates and placed in SIG IV (Mohammed *et al.* 1994).

Although isolates of *Armillaria* referred to as *A. heimii* have been considered as belonging to the same biological species (SIG II), they display considerable variation. This variation includes differences in their mating systems (Abomo-Ndongo *et al.* 1997), mycelial-mat morphology and rhizomorph characteristics (Mwenje and Ride 1993, Mohammed *et al.* 1994), optimal growth temperatures (Mohammed and Guillaumin 1993, Mohammed *et al.* 1994), randomly amplified polymorphic DNA (RAPD) patterns (Mohammed 1994, Otieno *et al.* 2003), isozyme electrophoresis profiles (Agustian *et al.* 1994, Mwenje and Ride 1997), internally transcribed spacer (ITS) and intergenic spacer (IGS) restriction fragment length polymorphisms (RFLPs) (Chillali *et al.* 1997, Otieno *et al.* 2003) and inter-simple sequence repeat (ISSR) polymorphisms (Otieno *et al.* 2003). Collectively, these studies have shown that the isolates can be differentiated into at least three sub-groups. In the light of these findings, *A. heimii* is referred to as *A. heimii sensu lato* and it possibly comprises several species (Mohammed and Guillaumin 1993).

In addition to *A. heimii* and *A. mellea*, studies on the taxonomy of African *Armillaria* spp. have included the fungus known as *A. fuscipes* Petch. This species was first reported from Sri Lanka (Ceylon) on *Acacia decurrens* (Petch 1909). After considering the micro-morphology of *A. fuscipes*, Chandra and Watling (1981) suggested that this taxon and *A. heimii* are conspecific, although they did not formalise this synonymy. Pegler (1986), based on overall basidiocarp morphology, reduced *A. heimii* to synonymy with *A. fuscipes* which was the earlier named species. Pegler (1986) also suggested that *A. fuscipes* had been introduced into Sri Lanka on tea. After examining the microscopic characteristics of the type specimen of *A. heimii*, Kile and Watling (1988) and Watling (1992) supported the prior taxonomic treatment of *A. heimii* and *A. fuscipes* by Pegler (1986). These authors, however, suggested that conspecificity of the two species should be verified using cultural and interfertility studies. Although the similarity in

basidiocarp morphology of *A. heimii* and *A. fuscipes* provides a strong case for their synonymy, this has not been generally accepted (Mohammed and Guillaumin 1993, Otieno *et al.* 2003). Notwithstanding the predilection for the name *A. heimii* in taxonomic literature, from the perspective of a morphological species concept there is no reason to afford it preference over *A. fuscipes*. Consequently, we use this name in the present study for the South African isolates but also for those from other parts of Africa that have been treated as *A. heimii* in earlier publications.

As mentioned above, several studies have raised the suspicion that *A. heimii sensu lato* from Africa, that we refer to as *A. fuscipes*, comprises several distinct species. Two recent studies (Coetzee *et al.* 2000a, Mwenje *et al.* 2003) confirmed this view. Phylogenetic analysis of IGS-1 sequence data showed that isolates of *A. fuscipes* from different African countries reside in two strongly resolved monophyletic groups. One of these groups includes isolates from South Africa, Zimbabwe (Group I of Mwenje and Ride 1996) and La Reunion and the other isolates from Zambia, Zimbabwe (Group II and III of Mwenje and Ride 1996) and Cameroon. These studies have suggested that the two groups represent at least two different species (Coetzee *et al.* 2000a, Mwenje *et al.* 2003). Some of the isolates included were, however, previously shown to represent the same somatic compatibility group, even though they belong to different phylogenetic lineages. The possibility, therefore, remains that the groups recognised by Coetzee *et al.* (2000a) and Mwenje *et al.* (2003), might reflect intraspecific genetic variation within *A. fuscipes*. Hence, the objective of this study was to re-evaluate the suggestion that *A. fuscipes* encompasses more than one species. This objective was accomplished by extending the number of isolates considered previously (Coetzee *et al.* 2000a, Mwenje *et al.* 2003) and conducting phylogenetic and genetic analysis of both ITS and IGS-1 sequence data.

## MATERIALS AND METHODS

### Fungal strains

*Armillaria* isolates used in this study originated from eight different countries in sub-Saharan Africa and were from a wide range of hosts (Table 1). All isolates other than those from South Africa and Ethiopia were from the collections of Dr. C. Mohammed (CSIRO, Forestry and Forestry Products, Hobart, Australia) and Prof. TC Harrington (Iowa State University, Ames, USA). These cultures are preserved in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

*Information pertaining to the fungal strains:*

Isolates that have been treated as *A. heimii* and placed in SIG II based on their basidiocarp morphology *in vitro*, phenotypic similarity and intraspecific somatic compatibility (Mohammed *et al* 1989, 1994, Abomo-Ndongo and Guillaumin 1997) are indicated in Table 1. The isolates from Ethiopia (CMW5844 and CMW5846) and South Africa (CMW2717 and CMW2740) were identified as *A. fuscipes* based on basidiocarp morphology and IGS-1 sequence data (Coetzee *et al*. 2000a, Gezahgne 2003). The Zimbabwean isolates were previously characterised based on their morphological, biochemical and IGS-1 sequence data and found to reside in three groups: Group I, CMW4874 and CMW10165; Group II, CMW4455 and CMW4456; Group III, CMW10115 and CMW10116 (Mwenje and Ride 1996, Mwenje *et al*. 2003). Isolates CMW4456 (from Group II) and CMW4874 (from Group I) were shown to be somatically compatible with isolates in SIG II (Abomo-Ndongo and Guillaumin 1997; Mohammed *et al* 1989, 1994).

**DNA extractions**

Isolates were grown in liquid MY (1.5% Malt extract and 0.2% Yeast extract) medium for 4 weeks in the dark at 24 °C. The mycelium was harvested using a strainer, lyophilised and ground to a fine powder in liquid nitrogen. Extraction buffer (1 mL) [100mM Tris-Cl pH 8.4; 1.4M NaCl; 25mM EDTA pH 8; 2% CTAB (hexadecyltrimethylammonium bromide)] was added to *ca*. 0.5 g powdered mycelium and incubated at 60 °C for 2 h. The mycelium powder-buffer suspension was divided into two parts and centrifuged (17 900 g, 20 min) to precipitate cell debris. Isoamyl alcohol: chloroform (1:24 v/v) extractions were performed on the aqueous phase until a clean interphase was obtained. Nucleic acids were precipitated by 96% ice-cold ethanol. The precipitate was collected by centrifugation (17 900 g, 30 min), washed with cold 70% ethanol, dried and dissolved in sterile distilled water. Contaminating RNA was removed by adding RNAse A (0.01 mg/µL) (Roche, South Africa).

**Amplification of the ITS and IGS-1 regions of the rDNA**

The intergenic spacer regions (ITS1 and ITS2) between the 3' end of the small subunit (SSU) and the 5' end of the large subunit (LSU) ribosomal RNA (rRNA) gene as well the first intergenic spacer region (IGS-1) between the 3' end of the LSU and the 5' end of the 5S gene were amplified using PCR. The ITS regions were amplified using primer pair ITS1 and ITS4 (White

*et al.* 1990). Primer pair P-1 (Hsiau 1996) and 5S-2B (Coetzee *et al.* 2000a) were used to amplify the IGS-1 region for the *Armillaria* isolates. Reaction conditions and the PCR reaction mix were the same as those previously described by Coetzee *et al.* (2000b). ITS and IGS-1 amplicons were visualised under UV illumination after electrophoresis on an agarose (Promega, Wisconsin) gel (0.8% wt/v) stained with ethidium bromide.

### DNA sequencing

ITS and IGS-1 DNA sequences were obtained using an ABI PRISM automated sequencer. A QIAquick PCR purification kit (QIAGEN, Germany) was used to purify PCR products from unincorporated nucleotides and primer dimers, prior to sequencing. Sequence reactions were carried out with the ABI Prism® BigDyeTM Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase, FS (Perkin Elmer, Warrington, UK) following the protocol supplied by the manufacturer. DNA sequences for the ITS region were generated using primers ITS1 and ITS4 as well as internal primers CS2B and CS3B (Coetzee *et al.* 2001). The IGS-1 region was sequenced using primers P-1, 5S-2B and internal primers MCP2, MCP2R, MCP3, MCP3R, 5S-3MC and 5S-4MCR (Coetzee *et al.* 2000a).

### Cloning of IGS-1 amplicons

IGS-1 PCR products from isolates that gave poor sequences (CMW4949 and CMW4950) were cloned into vector pCR®4-TOPO® after purification, as outlined above. Cloning reactions were done using a TOPO TA Cloning® Kit for Sequencing (Invitrogen life technologies, Carlsbad, California) with One Shot® TOP10 Chemically Competent *E. coli* cells following the manufacturer's directions. The IGS-1 region was amplified directly from transformed *E. coli* cells to verify positive inserts. PCR mixtures included dNTPs (250 µM each), *Taq* Polymerase (2.5 U) (Roche Diagnostics, Mannheim, Germany), PCR buffer with $MgCl_2$ (supplied by the manufacturer), primers P-1 and 5S-2B (0.1 µM each), brought to a final volume of 50 µL with water. PCR conditions were as follows: 1 cycle at 95 °C for 1 min (denaturation), 35 cycles of 60 °C for 30 s (primer annealing), 70 °C for 30 s (elongation) and 95 °C for 30 s (denaturation). A final elongation step was allowed at 70 °C for 7 min. PCR products were visualized under UV illumination on a 1% agarose gel stained with ethidium bromide. The IGS-1 insert from one clone that was successfully amplified from positively transformed cells was sequenced for each isolate as described above.

**Sequence and phylogenetic analyses**

Phylogenetic analyses were done on three data matrices: 1) *Armillaria* ITS including all African isolates and two representative sequences for each *Armillaria* spp. available on GenBank (accession numbers in Fig. 1); 2) African *Armillaria* ITS including ITS sequences determined in this study with *A. hinnulea* (AF394918 and AF329907) as outgroup taxon; 3) African *Armillaria* IGS-1 including sequences obtained in this study and sequences available on GenBank from previous studies (Table 1). Alignment was done using Clustal X (Thompson *et al.* 1997) and manually corrected using BioEdit Sequence Alignment Editor version 5.0.9 (Hall 1999). Indels larger than two base pairs in the African ITS and IGS-1 data matrices were coded using a multistate-character system (Coetzee *et al.* 2001).

Parsimony analyses were conducted on the African ITS and IGS-1 data matrices using PAUP* version 4.10 (Swofford 1998). Missing, parsimony uninformative and ambiguously aligned regions were excluded from the data sets before analyses. Gaps were treated as a fifth character, "newstate". Most parsimonious trees were generated by heuristic searches with random addition of sequences (100 replicates), TBR (tree bisection reconnection) branch swapping and MULPARS active. MaxTrees was set to auto-increase after 100 MP trees were generated and branches collapsed if negative branch lengths were obtained. Bootstrap analysis (1000 replicates) using the same settings as above but with *A. hinnulea* as reference taxon, and sequential addition of sequences was employed to obtain confidence of branch nodes (Felsenstein 1985) for trees generated from the African ITS data matrix.

Phylogenetic trees based on distance methods were generated for all data matrices using PAUP*. Missing data and ambiguously aligned or gapped regions were excluded from the data sets prior to analysis. Trees were obtained using a Neighbour-Joining tree building algorithm (Saitou and Nei 1987) that incorporated a Kimura 2-parameter nucleotide substitution model (Kimura 1980). Confidence values for branching nodes were determined for the African ITS and *Armillaria* ITS datasets using bootstrap analysis (1000 replicates).

Relative nucleic substitution rate heterogeneity among lineages based on ITS sequence data was determined for the African taxa based on a relative rate test (Robinson *et al.* 1998) using RRTree version 1.1 (Robinson-Rechavi and Huchon 2000). The distance method was based on a Kimura 2-parameter substitution model. Ambiguously aligned and missing data were excluded from the data matrix prior to the analysis using PAUP*. *Armillaria hinnulea* was used as outgroup taxon.

### Intra-specific nucleotide diversity and evolutionary distance comparisons

The intra-specific nucleotide diversity and evolutionary distance between the two African taxa were compared against those for geographically separated groups in *A. luteobubalina*, *A. mellea*, and *A. novae-zelandiae* (Table 2). Sequences for each species were aligned in a separate dataset using methods described above. Characters from the extreme 5' and 3' ends of the ITS sequences were deleted from some taxa to obtain individual datasets that had identical start and end positions. Missing and gapped regions were excluded prior to analyses. Mean nucleotide diversity ($\pi$) was calculated over all taxa for each data set and mean evolutionary distances between and within groups within a specific dataset using a Kimura 2-parameter nucleotide substitution model in MEGA version 2.1 (Kumar *et al.* 2001).

## RESULTS

### Amplification of ITS and IGS-1

The ITS region was successfully amplified for all African isolates. The ITS amplicon size for all isolates was approximately 650 base pairs (bp.). The IGS-1 region was successfully amplified for isolates CMW4871, CMW4873, CMW4949, CMW4950, CMW4953, CMW7187 and CMW7184. The IGS-1 amplicon sizes for these isolates were approximately 1200 bp.

### Phylogenetic analyses

#### *Armillaria ITS data matrix and NJ tree*

The *Armillaria* ITS data matrix included 52 taxa with 1183 character sites after alignment with inclusion of gaps. Eighty-eight missing and ambiguously aligned characters as well as 656 gaps were excluded prior to the analysis. The final analysis included 439 characters.

The NJ-tree (Fig. 1) generated from the *Armillaria* ITS dataset grouped the African isolates into a strongly supported (100% bootstrap) clade. The isolates incorporated in this African clade were further separated into two strongly supported sub-clades. Lengths for branches separating these two major groups were longer or nearly equal to those separating other *Armillaria* spp. in the NJ-tree.

*African datasets*

*African ITS data matrix:* This dataset included 880 characters after alignment by inserting gaps. The absolute lengths for the African taxa ranged between 625 to 630 bp. and for *A. hinnulea* between 851 and 853 bp. Ten indel regions equalling 241 characters were replaced with multistate characters. Ninety-four missing and ambiguously aligned characters were excluded prior to cladistic and distance analysis. After exclusion of parsimony-uninformative and constant characters, 129 characters were included in the parsimony analysis. Distance analysis was based on 515 characters after exclusion of gapped regions and multistate characters.

*African IGS-1 data matrix:* This dataset included 1259 characters after alignment with inclusion of gaps. The absolute lengths of the IGS-1 sequences for the isolates included in this analysis ranged between 851 and 1133 bp. Forty-six indel regions of 549 characters were replaced by multi-state characters, yielding a total of 756 characters available for analysis. Eighty-one missing and ambiguously aligned regions were excluded before cladistic and distance analysis. Parsimony analysis was based on 156 parsimony-informative characters after exclusion of 20 parsimony-uninformative and 580 constant characters. Distance analysis included 675 characters after exclusion of gaps and multi-state characters.

***African cladograms and Neighbour-Joining trees***

Heuristic searches on the African ITS data matrix yielded seven most parsimonious (MP) trees with similar topology. Two MP trees with similar topologies were generated from the African IGS-1 data matrix after a heuristic search. The overall topologies of the MP trees (Fig. 2) obtained from these two datasets were congruent. The general topology of the Neighbour-Joining (NJ) trees (Fig. 3) generated from the African ITS and IGS-1 data matrixes were similar and reflected those of the MP trees.

Parsimony and Neighbour-Joining trees generated from ITS sequence data separated the African isolates into two highly supported clades (labelled A and B) both with 100% bootstrap support (bs). Isolates in Clade A resided in three sub-groups ($A_1$, $A_2$ and $A_3$). Clade $A_1$ included isolates from Zimbabwe (CMW4874, CMW10165), South Africa (CMW2717, CMW2740), La Reunion (CMW3164, CMW4953), Malawi (CMW4871, CMW4873) and Tanzania (CMW4949, CMW4950) (MP: 65% bs, NJ: 91% bs). Isolates in group $A_2$ originated from Kenya (CMW7184, CMW7187) (MP: 99% bs, NJ: 89% bs). Clade $A_3$ included isolates from Ethiopia (CMW5844, CMW5846) (MP: 85% bs, NJ: 99% bs). Clade B included isolates from Cameroon

(CMW3152), Zambia (CMW3173) and Zimbabwe (CMW4455, CMW4456). Two isolates from Zimbabwe (CMW10115, CMW10116) resided in a strongly supported $B_1$ sub-clade (MP: 99% bs, NJ: 94% bs).

***Relative rate heterogeneity test***

Treating individual isolates as representing independent lineages yielded a $p = 0.244805$. Tests conducted after grouping isolates according to their associated clades, and then treating the clades as independent lineages, yielded a $p = 0.24477$. The relative rate test, therefore, indicated that substitution rate heterogeneity in the ITS regions among isolates included in this study is not statistically significant.

**Intra-specific sequence diversity and evolutionary distance comparisons**

Mean sequence diversity in the ITS sequence data sets was the highest for the African taxa (0.0368 ± 0.0056) followed by *A. mellea* (0.0321 ± 0. 0042), *A. novae-zelandiae* (0.0193 ± 0.0035), *A. luteobubalina* (0.0089 ± 0.0021) (Fig. 4). The overall mean sequence diversity in the IGS-1 datasets was the highest for the African taxa (0.0638 ± 0.0067), followed by *A. mellea* (0.0537 ± 0.0064) (Fig. 4). Evolutionary distances between the two African groups were the highest in both ITS and IGS-1 sequence analysis (Fig. 4). The evolutionary distance between the two African groups A and B based on ITS data was 1.8 to 2.4 times greater than between the groups in *A. mellea*, 4.2 to 8.8 times greater than between the groups in *A. novae-zelandiae* and 11.3 times greater than the groups in *A. luteobubalina*. The distance between the two African groups based on IGS-1 sequence data was 1.5 to 2.9 times greater than between groups in *A. mellea*.

## DISCUSSION

An overall objective of this study was to test two opposing views regarding the taxonomy of *A. fuscipes*, which we have treated as synonym of *A. heimii*, from Africa. Using mating and somatic compatibility tests, isolates have been shown to represent a single somatic compatibility group, and thus the same biological species (Mohammed *et al.* 1989, 1994, Abomo-Ndongo and Guillaumin 1997). In contrast, phylogenetic studies have suggested that *A. fuscipes* includes more than one species (Coetzee *et al.* 2000a). Results of the present study provide additional evidence supporting the view that *A. fuscipes* represent at least two species.

Results of this study show that isolates representing *A. fuscipes*, also referred to as *A. heimii* or somatic incompatibility group II (Mohammed *et al.* 1989, 1994, Abomo-Ndongo and Guillaumin 1997), all have an inverted 5S gene. The IGS-1 region of seven isolates was amplified in this study using primer 5S2B that binds to the complimentary 5' position of the inverted 5S gene (Coetzee *et al.* 2000a). Successful amplification with this primer thus indicates that the 5S gene is inverted for these isolates. Inversion of the 5S gene has previously been reported for other isolates included in this study (Coetzee *et al.* 2000a, Mwenje *et al.* 2003). This study, together with those on other *Armillaria* spp. (Anderson and Stasovski 1992, Terashima *et al.* 1998, Coetzee *et al.* 2001, 2003), indicates that this phenomenon is restricted to isolates representing *A. fuscipes*. Inversion of the 5S gene was previously reported only for *Coprinus comatus* (Mull.:Fr.) S.F. Gray (Cassidy and Pukkila 1987) and is therefore highly unusual in basidiomycetes.

Inversion of the 5S gene renders the IGS-1 region unsuitable for phylogenetic analyses aimed at comparing the African isolates with the other species of *Armillaria*. This is because there are no other closely related taxa that have this 5S gene inversion and there is thus also no appropriate outgroup taxon for a phylogenetic analysis using only the sequences for African isolates. For this reason phylogenetic analyses conducted in this study to determine the relationships between the Africana isolates and other *Armillaria* spp. were based only on ITS sequence data.

The NJ-tree generated from the ITS data matrix grouped the African taxa in a strongly supported cluster that was separated from other *Armillaria* spp. included in this study. The length of the branch connecting the ancestral node of the African isolates with the basal node was exceptionally long in comparison with other branches in the NJ-tree. Nucleic acid substitution rate homogeneity among lineages was not tested for taxa in the *Armillaria* ITS data set; consequently it is uncertain if all lineages presented in the NJ-tree evolved at the same evolutionary rate. However, interpretation of the NJ-tree suggests strongly that the African group has undergone a very long period of independent evolution from the common *Armillaria* ancestor.

Cladograms and NJ-trees generated from the ITS datasets in this study separated the African isolates into two highly supported sister groups. Some biogeographic structure was observed with isolates from Kenya and Ethiopia grouping in two different sub-groups within one of the

sister groups. A third sub-group within the other sister group represented two isolates from Zimbabwe that are distinct from the other Zimbabwean isolates in terms of their cultural, molecular and biochemical characteristics (Mwenje and Ride 1996, Mwenje *et al.* 2003). Cladograms and NJ-trees generated from the IGS-1 data set yielded topologies similar to those from the ITS data sets. Because of the absence of an outgroup, it was, however, not possible to gain statistical support for these results.

Isolates from Cameroon, La Reunion, Malawi, Tanzania, Zambia and Zimbabwe that have been treated as *A. heimii* (Mohammed *et al.* 1989, 1994, Abomo-Ndongo and Guillaumin 1997), and considered here to be *A. fuscipes*, resided in two phylogenetic groups. The question emerging from our results is, therefore, whether these two African lineages represent infraspecific taxa or distinct species displaying considerable biological similarity. In an attempt to address this question, we regarded the African isolates as a single population and investigated the genetic variation among them. We considered the evolutionary distances within and between these two phylogenetic groups, which are sympatric, and compared them with those determined in this study for allopatric groups in *A. luteobubalina, A. mellea* and *A. novae-zelandiae.*

Analyses of the ITS and IGS-1 sequence data revealed variation within the sympatric African taxa that was nearly equivalent to or higher than that within the heterothallic allopatric global populations for the other *Armillaria* spp. included in this study. Earlier studies have shown that African isolates included in this study are homothallic (Mohammed *et al.* 1989, Abomo-Ndongo *et al.* 1997), with exception of the isolate from Cameroon that is heterothallic (Mohammed *et al.* 1989, Abomo-Ndongo *et al.* 1997). The sexual system is not known for isolates from South Africa, Kenya, Ethiopia and some of the Zimbabwean isolates. Homothallic (self-fertilising) species maintain low intraspecific genetic variation and this reduces the amount of variation with every new generation. In contrast, heterothallic species display higher levels of genetic variation as a result of gene flow and subsequent recombination between individuals. Thus, the overall intraspecific sequence diversity would be lower for homothallic than for heterothallic species. The fact that isolates in the African group, most of which are known to be homothallic, display higher nucleotide diversity than the heterothallic populations to which they were compared suggest that this group does not represent a single species.

The distances between the two major African lineages emerging from the ITS and IGS-1 sequence data were 1.8 to 8.8 times greater than between the allopatric groups in *A.*

*luteobubalina, A. mellea and A. novae-zelandiae* included in this study. Furthermore, the distances within the two African groups were generally lower than those within the sympatric groups of *A. mellea*, *A. luteobubalina* and *A. novae-zelandiae*. Variation in the tandem arrays, especially within the ITS and IGS region, are usually observed between species, whilst they are relatively conserved among individuals of the same species (Hillis and Dixon 1991). Concerted evolution occurs through the processes of unequal crossing over and/or gene conversion (Dover 1982, Arnheim 1983). Because of concerted evolution, mutations occurring within the rDNA spacer and gene regions are homogenised throughout the tandem array and become fixed in populations characterised by unrestricted gene flow, thereby maintaining low intraspecific variation (Hillis and Dixon 1991). It is, therefore, reasonable to expect that if the isolates from the two African groups represent the same species, with unrestricted gene flow between the groups, mutations within the ITS and IGS-1 regions would be homogenised, yielding low intergroup variation. Hence, the evolutionary distances between the two African groups should be shorter than between allopatric populations belonging to the other *Armillaria* spp. The results, however, indicated that the ITS and IGS-1 sequences are conserved within, but highly variable between the two African groups. Mutations that occurred in the ITS and IGS-1 regions of isolates from one group did not become fixed in isolates from the other group. Data from this study, therefore, suggests a lack of gene flow and subsequent genetic recombination between isolates from the respective groups in their natural environments, despite their being somatically compatible *in vitro*.

The observed phylogenetic partition and lack of genetic recombination in ITS and IGS-1 loci for the African isolates could be attributed to their homothallic nature. If this is the case, the two major phylogenetic lineages could represent clonal lineages within a species. Neighbour-Joining trees generated from the *Armillaria* ITS data matrix showed, however, that branches connecting the two main clades with the ancestral node are longer than or equivalent in length to branches separating species from Australasia, South America and the Northern Hemisphere. Relative rate heterogeneity tests indicated that the two lineages evolved independently, and at the same rate, from their common ancestor. These results, together with the geographic distribution of the African taxa and the sister relationships of the two main clades, indicate that sympatric speciation has occurred and that the two lineages represent two closely related species.

Recognition of the two main phylogenetic lineages emerging from this study as discrete species led to a decision to search previous publications for diagnostic characters linked to isolates used

in this study. Characters identified during this search included sexual systems (Mohammed *et al.* 1989, Abomo-Ndongo *et al.* 1997); temperature sensitivity (Guillaumin 1992), groupings according to protein, esterase and RAPD analysis as well as Southern hybridization (Mohammed 1992); groupings based on physiology and morphology (Guillaumin 1992); grouping of isolates from Zimbabwe using morphological and biochemical characteristics (Mwenje and Ride 1996); and IGS-1 amplicon sizes (Coetzee *et al.* 2000a, Mwenje *et al.* 2003, and this study) (Fig. 2). Amplicon size differences and temperature sensitivity were found to be the most diagnostic characters for recognizing the two species. Isolates residing in the two major phylogenetic lineages can be distinguished by amplicon sizes of approximately 1200 bp. and 900 bp, respectively. Although temperature sensitivity has been reported for only few isolates, there is a strong indication that isolates residing in the one phylogenetic lineage (Clade A) are thermophobic whereas those in the other lineage (Clade B) are thermophilic.

Phylogenetic trees generated from ITS and IGS-1 sequence data in this study clearly separated the isolates into two major lineages. If the biological species concept is adopted, isolates within these lineages would represent the same species. Comparisons in terms of basidiocarp morphology would probably lead to the same conclusion. Applying the morphological species concept, but taking into account biological characteristics other than basidiocarp morphology, however, yield results suggesting that they might represent different species. This possibility is confirmed if the phylogenetic species concept is employed. One of these two lineages (Clade A) represents the widely distributed *A. fuscipes,* which has also been referred to as *A. heimii* in some previous studies. Isolates residing in the second phylogenetic lineage (Clade B) represent an as yet unnamed species.

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TABLE 1: *Armillaria* isolates used in this study.

| Culture number [a] | Alternative number | Host | Origin | Collector | IGS[b] GenBank | ITS[b] GenBank |
|---|---|---|---|---|---|---|
| CMW2717 | A04-SA | *P. elliottii* | Sabie, South Africa | Wingfield, M.J. | AF204821 | ✓ |
| CMW2740 | B07-SA | *P. patula* | Entabeni, South Africa | Wingfield, M.J. | AF204822 | ✓ |
| CMW3152[c] | CA1, B935 | Unknown | Western Province, Cameroon | Watling, R. | AF204826 | ✓ |
| CMW3164[c] | LR3, B933 | *Pelargonium asperum* | Saint-Denis, La Reunion | Fabrègue, C. | AF204824 | ✓ |
| CMW3173[c] | ZM1, B932 | *Tectona grandis* | Dola Hill, Zambia | Ivory, M. | AF204825 | ✓ |
| CWM4455 | 40 | *Camellia sinensis* | Eastern Highlands Estates, Zimbabwe | Mwenje, E. | AF489486 | ✓ |
| CMW4456[c] | Z1 | *Brachystegia utilis* | Maswera, Zimbabwe | Ivory, M. | AF489485 | ✓ |
| CMW4871[c] | M1 | *Widdringtonia whytei* | Zomba Mts., Malawi | Ivory, M. | ✓ | ✓ |
| CMW4873[c] | M3 | Indidenous shrub | Zomba Mts., Malawi | Ivory, M. | ✓ | ✓ |
| CMW4874[c] | Z2 | *Araucaria cunninghamii* | Stapleford, Zimbabwe | Ivory, M. | AF489481 | ✓ |
| CMW4949[c] | T1 | *Pinus elliottii* | Lushoto, Tanzania | Ivory, M. | ✓ | ✓ |
| CMW4950[c] | T2 | *Pinus strobes* | Lushoto, Tanzania | Ivory, M. | ✓ | ✓ |

TABLE 1 (continued)

| Culture number [a] | Alternative number | Host | Origin | Collector | IGS GenBank | ITS GenBank |
|---|---|---|---|---|---|---|
| CMW4953 [c] | LR2 | *Pelargonium asperum* | Saint-Denis, La Reunion | Fabrègue, C. | ✓ | ✓ |
| CMW5844 | WG1I | *P. patula* | Wondo Genet, Ethiopia | Alemu Gezahgne & Roux, J. | AY172032 | ✓ |
| CMW5846 | WG2E | *P. patula* | Wondo Genet, Ethiopia | Alemu Gezahgne & Roux, J. | AY172030 | ✓ |
| CMW7184 | K52 | *Cypress sp.* | Kenya | Mwangi, L. | ✓ | ✓ |
| CMW7187 | K65 | *Camellia sinensis* | Kenya | Mwangi, L. | ✓ | ✓ |
| CMW10115 | 55 | *Acacia albida* | Harare, Zimbabwe | Mwenje, E. | AF489483 | ✓ |
| CMW10116 | 56 | *Newtonia buchananii* | Harare, Zimbabwe | Mwenje, E. | AF489484 | ✓ |
| CMW10165 | P7 | *Prunus persica* | Chimanimani, Zimbabwe | Mwenje, E. | AF489482 | ✓ |

[a]CMW numbers refer to the culture collection numbers of the Tree Pathology Co-operative Programme (TPCP), FABI, UP, Pretoria.

[b] Tick mark (✓) denotes sequences derived in this study.

[c] Isolates used in Mohammed *et al.* 1989, 1994, Abomo-Ndongo and Guillaumin 1997.

TABLE 2: GenBank numbers, origin and grouping of *Armillaria* spp. included in intra-specific nucleotide diversity and evolutionary distance comparisons.

| Species | Group | Origin | GenBank accession no. ITS | GenBank accession no. IGS-1 | Published by |
|---|---|---|---|---|---|
| *A. luteobubalina* | Australia (west) | WA, Australia | AF329913 | - | Coetzee *et al.* (2001) |
| | | Cape Arid, WA, Australia | AF454741 | - | Dunne *et al.* (2002) |
| | | Popanyinning, WA, Australia | AF454742 | - | " |
| | Australia (east) | VIC, Australia | AF329909 | - | Coetzee *et al.* (2001) |
| | | VIC, Australia | AF329910 | - | " |
| | | South Australia | AF329912 | - | " |
| | | Traralgon, VIC, Australia | AF454743 | - | Dunne *et al.* (2002) |
| *A. mellea* | Europe | Cambs Co., England | AF163578 | AF162602 | Coetzee *et al.* (2000) |
| | | Hungary | AF163581 | AF163605 | " |
| | | Iran | AF163583 | AF163606 | " |
| | | France | AF163585 | AF163600 | " |
| | USA (west) | Orinda, CA, USA | AF163595 | AF163608 | " |
| | | Berkeley, CA, USA | AF163596 | AF163607 | " |
| | | CA, USA | AF163597 | AF163609 | " |
| | USA (east) | Durham, NH, USA | AF163587 | AF163616 | " |
| | | Durham, NH, USA | AF163588 | AF163617 | " |

TABLE 2 (continued)

| Species | Group | Origin | GenBank accession no. ITS | GenBank accession no. IGS-1 | Published by |
|---|---|---|---|---|---|
| *A. mellea* | USA (east) | Boston, MA, USA | AF163589 | AF163614 | Coetzee *et al.* (2000) |
| | | Provincetown, MA, USA | AF163590 | AF163615 | " |
| | Asia | South Korea | AF163591 | AF163611 | " |
| | | South Korea | AF163592 | AF163612 | |
| | | South Korea | AF163593 | AF163613 | " |
| | | Japan | AF163594 | AF163610 | " |
| *A. novae-zelandiae* | Australia | Australia | AF329923 | - | Coetzee *et al* (2001) |
| | | Mt. Wellington, Tasmania | AF454739 | - | Dunne *et al.* (2002) |
| | New Zealand | South Island, New Zealand | AF329925 | - | Coetzee *et al* (2001) |
| | | North Island, New Zealand | AF239926 | - | " |
| | South America | Grand Isla de Chiloe, Chile | AF448417 | - | Coetzee *et al.* (2003) |
| | | Grand Isla de Chiloe, Chile | AF448418 | - | " |

**Figure 1.** Neighbour-Joining tree generated from the *Armillaria* ITS data matrix. Numbers in brackets are GenBank accession numbers. Bootstrap values are given above the tree branches. Dots (•) denote isolates previously shown to belong to SIG II (Mohammed *et al.* 1989, 1994, Abomo-Ndongo and Guillaumin 1997).

ITS NJ tree

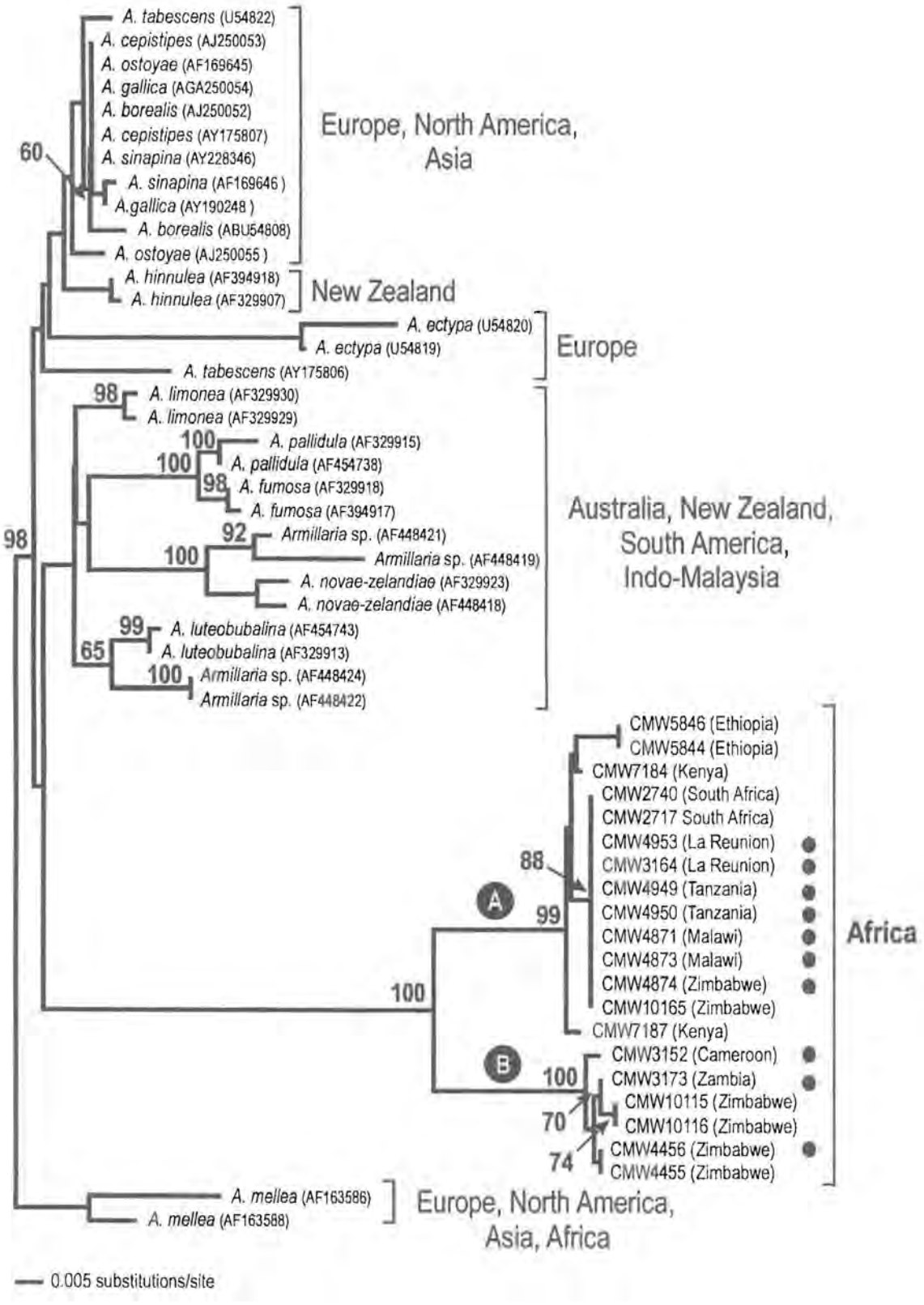

A. tabescens (U54822)
A. cepistipes (AJ250053)
A. ostoyae (AF169645)
A. gallica (AGA250054)
A. borealis (AJ250052)
A. cepistipes (AY175807)
A. sinapina (AY228346)
A. sinapina (AF169646 )
A.gallica (AY190248 )
A. borealis (ABU54808)
A. ostoyae (AJ250055 )
Europe, North America, Asia
A. hinnulea (AF394918)
A. hinnulea (AF329907)
New Zealand
A. ectypa (U54820)
A. ectypa (U54819)
Europe
A. tabescens (AY175806)
A. limonea (AF329930)
A. limonea (AF329929)
A. pallidula (AF329915)
A. pallidula (AF454738)
A. fumosa (AF329918)
A. fumosa (AF394917)
Armillaria sp. (AF448421)
Armillaria sp. (AF448419)
A. novae-zelandiae (AF329923)
A. novae-zelandiae (AF448418)
A. luteobubalina (AF454743)
A. luteobubalina (AF329913)
Armillaria sp. (AF448424)
Armillaria sp. (AF448422)
Australia, New Zealand, South America, Indo-Malaysia
CMW5846 (Ethiopia)
CMW5844 (Ethiopia)
CMW7184 (Kenya)
CMW2740 (South Africa)
CMW2717 South Africa)
CMW4953 (La Reunion)
CMW3164 (La Reunion)
CMW4949 (Tanzania)
CMW4950 (Tanzania)
CMW4871 (Malawi)
CMW4873 (Malawi)
CMW4874 (Zimbabwe)
CMW10165 (Zimbabwe)
CMW7187 (Kenya)
CMW3152 (Cameroon)
CMW3173 (Zambia)
CMW10115 (Zimbabwe)
CMW10116 (Zimbabwe)
CMW4456 (Zimbabwe)
CMW4455 (Zimbabwe)
Africa
A. mellea (AF163586)
A. mellea (AF163588)
Europe, North America, Asia, Africa
0.005 substitutions/site

**Figure 2.** Cladograms generated from the African ITS and IGS-1 data matrixes. ITS MP tree: One of seven most parsimonious (MP) trees obtained from the ITS dataset, tree length = 161 steps, consistency index (CI) = 0.959 and retention index (RI) = 0.983. IGS-1 MP tree: One of two MP trees obtained from the IGS-1 data matrix, tree length = 187 steps, CI = 0.936 and RI = 0.983. Bootstrap values are given below and branch lengths above the tree branches. Additional characteristics: I) isolate numbers from previous publications, II) sexual system; Ψ = homothallic and ∂ = heterothallic (Mohammed *et al.* 1989, Abomo-Ndongo *et al.* 1997), III) temperature sensitivity; ❄ = thermophobic and ☼ = thermophilic (Guillaumin 1992), IV) grouping of African isolates based on protein, esterase and RAPD analysis as well as Southern hybridization (Mohammed 1992), V) grouping of African isolates according to their physiological, morphological and sexual systems (Guillaumin 1992), VI) grouping of Zimbabwean isolates based on their morphological and biochemical characteristics (Mwenje and Ride 1996) and VII) IGS-1 amplicon sizes in bp. (Coetzee *et al.* 2000a, Mwenje *et al.* 2003, and this study).

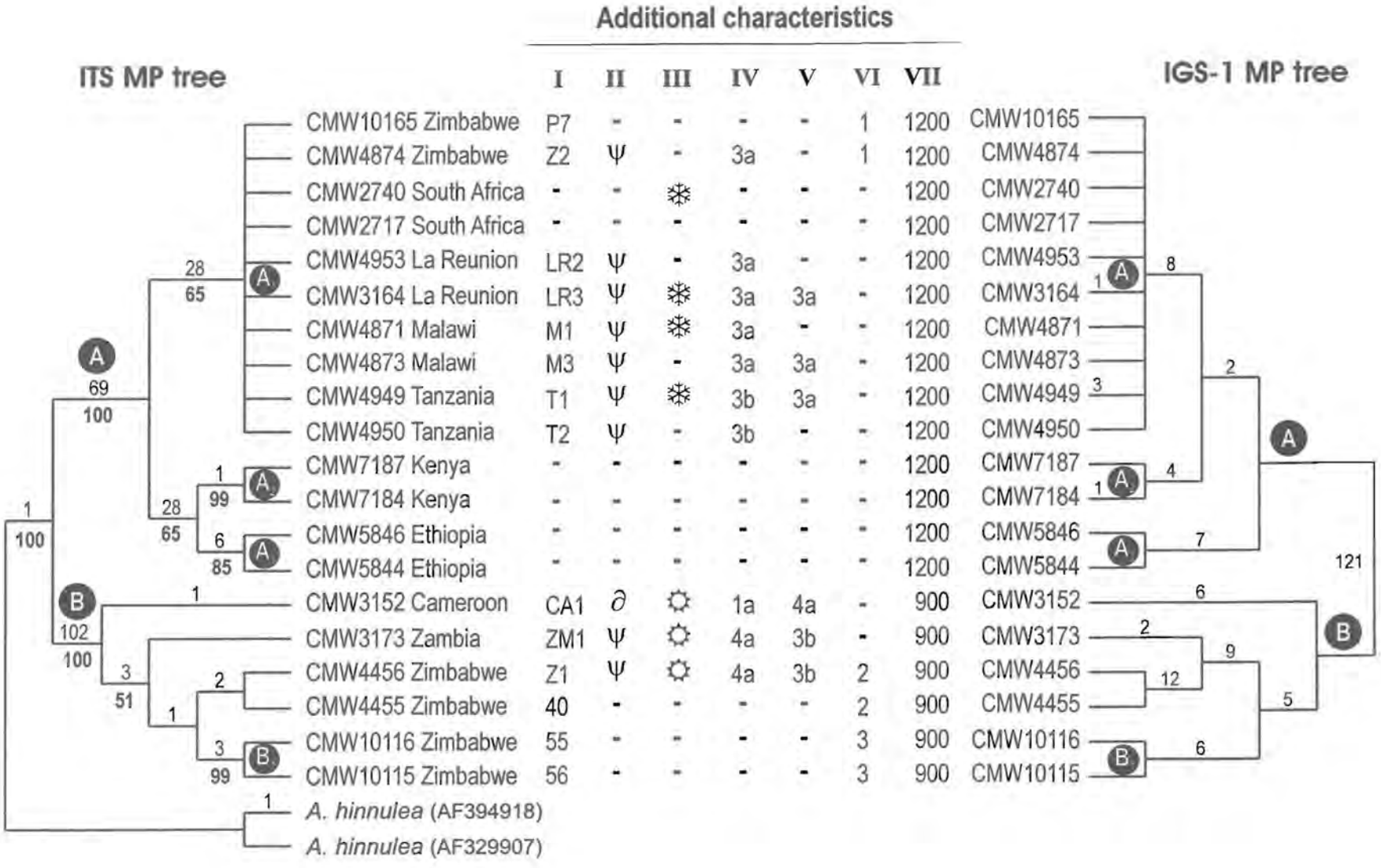


**Figure 3.** Neighbour-Joining trees generated from the African ITS and IGS-1 data matrixes. Bootstrap values are given below the tree branches.

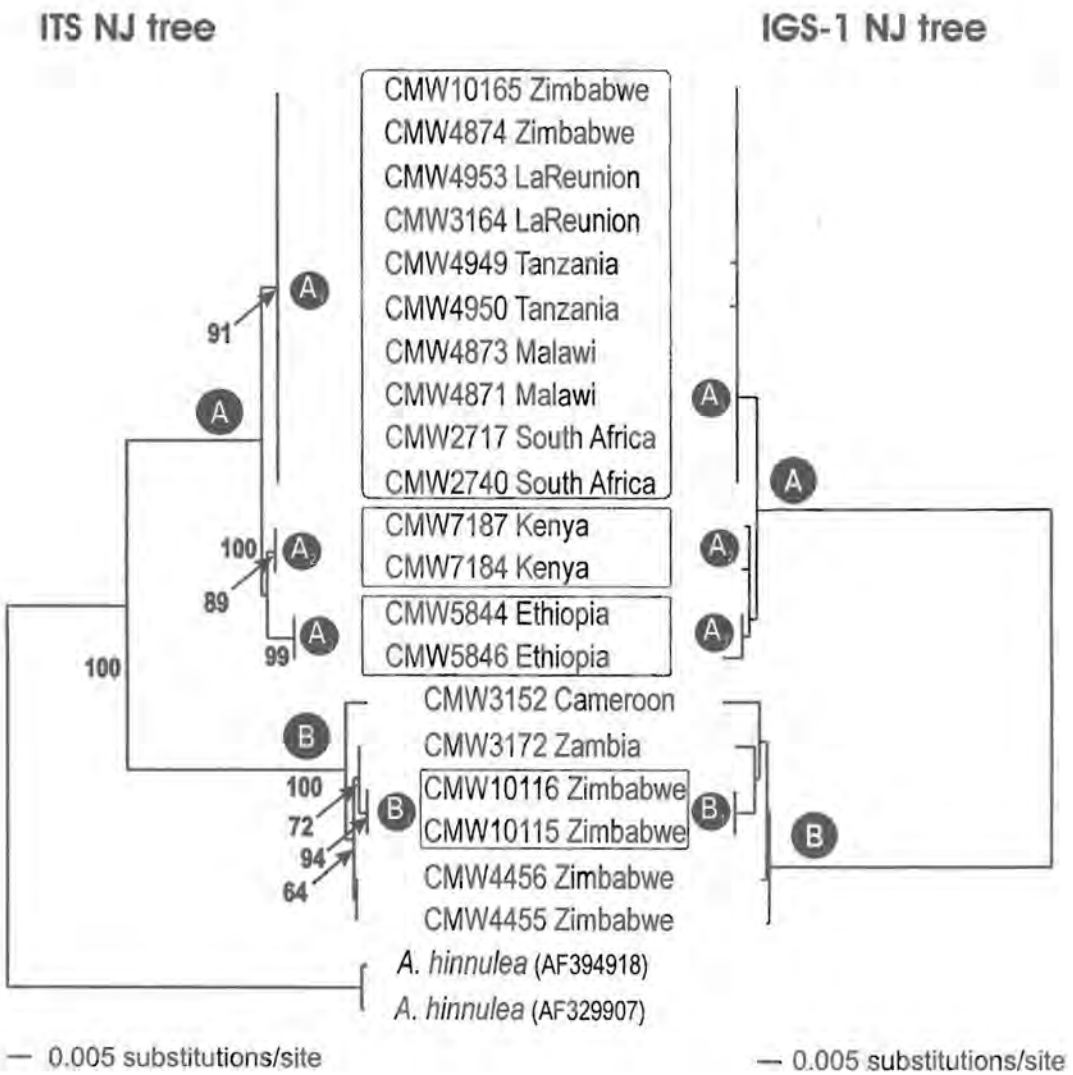
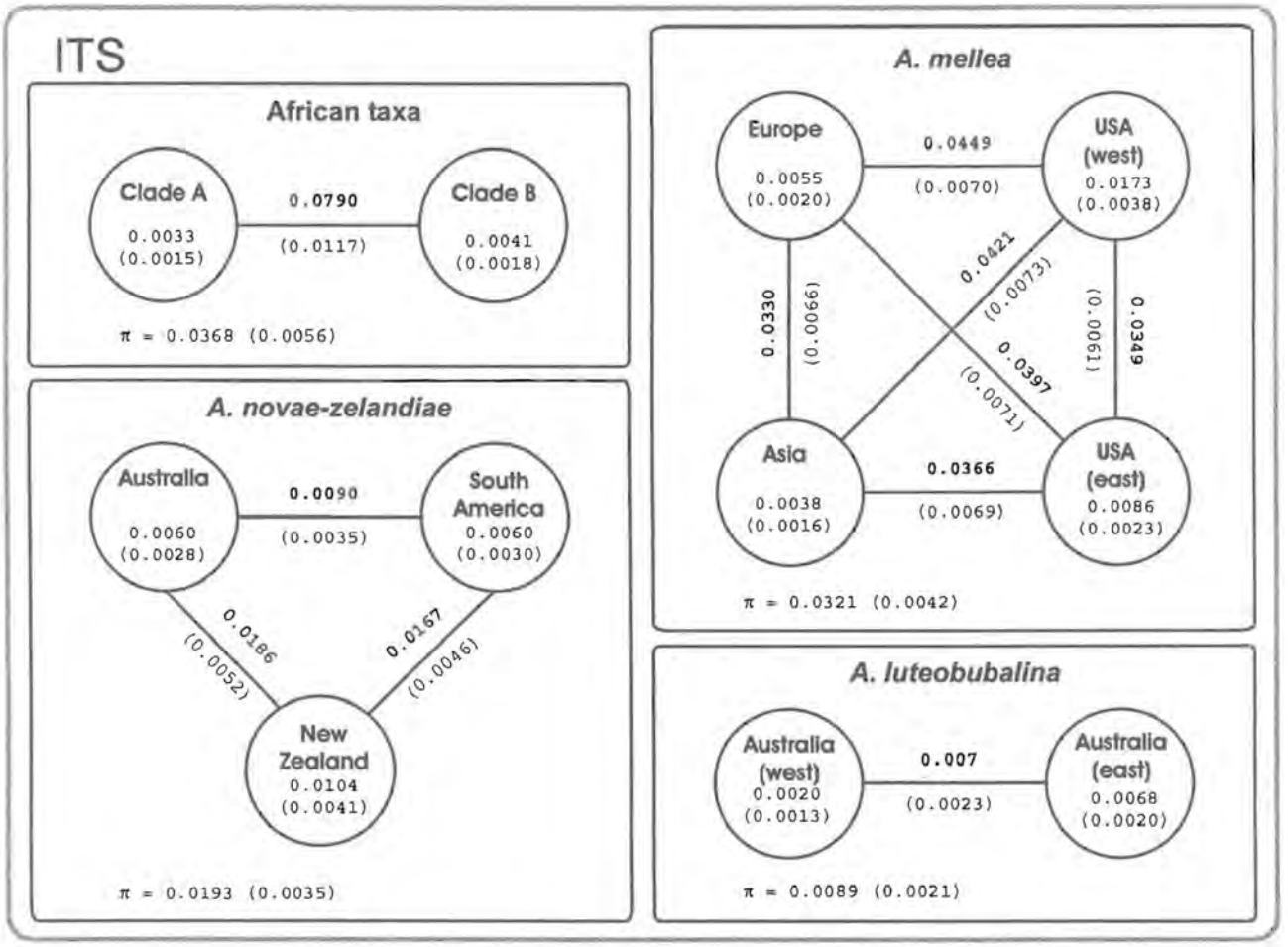
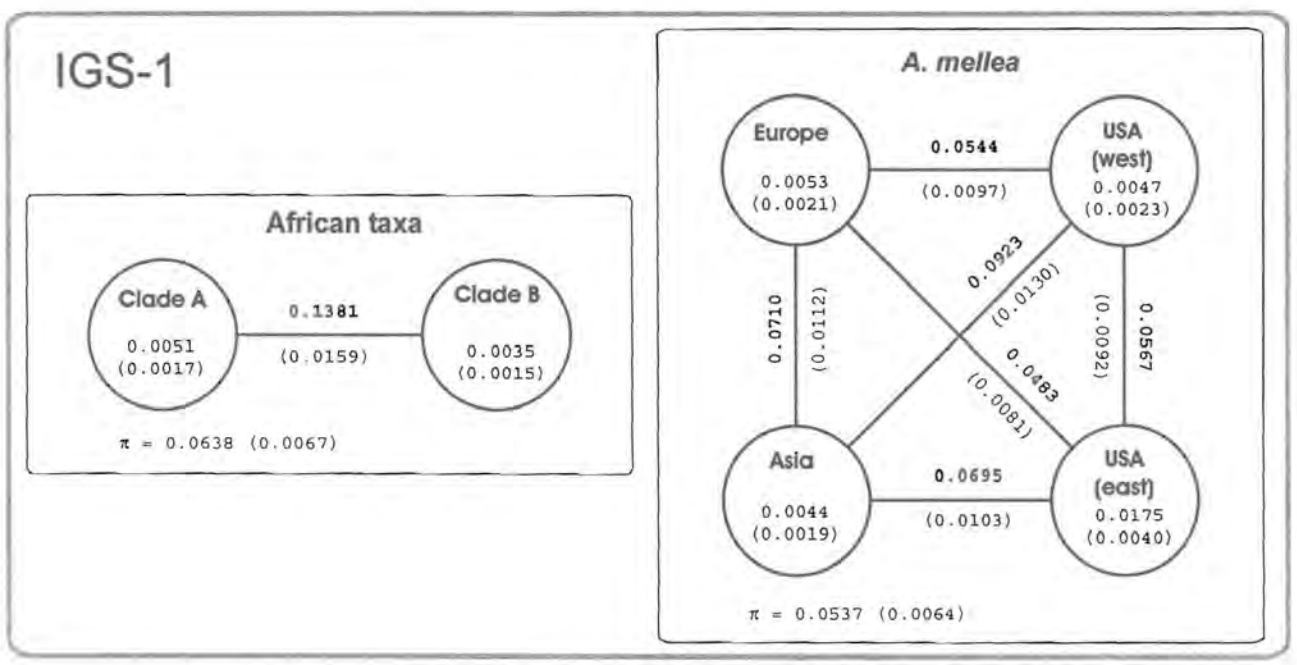

ITS NJ tree
IGS-1 NJ tree
CMW10165 Zimbabwe
CMW4874 Zimbabwe
CMW4953 LaReunion
CMW3164 LaReunion
CMW4949 Tanzania
CMW4950 Tanzania
CMW4873 Malawi
CMW4871 Malawi
CMW2717 South Africa
CMW2740 South Africa
CMW7187 Kenya
CMW7184 Kenya
CMW5844 Ethiopia
CMW5846 Ethiopia
CMW3152 Cameroon
CMW3172 Zambia
CMW10116 Zimbabwe
CMW10115 Zimbabwe
CMW4456 Zimbabwe
CMW4455 Zimbabwe
A. hinnulea (AF394918)
A. hinnulea (AF329907)
A
A1
A2
B
B1
91
100
89
99
100
100
72
94
64
— 0.005 substitutions/site
— 0.005 substitutions/site

**Figure 4.** Nucleotide diversity ($\pi$) within global populations and mean evolutionary distances between and within groups based on ITS and IGS-1 sequence data. Values within circles are the intragroup distance and those above the lines connecting the groups, the intergroup distances. Standard deviations are presented in brackets.


ITS
African taxa
Clade A
0.0033
(0.0015)
0.0790
(0.0117)
Clade B
0.0041
(0.0018)
π = 0.0368 (0.0056)
A. novae-zelandiae
Australia
0.0060
(0.0028)
0.0090
(0.0035)
South America
0.0060
(0.0030)
0.0186
(0.0052)
0.0167
(0.0046)
New Zealand
0.0104
(0.0041)
π = 0.0193 (0.0035)
A. mellea
Europe
0.0055
(0.0020)
0.0449
(0.0070)
USA (west)
0.0173
(0.0038)
0.0330
(0.0066)
0.0421
(0.0073)
0.0397
(0.0071)
0.0349
(0.0061)
Asia
0.0038
(0.0016)
0.0366
(0.0069)
USA (east)
0.0086
(0.0023)
π = 0.0321 (0.0042)
A. luteobubalina
Australia (west)
0.0020
(0.0013)
0.007
(0.0023)
Australia (east)
0.0068
(0.0020)
π = 0.0089 (0.0021)


IGS-1
African taxa
Clade A
0.0051
(0.0017)
0.1381
(0.0159)
Clade B
0.0035
(0.0015)
π = 0.0638 (0.0067)
A. mellea
Europe
0.0053
(0.0021)
0.0544
(0.0097)
USA (west)
0.0047
(0.0023)
0.0710
(0.0112)
0.0923
(0.0130)
0.0483
(0.0081)
0.0567
(0.0092)
Asia
0.0044
(0.0019)
0.0695
(0.0103)
USA (east)
0.0175
(0.0040)
π = 0.0537 (0.0064)

# CHAPTER SIX

# DISCOVERY OF TWO NORTHERN HEMISPHERE *ARMILLARIA* SPECIES ON PROTEACEAE IN SOUTH AFRICA

Published: *Plant Disease* **52**: 604 – 612 (2003)

# DISCOVERY OF TWO NORTHERN HEMISPHERE *ARMILLARIA* SPECIES ON PROTEACEAE IN SOUTH AFRICA

## ABSTRACT

Armillaria root rot symptoms were observed on native *Protea* and *Leucadendron* (Proteaceae) species in Kirstenbosch Botanical Gardens in the Western Cape Province of South Africa. Intergenic spacer (IGS)-1 polymerase chain reaction (PCR) restriction fragment-length polymorphisms (RFLP) profiling indicated the presence of at least two *Armillaria* species. The profiles of two isolates were identical to those of *A. mellea* s. str., originating in Europe. Phylogenetic analyses incorporating internal transcribed spacer (ITS) and IGS-1 sequence data identified the remaining isolates as closely related to *A. calvescens*, *A. gallica, A. jezoensis* and *A. sinapina*. These isolates displayed mating compatibility with *A. gallica*. From the RFLP profiles, sequencing results and sexual compatibility studies, it is concluded that the two species on Proteaceae in Kirstenbosch represent *A. mellea* and *A. gallica*. These are Northern Hemisphere fungi that have apparently been accidentally introduced into South Africa. This is the second report of *Armillaria* being introduced into South Africa. The introduction probably occurred early in the colonization of Cape Town, when potted plants from Europe were used to establish gardens.

***Keywords:*** *Armillaria gallica*, *Armillaria mellea*, IGS, ITS, mating compatibility tests, RFLP.

## INTRODUCTION

The Proteaceae represent one of the most interesting and prominent families of the flowering plants in the Southern Hemisphere. In the South-Western Cape region (fynbos biome) of South Africa alone, the family encompasses 14 genera and 330 species (Rebelo 1995). Disease reports dating back to the beginning of the 1900's have indicated that native Proteaceae in South Africa are affected by a large number of plant pathogens, mainly host-specific. These include pathogens causing leaf speck, leaf blotch, leaf spot, shoot and stem diseases, as well as soil-borne diseases (Knox-Davies *et al.* 1987, Taylor and Crous 2000, Denman *et al.* 2003). Amongst the most important root rot pathogens known on native Proteaceae is the omnivorous oomycete *Phytophthora cinnamomi,* which has an extremely wide host range on this family of plants (Von Broembsen 1984). Recent studies have also suggested that *P. cinnamomi* has been introduced into South Africa (Linde *et al.* 1997), and this might account for the very high levels of susceptibility of Proteaceae occurring in this region.

The present study concerns dying *Protea* and *Leucadendron* plants that were encountered in planted beds of the internationally renowned Kirstenbosch Botanical Gardens in Western Cape Province, in May 2000. In South Africa, dying Proteaceae with obvious root disease are generally attributed to *P. cinnamomi* infections. In a disease report by Denman *et al.* (2000) roots of the affected plants were blackened and lacked feeder roots typical of Phytophthora root rot. Removal of the bark, however, revealed white mycelial fans in the cambial region, characteristic of the root-infecting pathogen *Armillaria.* Basidiocarps of *Armillaria* were not found in the vicinity of the infected plants, making field identification of the *Armillaria* species impossible.

Armillaria root rot is a well-known problem on Proteaceae in different regions of the world. These include Australia (Porter *et al.* 1996), California (Farr *et al.* 1989), Hawaii (Laemmlen and Bega 1974), Kenya (Denman *et al.* 2000), Madeira (Moura and Rodriques 2001), New Zealand (Pennycook 1989), Tanzania (Denman *et al.* 2000) and Zimbabwe (Masuka *et al.* 1998). In countries where this disease occurs in commercial protea cut-flower plantations, losses are of economic significance to the producers. In South Africa, Armillaria root rot of Proteaceae has

been reported only once, but this was on *Grevillea robusta* (Doidge 1950), a tree species of Australian origin.

Identification of *Armillaria* species based on morphology is generally considered to be difficult. This is because basidiocarps of the fungus are short-lived and infrequently produced. Sexual compatibility tests between haploid tester strains of known identity and haploid or diploid field isolates provide an alternative to identification based on basidiocarp morphology (Korhonen 1978, Anderson and Ullrich 1979). These tests gained much acceptance due to their simplicity, but they are time-consuming and results are often ambiguous. This is especially true of diploid field isolates. Molecular-based identification techniques offer an effective alternative to sexual compatibility tests due to their time-efficient, relatively simple and informative nature (Harrington and Wingfield 1995, Coetzee *et al.* 2003).

A preliminary report on the discovery of Armillaria root rot on Proteaceae in the Kirstenbosch Botanical Gardens of South Africa was published by Denman *et al.* (2000). This report discussed general taxonomy, epidemiology and distribution of *Armillaria* on Proteaceae. The species causing the disease, however, could not be identified at the time. The aim of the present study was to identify the species on affected *Protea* and *Leucadendron* species in Kirstenbosch Botanical Gardens, based on intergenic spacer (IGS)-1 restriction fragment-length polymorphism (RFLP) comparisons, their phylogenetic relationships using IGS-1 and internal transcribed spacer (ITS) sequence data, and their sexual compatibility with other species of *Armillaria*.

## MATERIALS AND METHODS

### Isolation and cultivation of fungal isolates

Small pieces of white mycelium were removed from below the bark on the roots of dead and dying *Leucadendron* and *Protea* species from Kirstenbosch Botanical Gardens (33°59'S, 18°26'E, altitude 89 m) and placed on selective Dichloran-Benomyl-Streptomycin (DBS) medium (Harrington *et al.* 1992). Isolates were then incubated in the dark at 24 °C for 2 weeks. Rhizomorph tips that developed from the primary cultures were transferred to DBS-medium and further incubated under the same conditions. This procedure was repeated until pure cultures were obtained. Cultures were maintained on malt extract yeast agar (MYA): 1% malt extract, 0.2% yeast extract and 1.5% agar (Biolab, Midrand, Johannesburg, Republic of South Africa).

### DNA extractions

Cultures were grown in liquid MY medium (1% malt extract, 0.2% yeast extract) for 4 weeks in the dark at 24 °C. Mycelium was harvested using a sterilized metal strainer, frozen at -70 °C for 20 min and lyophilized. The dry mycelium was ground to a fine powder in liquid nitrogen and stored at -70 °C. DNA extraction from the powdered mycelium was performed using the method described by Coetzee *et al.* (2000b).

### PCR amplification

The ITS regions ITS-1 and ITS-2 and the 5.8S gene, situated between the small and large subunits of the ribosomal DNA operon, were amplified using the primer set ITS1/ITS4 (White *et al.* 1990). The intergenic spacer region one (IGS-1) of the rRNA operon was amplified using primer sets P-1 (Hsiau 1996) and 5S-B (Coetzee *et al.* 2000a), and CLR12R (Veldman *et al.* 1981) and O-1 (Duchesne and Anderson 1990). Primers P-1 and O-1 were alternatively used to obtain IGS-1 PCR fragments for RFLP comparison with profiles published by Coetzee *et al.* (2001). The PCR reaction mixture (50 µL, final volume) included dNTP (200 µM of each), *Taq* DNA Polymerase (2.5 U) (Roche Diagnostics, Mannheim, Germany), *Taq* DNA Polymerase buffer containing $MgCl_2$ (supplied with the enzyme), additional $MgCl_2$ (2.5 mM), 0.1 µM of each primer, and approximately 80 ng of template DNA. The thermocycling (Perkin Elmer 9600) conditions were an initial denaturation at 94 °C for 2 min, followed by 30 cycles of 58 °C (ITS amplification) or 64 °C (IGS-1 amplification) for 30 s (annealing), 72 °C for 30 s (elongation) and 94 °C for 30 s (denaturation). A final elongation was allowed for 7 min at 72 °C. The quality and sizes of ITS and IGS-1 PCR products were determined on an agarose gel (1% agarose) stained with ethidium bromide and visualized under UV illumination.

### Restriction enzyme digestion of the PCR products

The IGS-1 amplicons were digested with the restriction endonuclease *Alu*I. Ten units of restriction enzyme were added to the amplified PCR fragments within the PCR reaction mix (20 µL) and incubated at 37 °C for 6 h. Resulting restriction fragments were separated on 2.5% agarose gel stained with ethidium bromide and visualized under UV illumination. Both

CLR12R/O-1 and P-1/O-1 RFLP profiles were compared with those previously observed for *Armillaria* species from North America, Europe and Asia.

### DNA sequencing and sequence analysis

Sequences for the ITS and the IGS-1 DNA regions were determined using an ABI PRISM™377 DNA automated sequencer. The ITS regions were sequenced in both directions using primers ITS1 and ITS4 and internal primers CS2B and CS3B (Coetzee *et al.* 2000b). The IGS-1 region was sequenced using primers CLR12R and O-1 as well as internal primers MCO-2 and MCO-2R (Coetzee *et al.* 2000b). The sequence reactions were carried out using an ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerases, FS (Applied Biosystems, Johannesburg, RSA) according to the manufacturer's directions.

The ITS and IGS-1 sequence data available for *Armillaria* spp. were obtained from GenBank (Table 1). These sequences were aligned against ITS and IGS-1 sequences for isolates CMW7202 and CMW7204 [GenBank accession numbers: AY190247 and AY190248 (ITS); AY190245 and AY190246 (IGS-1)] from Kirstenbosch Botanical Gardens using Clustal W (Thompson *et al.* 1994) and manually adjusted. Missing and ambiguously aligned regions were excluded before analysing the ITS and IGS-1 data sets. Distance and parsimony phylogenetic analyses were performed to determine the identity and phylogenetic relationships between the isolates from Kirstenbosch and other *Armillaria* spp. Distance analyses were based on the Neighbour-Joining search algorithm using the BioNJ method and the HKY85 nucleotide substitution model (Hasegawa *et al.* 1985). Phylogenetic trees were generated in parsimony analysis by using the heuristic search method with TBR (tree bisection reconnection) branch swapping, MULPARS active, random addition of sequences (100 replicates) and gaps treated as missing data. Bootstrap analysis (1000 replicates) was carried out to determine the support at each branching point on the phylogenetic trees. Trees generated from the ITS and IGS-1 data sets were rooted with *A. tabescens* as monophyletic sister outgroup.

### Diploid-haploid compatibility tests

Pairings between diploid isolates from Kirstenbosch Botanical Gardens were made with *Armillaria* haploid tester strains to confirm the results emerging from DNA based identification. Compatible reactions between the diploid isolates and the haploid tester strains were determined

using the methods of Rizzo and Harrington (1992). In this study mycelial plugs of two representative diploid cultures from Kirstenbosch Botanical Gardens (CMW7202 and CMW7204) were paired in all combinations with North American and European haploid tester strains. Paired cultures were incubated for 4 weeks at 24 °C in the dark prior to evaluating the results.

## RESULTS

### Fungal isolations and cultures

Eighteen isolates were obtained from dead and dying Proteaceae in Kirstenbosch Botanical Gardens. Infected plants included *Leucadendron argenteum*, *L. gandogeri*, *L. grandiflorum*, *Protea longifolia*, *P. eximia* and *P. scolymocephala*. Isolates obtained from these plants are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa. Representative isolates have also been deposited at the Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands.

### PCR amplification and RFLP analysis

The IGS-1 and ITS regions were successfully amplified for all the isolates. PCR amplification with primer set P-1/5S2B yielded no amplicons for any of the isolates included in this study. The IGS-1 amplicon size (using primers CLR12R/O-1) was 900 bp for all isolates with the exception of CMW7206 and CMW7207 which had an IGS-1 amplicon of 800 bp. The ITS region was 800 bp in size for all the isolates in this study.

Two different IGS-1/RFLP profiles were obtained for the isolates from Kirstenbosch Botanical Gardens and they were therefore separated into two groups. The first group consisted of isolates CMW7206 and CMW7207 and had fragment sizes of 215, 175 and 150 bp after digestion of their P-1/0-1 amplicons with *Alu*I. These fragment sizes were identical to those of *A. mellea* s. str. (Coetzee *et al.* 2001).

Isolates in the second group showed RFLP profiles with fragment sizes of 427 (417-441), 236 (233-238) and 183 (180-185) bp after digestion of their CLR12R/O-1 amplicons. Exact fragment sizes were calculated by mapping the restriction sites on the IGS-1 sequence data, and

yielded values of 397, 233 and 182 bp. These RFLP fragment sizes were closest to those published for *A. sinapina* (White *et al.* 1998), *A. calvescens* (Kim *et al.* 2001), *A. jezoensis* (Terashima *et al.* 1998b) and *A. gallica* (Harrington and Wingfield, 1995, Banik *et al.* 1996, White *et al.* 1998). The identity of isolates in this group could not therefore be determined by means of their RFLP profiles. Two representative isolates (CMW7202 and CMW7204) were subsequently chosen from this group and subjected to further investigation.

## DNA sequence analysis

### *IGS-1 sequence data*

The number of characters included in the IGS-1 data set after alignment was 750. The number of characters included in distance analysis was 483 after exclusion of missing and ambiguously aligned regions, and 147 parsimony informative characters were included in parsimony analysis. Trees generated after heuristic and Neighbour-Joining analyses grouped the Kirstenbosch isolates, CMW7202 and CMW7204, within a clade that included *A. cepistipes*, *A. jezoensis*, *A. sinapina* and *A. singula* from Japan (51% bootstrap support for the NJ tree and 61% for the MP tree) (Fig. 1). These two Kirstenbosch isolates formed a strongly supported (97% bootstrap support for the NJ tree; 83% for the MP tree) monophyletic group with *A. sinapina* and *A. cepistipes* within the Japanese clade.

### *ITS sequence data*

The ITS data set included 899 characters after alignment by inserting gaps. There were 715 characters included in distance analysis. Parsimony analysis was based on 31 parsimony informative characters. Neighbour-Joining trees generated from the ITS data placed the Kirstenbosch isolates (CMW7202 and CMW7204) as sister group to *A. gallica* (U54812) with a bootstrap support of 71% (Fig. 2a). These isolates from Kirstenbosch formed a monophyletic group with *A. gallica* (U54812) in parsimony analysis, supported by a 59% bootstrap value. The Kirstenbosch isolates (CMW7202 and CMW7204), together with *A. gallica* (U54812) formed a sister group with other representative isolates of this species, although this relationship did not have strong bootstrap support (Fig. 2b).

### Diploid-haploid compatibility tests

The choice of haploid testers strains used in the mating study was based on the close phylogenetic relationship between the Kirstenbosch isolates (CMW7202 and CMW7204) and *A. cepistipes*, *A. sinapina* and *A. gallica*. *Armillaria gemina*, which is not closely related to the Kirstenbosch isolates, was included as negative control. Tester strains of *A. gallica* (CMW3163 and CMW6902) were sexually compatible with the Kirstenbosch isolates (CMW7202 and CMW7204), and their culture morphology changed to brown pigmented and depressed mycelia, indicating successful diploidisation (Fig. 3a). Sub-cultures made from three different areas from this diploidised tester retained the overall diploid culture morphology but some white aerial mycelium was observed (Fig. 3b). No sexually compatible interaction was observed between the Kirstenbosch isolates and the haploid tester strains of *A. sinapina* (CMW3156), *A. cepistipes* (CMW3161) and *A. gemina* (CMW3181) (Fig. 3a). The tester strains of these species retained their typical haploid white and fluffy aerial mycelium when paired with the diploid isolates from Kirstenbosch. After pairing, the culture morphology of the two haploid *A. gallica* tester strains was transformed from white and fluffy abundant aerial mycelium to crustose depressed pigmented mycelium, indicating successful diploidisation (Fig. 3a).

## DISCUSSION

Results of this study have shown that two species of *Armillaria* are implicated in the death of *Protea* and *Leucadendron* species in the historically and internationally important Kirstenbosch Botanical Gardens of South Africa. These species, *A. mellea* and *A. gallica,* are both known to be native to the Northern Hemisphere, so have clearly been introduced into the gardens. This finding is intriguing, although not without precedent in the Cape Province of South Africa. It was recently shown that *Armillaria mellea* s. str. was introduced into the Dutch East India Company Gardens in the centre of Cape Town approximately 300 years ago (Coetzee *et al.* 2001). This was probably with citrus plants brought from Europe to provide a source of vitamin C for sailors. In the present study, an additional Northern Hemisphere species, *A. gallica*, was identified. It is reasonable to assume that this species was introduced during the early settlement of Cape Town, as it is known that potted plants were introduced into the area during this period.

The original expectation was that the *Armillaria* species in Kirstenbosch would represent an African species. Primer set P-1/5S2B was previously reported to amplify the IGS-1 region of

African *Armillaria* species, therefore it was initially utilized (Coetzee *et al.* 2000a). However, PCR amplification of the IGS-1 region for all Kirstenbosch isolates, using this primer set, produced negative results. In contrast, primer set CLR12R/O-1 resulted in successful amplification of the IGS-1 region in all isolates. Primer set P-1/5S2B amplifies the IGS-1 region only when the 5S gene is inverted in relation to the other genes in the rRNA operon, as was found in native African *Armillaria* species (Coetzee *et al.* 2000a). Negative results provided us with early evidence that a non-African *Armillaria* species was present in Kirstenbosch Botanical Gardens.

The PCR-RFLP method developed by Harrington and Wingfield (1995) for rapid identification of *Armillaria* species was used in a preliminary analysis to determine the identity of the species present in Kirstenbosch Botanical Gardens. Two different IGS-1/RFLP profiles were observed for the isolates. Profiles obtained for two of the isolates were identical to *A. mellea* s. str. from Europe, as well as to those from the Company Gardens (Coetzee *et al.* 2001). These two isolates therefore represent *A. mellea* s. str. This is the second report of the fungus in a cultivated national heritage garden of South Africa.

*Armillaria mellea* s. str. is one of the most aggressive species in the genus, and tends not to be host-specific (Gregory *et al.* 1991). This fungus is restricted to the Northern Hemisphere, and its occurrence in the Company Gardens of Cape Town is the only previously recorded exception (Coetzee *et al.* 2001). One possible explanation for the presence of *A. mellea* s. str. in the Kirstenbosch Botanical Gardens is that it has spread from the Company Gardens, where it sporulates profusely. These areas are only a few kilometres apart, and such spread could have occurred via basidiospores. If this is the case, it would suggest that other occurrences of this European fungus might be encountered in the Cape Peninsula in the future. An alternative explanation is that the fungus was introduced independently into Kirstenbosch, with plants from the Northern Hemisphere. Additional isolates and genetic fingerprinting will be necessary to resolve this intriguing question.

The remaining 16 isolates from Kirstenbosch Botanical Gardens had identical IGS-1/RFLP profiles, suggesting that they all represent the same species. The identity of these isolates, however, was uncertain due to the similarity between their RFLP profiles and previously reported IGS-1/RFLP profiles of *A. calvescens, A. gallica, A. sinapina* and *A. jezoensis* (Harrington and Wingfield, 1995, Banik *et al.* 1996, Terashima *et al.* 1998b, White *et al.* 1998,

Kim *et al.* 2001). Phylogenetic trees generated from IGS-1 sequence data indicated that these isolates are closely related to *A. sinapina* and *A. cepistipes* from Japan. Terashima *et al.* (1998a) found that *A. sinapina, A. cepistipes, A. singula* and *A. jezoensis* from Japan grouped together in a strongly supported sub-clade (Japanese clade), which formed part of a major clade including *A. sinapina, A. cepistipes* and other *Armillaria* species from Europe and North America. In the present study, *Armillaria* isolates from Kirstenbosch, other than those representing *A. mellea*, grouped within this Asian clade. They are clearly of the same geographical lineage and it is, therefore, believed that they were introduced into Kirstenbosch Botanical Gardens from Japan.

Neighbour-Joining and parsimony trees generated from ITS sequence data indicated that the *Armillaria* isolates from Kirstenbosch are closely related to *A. gallica,* and might represent this species. This relationship, however, was not reflected in the IGS-1 phylogenetic trees. This discrepancy between ITS and IGS-1 data might be explained by the fact that GenBank contains IGS-1 sequence data for only one Japanese *A. gallica* isolate, and intraspecific variation could have rendered this isolate unrepresentative of other Japanese *A. gallica* isolates. In the present study, as well as that of Terashima *et al.* (1998a), this isolate grouped with *A. nabsnona* from North America and not with other *Armillaria* species within the Asian clade, as might have been expected. Discounting IGS-1 data, the results from ITS phylogenetic analyses strongly suggest that the Kirstenbosch isolates represent *A. gallica.*

In order to confirm the identity of the majority of isolates from Kirstenbosch Botanical Gardens as *A. gallica,* sexual compatibility tests were conducted. When two representative isolates were paired with haploid tester strains of *A. gallica,* a strong positive interaction emerged. These results indicate that the predominant *Armillaria* species in Kirstenbosch Botanical Gardens represents *A. gallica.*

*Armillaria gallica* has previously been reported from areas at low altitudes and at southern latitudes in North America, Europe and Japan (Guillaumin *et al.* 1989, Harrington and Rizzo 1993, Ota *et al.* 1998). This species is considered to be a weak pathogen but can act as secondary agent in mortality of hosts affected by biotic and abiotic stress (Rishbeth 1982, Gregory 1985). It is known that the *Protea* and *Leucadendron* species in Kirstenbosch Botanical Gardens are continually subjected to infection by *Phytophthora cinnamomi* (H. Jamieson, Kirstenbosch Botanical Gardens, South Africa, personal communication) and this could have provided the necessary predisposition to favour infection by *A. gallica.*

All evidence available to us suggests that *A. gallica* in Kirstenbosch Botanical Gardens would have originated in Asia. Although this garden was formally established in 1913, its history goes back to the early 1800s, when it was utilized as farmland. Chestnuts, oaks, fruit trees and vines were planted and cultivated at various stages on this land during that period. The introduction of *Armillaria* species would have been most likely to occur via soil with plants. Although the movement of potted plants has been restricted for many years, it is likely that they would have been introduced during the early establishment of Cape Town.

The discovery of *A. mellea* in the Company Gardens in Cape Town, was considered most unusual (Coetzee *et al.* 2001). This was particularly because root inhabiting basidiomycetes are not generally considered to be common invaders of new areas. Results of the present study suggest that such introductions during the early European colonization of South Africa might have been much more common than was previously realized. There are clearly fascinating discoveries to be made in this area of research, and many lessons to be learned in terms of global distribution of pathogens and quarantine procedures.

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TABLE 1: List of ITS and IGS-1 sequence obtained from GenBank for phylogenetic analyses.

| Species | GenBank number | Origin | Published by |
|---|---|---|---|
| **ITS data set** | | | |
| *A. borealis* | U54808 | Finland | Chillali *et al.* (1998) |
| | U54809 | France | " |
| | AJ250052 | Finland | Schmidt *et al.* (unpublished) |
| *A. cepistipes* | U54810 | France | Chillali *et al* (1998) |
| | U54811 | " | " |
| | AJ250053 | Poland | Schmidt *et al.* (unpublished) |
| *A. gallica* | U54812 | France | Chillali *et al.* (1998) |
| | U54814 | " | " |
| | AJ250054 | Italy | Schmidt *et al.* (unpublished) |
| *A. ostoyae* | U54813 | France | Chillali *et al.* (1998) |
| | U54815 | " | " |
| | U54816 | " | " |
| *A. sinapina* | AF169646 | NY, USA | Coetzee *et al.* (2000b) |
| *A. tabescens* | U54821 | France | Chillali *et al.* (1998) |
| | U54822 | Italy | " |
| **IGS-1 data set** | | | |
| *A. borealis* | AF243055 | Finland | Anderson and Stasovski (1992) |
| | AF243056 | Munich, Germany | " |
| *A. calvescens* | AF243070 | VT, USA | " |
| | AF243071 | " | " |
| *A. cepistipes* | AF243067 | France | " |
| | AF243068 | Helsinki, Finland | " |
| | AF243069 | Tampere, Finland | " |
| | D89919 | Mie, Japan | Terashima *et al.* (1998a) |
| *A. gemina* | AF243053 | VT, USA | Anderson and Stasovski (1992) |
| | AF243054 | VT, USA | " |
| *A. jezoensis* | D89921 | Hokkaido, Japan | Terashima *et al.* (1998a) |
| *A. gallica* | AF243064 | MI, USA | Anderson and Stasovski (1992) |

TABLE 1 (continued).

| Species | GenBank number | Origin | Published by |
|---|---|---|---|
| | AF243066 | VT, USA | Anderson and Stasovski (1992) |
| | D89920 | Hokkaido, Japan | Terashima *et al.* (1998a) |
| *A. nabsnona* | AF243059 | ID, USA | Anderson and Stasovski 1992 |
| | AF243060 | BC, Canada | " |
| NABS X | AF243061 | BC, Canada | " |
| | AF243062 | ID, USA | " |
| *A. ostoyae* | AF243051 | Denmark | " |
| | AF243052 | Michigan, USA | " |
| *A. sinapina* | AF243057 | BC, Canada | " |
| | AF243058 | NY, USA | " |
| | D89925 | Hokkaido, Japan | Terashima *et al.* (1998a) |
| *A. singula* | D89926 | Hokkaido, Japan | " |

**Figure 1.** Phylogenetic trees generated based on IGS-1 sequence data. Bootstrap values are indicated above the tree branches. Grey blocks indicate the grouping of the *Armillaria* isolates from Kirstenbosch with *A. sinapina* and *A. cepistipes* from Japan. a) Neighbour-Joining (NJ) tree generated from the data matrix. Branch lengths and scale bar below the tree correspond to distances measured as the proportion of the nucleotide substitutions between sequences. b) One of 12 most parsimonious (MP) trees with branch lengths indicated below the branches. Tree length (*TL*) = 193 steps, consistency index (*CI*) = 0.839 and retention index (*RI*) = 0.915. Abbreviations: OST (*A. ostoyae*), GEM (*A. gemina*), BOR (*A. borealis*), SIN (*A. sinapina*), CEP (*A. cepistipes*), NBX (NABS X), GAL (*A. gallica*), CAL (*A. calvescens*), JEZ (*A. jezoensis*), GUL (*A. singula*), NAB (*A. nabsnona*), TAB (*A. tabescens*) and KIR (*Armillaria* isolates from Kirstenbosch).

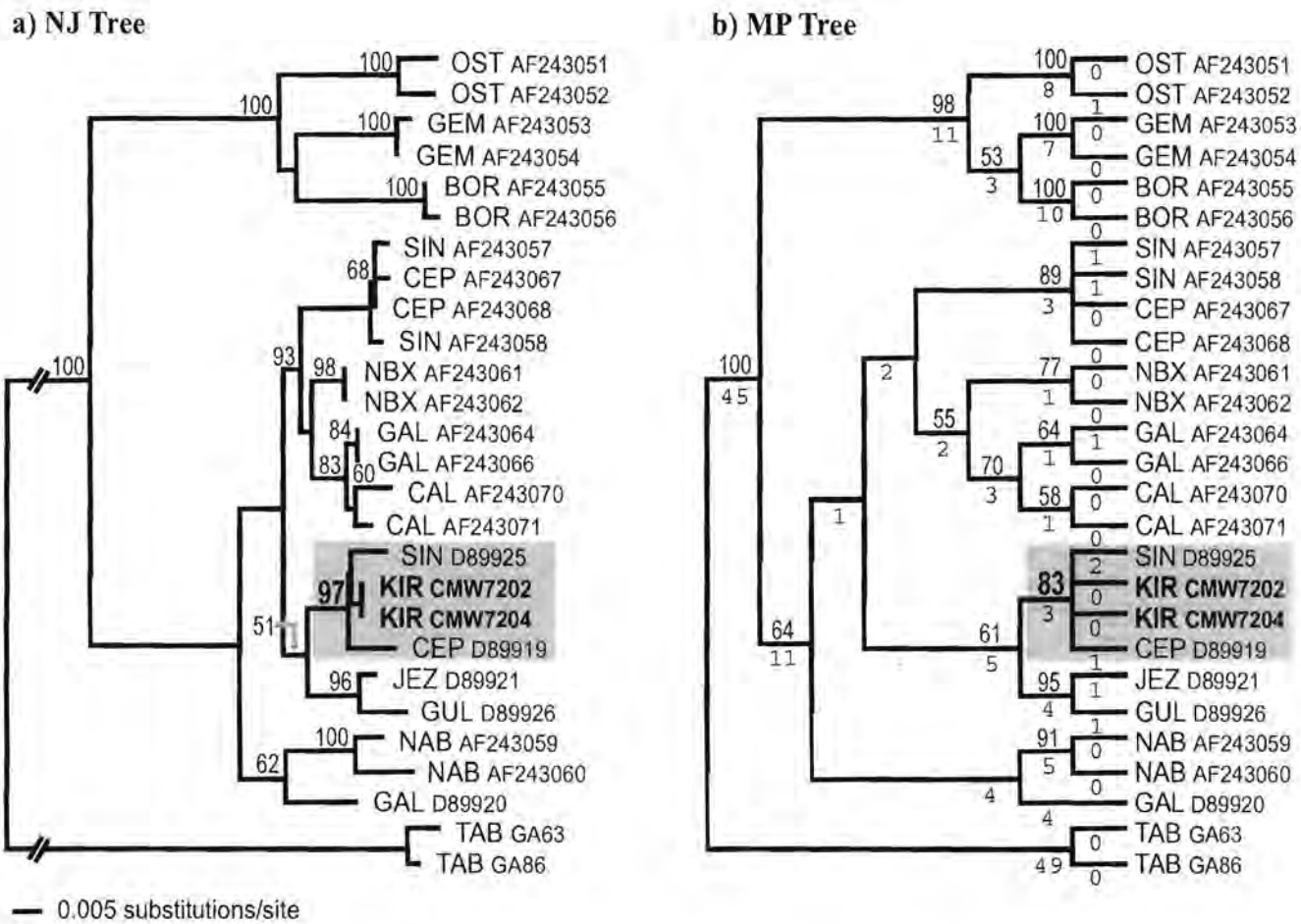
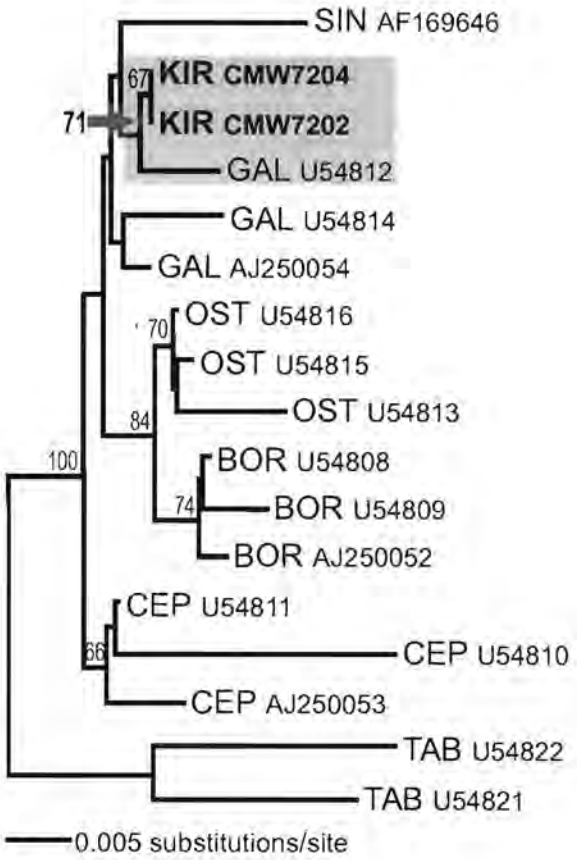

a) NJ Tree
OST AF243051
OST AF243052
GEM AF243053
GEM AF243054
BOR AF243055
BOR AF243056
SIN AF243057
CEP AF243067
CEP AF243068
SIN AF243058
NBX AF243061
NBX AF243062
GAL AF243064
GAL AF243066
CAL AF243070
CAL AF243071
SIN D89925
KIR CMW7202
KIR CMW7204
CEP D89919
JEZ D89921
GUL D89926
NAB AF243059
NAB AF243060
GAL D89920
TAB GA63
TAB GA86
0.005 substitutions/site
b) MP Tree
OST AF243051
OST AF243052
GEM AF243053
GEM AF243054
BOR AF243055
BOR AF243056
SIN AF243057
SIN AF243058
CEP AF243067
CEP AF243068
NBX AF243061
NBX AF243062
GAL AF243064
GAL AF243066
CAL AF243070
CAL AF243071
SIN D89925
KIR CMW7202
KIR CMW7204
CEP D89919
JEZ D89921
GUL D89926
NAB AF243059
NAB AF243060
GAL D89920
TAB GA63
TAB GA86

**Figure 2.** Phylogenetic trees generated based on ITS sequence data. Bootstrap values are indicated above the tree branches and grey blocks indicate the grouping of the *Armillaria* isolates from Kirstenbosch with *A. gallica*. a) NJ tree generated from the data matrix. Branch lengths and scale bar corresponds to the distance as a proportion of nucleotide substitutions between sequences. b) One of 138 MP trees with *TL* = 51 steps, *CI* = 0.667 and *RI* = 0.757. Branch lengths are indicated below the branches. Abbreviations are the same as in Fig. 2.

**a) NJ Tree**




**b) MP Tree**

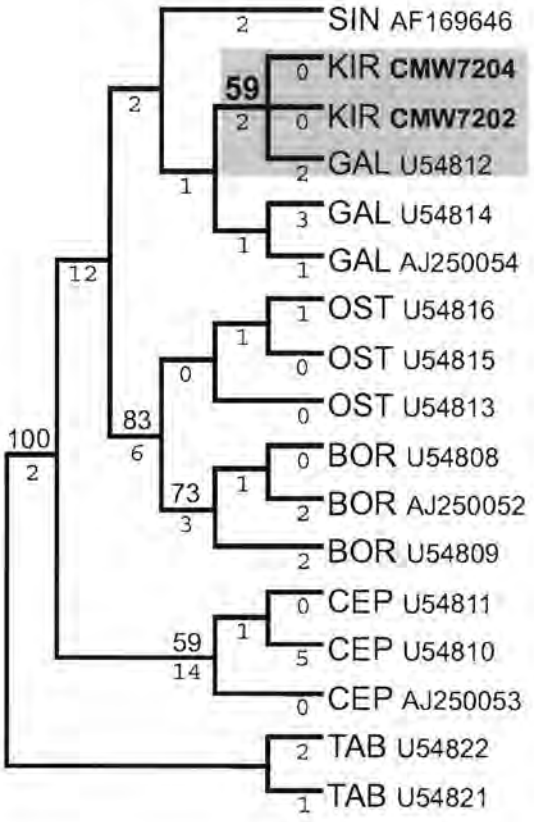
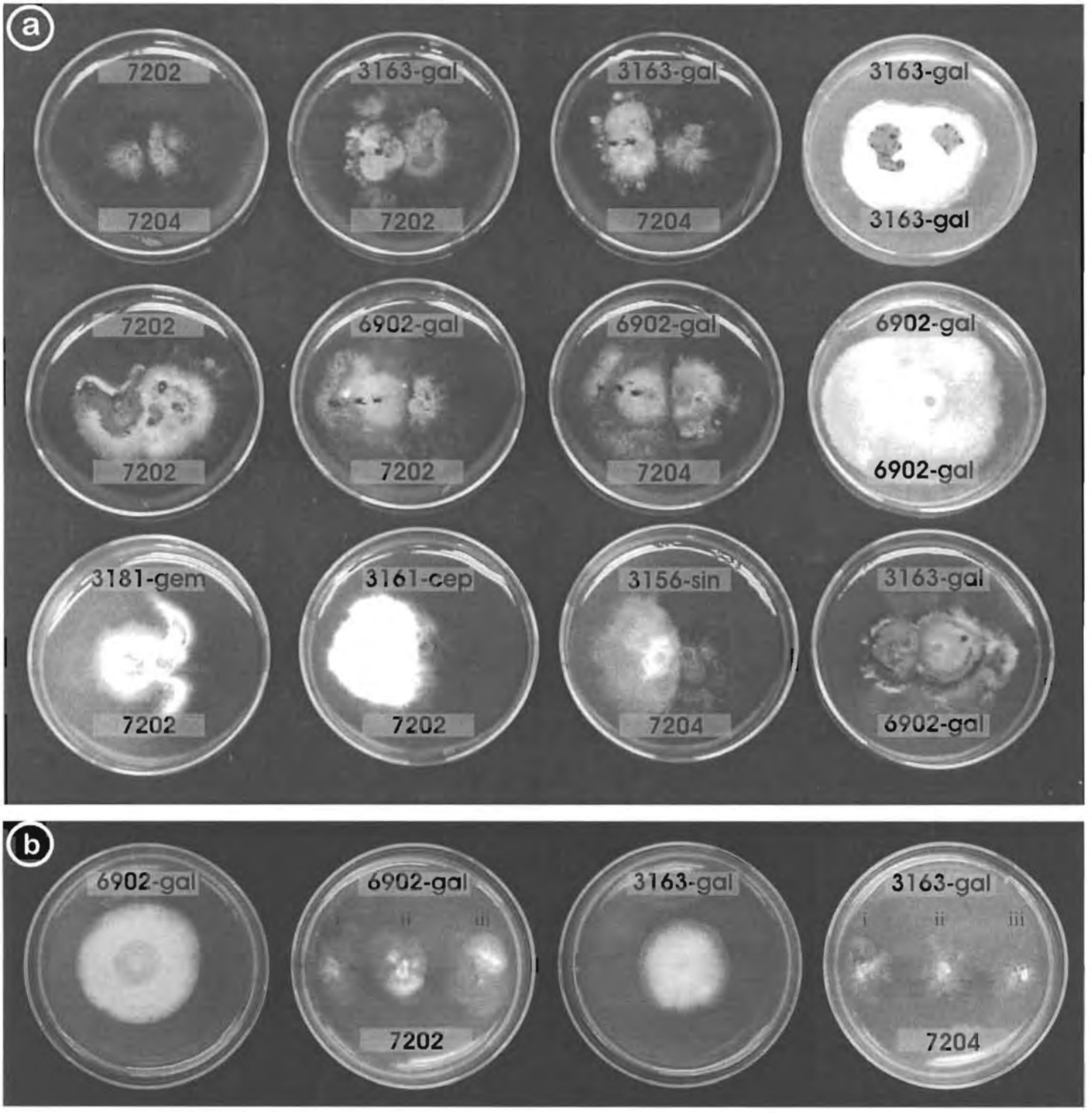


**Figure 3.** Sexual compatibility tests. a) Interaction between tester strains and isolates from Kirstenbosch. Numbers at the top and bottom of the Petri dish pertain to the inoculum at the left and right, respectively. b) Morphology of the secondary cultures made from the putative diploidized haploid tester strain. Inoculum at the left (i) was taken from the point behind the interaction between the tester and the isolate from Kirstenbosch Botanical Gardens. Middle inoculum (ii) was taken from a point away from the area of interaction and the inoculum at the right (iii) originates from the periphery of the tester culture.


a
7202
7204
3163-gal
7202
3163-gal
7204
3163-gal
3163-gal
7202
7202
6902-gal
7202
6902-gal
7204
6902-gal
6902-gal
3181-gem
7202
3161-cep
7202
3156-sin
7204
3163-gal
6902-gal
b
6902-gal
6902-gal
i ii iii
7202
3163-gal
3163-gal
i ii iii
7204

# CHAPTER SEVEN

## IDENTIFICATION OF *ARMILLARIA* ISOLATES FROM BHUTAN BASED ON DNA SEQUENCE COMPARISONS

Accepted: *Plant Pathology* (2003)

# IDENTIFICATION OF *ARMILLARIA* ISOLATES FROM BHUTAN BASED ON DNA SEQUENCE COMPARISONS

## ABSTRACT

Armillaria root rot is a serious disease in fir and mixed conifer forests of Bhutan, Eastern Himalayas. The species causing this disease have, however, never been identified. The aim of this study was to identify field isolates collected at four localities in Bhutan. Identification was based on RFLP analysis of the IGS-1 region, comparisons of ITS and IGS-1 sequence data with those available on GenBank, cladistic analyses and sexual compatibility studies. Isolates were found to reside in two distinct RFLP groups. RFLP GROUP 1 isolates from *Pinus wallichiana* at Yusipang had RFLP profiles and IGS-1 sequences similar to those of *A. mellea* subsp. *nipponica*. Although ITS sequence data are not available for *A. mellea* subsp. *nipponica*, sequences from this DNA region were most similar to the closely related *A. mellea* from Asia. The RFLP profile and IGS-1 sequences for RFLP GROUP 2 isolates from *Abies densa* at Changaphug, *Tsuga dumosa* at Chimithanka as well as *Picea spinulosa* and *T. dumosa* in the Phobjikha valley were similar to those published for *A. borealis, A. cepistipes, A. gemina* and *A. ostoyae*. Parsimony analysis based on IGS-1 and ITS sequence data placed these isolates in a clade that included *A. calvescens, A. cepistipes, A. gallica, A. jezoensis, A. sinapina* and *A. singula*. The isolates were, however, sexually incompatible with tester strains of *A. calvescens, A. cepistipes, A. gallica* and *A. sinapina*. Although closely related to these species they appear to represent a distinct taxon that we will refer to as Bhutanese Phylogenetic Species I (BPS I) until basidiocarps are found and the species can be described.

***Keywords:*** Armillaria root rot, *Armillaria mellea*, RFLP, IGS, ITS, biological species, phylogenetic species, Himalayas, Bhutan.

## INTRODUCTION

Armillaria root rot is caused by various species of *Armillaria* (Tricholomataceae, Agaricales, Basidiomycetes). These fungi are pathogens occurring throughout temperate and most tropical regions of the world (Hood *et al.* 1991). *Armillaria* spp. survive as pathogens, saprobes or perthotrophs on woody trees and shrubs and tend not to show species-specific interactions with their hosts (Gregory *et al.* 1991, Termorshuizen 2001). These survival strategies make *Armillaria* spp. serious pathogens capable of inflicting severe losses in forests and plantations.

Historically, plant pathologists attributed Armillaria root rot to the single species *A. mellea,* based on the assumption that this is a highly pleomorphic species (Singer 1956). This view changed with the emergence of a biological species concept for *Armillaria* and the subsequent identification of new biological species in Europe and North America (Korhonen 1978, Ullrich and Anderson 1978, Anderson and Ullrich 1979). Based on morphological differences and sexual compatibility interactions, at least 36 species are now accepted in *Armillaria* (Volk and Burdsall 1995).

A contemporary approach to the identification of *Armillaria* spp. has been to use DNA-based characteristics. Consequently, restriction fragment length polymorphism (RFLP) profiles (Harrington and Wingfield 1995) and DNA sequence data from the internal transcribed spacer (ITS) (Coetzee *et al.* 2000, 2001) as well as the intergenic spacer region one (IGS-1) (Anderson and Stasovski 1992) of the rRNA operon, have become available for most commonly-known *Armillaria* spp. This has facilitated rapid identification of field isolates for which basidiocarps are not available.

The Kingdom of Bhutan is a small land-locked country, located in the Eastern Himalayas between China and India. The total area is 47 010 $km^2$ with 64.2% covered by forest (FAO 2001). The dense forest cover of Bhutan is exceptional for Southern and South-Eastern Asia that has generally been severely deforested. Forests are of immense socio-economic and ecological importance to Bhutan. Diseases affecting this natural resource, therefore, pose a great threat to the economic and social well-being of the country.

Very little is known regarding diseases in Bhutanese forests. Recent surveys have recorded a number of diseases of which Armillaria root rot was commonly encountered (Donaubauer 1986, 1993, Nedomlel 1994, Tshering and Chhetri 2000, Kirisits *et al.* 2002). Based on basidiocarp morphology Nedomlel (1994) recorded the presence of *A. ostoyae* in Bhutan. Apart from this record, virtually nothing is known regarding the identity of the *Armillaria* spp. causing root rot in conifer forests of this Himalayan country.

During the course of a survey of tree diseases in 2001 (Kirisits *et al.* 2002), typical symptoms and signs of Armillaria root rot were found in various conifer forests in Bhutan. These symptoms and signs included trees dying in patches and white mycelial mats below the bark, at the bases of dead and dying trees (Morrison *et al.* 1991). Rhizomorphs were also present in the soil and under the bark of dead and dying trees. Although basidiocarps were never encountered, it was possible to obtain diploid *Armillaria* isolates from dying trees. The aim of this study was, therefore, to identify field isolates from Bhutan using RFLP and DNA sequence data. In addition, results from these DNA based studies were evaluated using sexual compatibility tests with appropriate haploid tester strains.

## MATERIALS AND METHODS

### Collection sites

A total of thirteen *Armillaria* isolates were collected from trees in fir and mixed conifer forests at four locations in Bhutan, during July of 2001 (Table 1). Collection sites included Changaphug, Yusipang and Chimithankha in the Western part of the country and the Phobjikha valley in Central Bhutan (Fig. 1). The high altitude forests at Changaphug that consist of Eastern Himalayan fir (*Abies densa*), suffered severely from a disease syndrome, known as fir decline (Donaubauer 1993), in the 1980's, which resulted in the death of the majority of the trees at this site. This dramatic and wide-spread decline of fir in Western Bhutan was thought to be primarily caused by prolonged drought, but various biotic agents, including *Armillaria* spp., were suggested to be involved as contributing factors in the syndrome (Donaubauer 1986, 1987, 1993, Ciesla and Donaubauer 1994). In the Phobjikha valley, isolates were collected in a stand of Eastern Himalayan spruce (*Picea spinulosa*), suffering from a local outbreak of the bark beetle *Ips schmutzenhoferi* (Schmutzenhofer 1988, Kirisits *et al.* 2002). Obvious signs of Armillaria root rot were present on spruce trees, attacked by *I. schmutzenhoferi*. In addition to spruce, one

isolate was collected from Himalayan hemlock (*Tsuga dumosa*) in the Phobjikha valley. At Yusipang and Chimithankha, isolates were collected from Himalayan blue pine (*Pinus wallichiana*) and Himalayan hemlock, respectively. Armillaria root rot was not obvious on living trees at the latter sites but the isolates were included to gain a broader view of the occurrence and species composition of *Armillaria* spp. in Bhutan.

### Fungal isolation and cultivation

Isolates were obtained either from mycelial fans or from rhizomorphs found between the bark and the wood of dying trees or on stumps. Small samples from the mycelial fans were placed on selective DBS (Dichloran-Benomyl-Streptomycin) medium (Harrington *et al.* 1992) and incubated at about 20 °C in artificial light for 2 weeks. Rhizomorphs from infected trees or stumps were surface sterilized in 96% ethanol for 1 min; small pieces from the inner parts were excised and placed on MA (2% Malt extract and 1.6% Agar) or selective DBS medium. Mycelium or rhizomorph tips, growing from primary isolates, were transferred to fresh medium and incubated. This procedure was repeated until pure cultures were obtained. Pure cultures were maintained on MYA (1.5% Malt extract, 0.2% Yeast extract and 1.5% Agar) medium. All isolates obtained from Bhutan are maintained in the culture collections of the Forestry and Agricultural Biotechnology Institute (FABI) (CMW), University of Pretoria, Pretoria, South Africa and the Institute of Forest Entomology, Forest Pathology and Forest Protection (IFFF), Universität für Bodenkultur Wien (BOKU), Vienna, Austria.

### DNA extraction

*Armillaria* isolates were grown in liquid MY (1% Malt and 2% Yeast extract) medium at 24 °C for four weeks in the dark. Mycelium was harvested using a sterile metal strainer, frozen at –70 °C for 20 min and lyophilized. The freeze-dried mycelium was then ground to a fine powder in liquid nitrogen. DNA extraction from the powdered mycelium followed the method described by Coetzee *et al.* (2000).

### Amplification of the ITS and IGS-1 regions

The ITS region (ITS1, 5.8S and ITS2) of the rRNA operon was amplified using primer set ITS1/ITS4 (White *et al.* 1990). The IGS-1 region was amplified with primers CLR12R

(Veldman *et al.* 1981) and O-1 (Duchesne and Anderson 1990). The PCR mixture and conditions for amplification of the ITS and IGS-1 regions were the same as those described by Coetzee *et al.* (2000). Amplified ITS and IGS-1 PCR products were visualized on an agarose gel (1% agar) stained with ethidium bromide under UV illumination.

### RFLP analysis of the IGS-1

Restriction enzyme digestion was done after PCR reactions by adding 10 U of the endo-nuclease *Alu*I to unpurified PCR mix (20 µL) containing the IGS-1 amplicons. DNA fragments were separated on an agarose gel (3%) stained with ethidium bromide and visualized under UV illumination. RFLP fragment sizes larger than 100 bp. were determined with GelFrag version 2.0.5 (National Centre for Super Computing Applications, University of Illinois at Urbana Champaign). RFLP profiles obtained for the isolates were compared with those previously published for various *Armillaria* spp. from Asia, Europe and North America (Harrington and Wingfield 1995, Schulze *et al.* 1995, Banik *et al.* 1996, Volk *et al.* 1996, Coetzee 1997, Chillali *et al.* 1998, Frontz *et al.* 1998, Terashima *et al.* 1998, White *et al.* 1998, Pérez Sierra *et al.* 1999, Coetzee *et al.* 2000, Kim *et al.* 2000, 2001).

### DNA sequencing

DNA sequences for the ITS and IGS-1 regions were obtained using an ABI PRISM™ automated sequencer. PCR products were purified from unincorporated nucleotides and primer dimers prior to sequencing using a QIAquick PCR purification kit (QIAGEN, Germany) and eluted with 50 µL water. Sequence reactions were carried out with the ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase, FS (Perkin Elmer, Warrington, UK) following the protocol supplied by the manufacturer. The ITS region was sequenced in both directions using primers ITS1 and ITS4 as well as internal primers CS2B and CS3B (Coetzee *et al.* 2001). DNA sequences for the IGS-1 region were determined with primers P-1 (Hsiau 1996), O-1 and primers MCO2 and MCO2R (Coetzee *et al.* 2000) that anneal to a region in the middle of the IGS-1 region.

### Cloning of IGS-1 amplicons

IGS-1 PCR products from isolates that gave poor sequencing results were cloned into vector pCR®4-TOPO® after purification, as outlined above. Cloning reactions were done using a TOPO TA Cloning® Kit for Sequencing (Invitrogen Life Technologies, Carlsbad, California) with One Shot® TOP10 Chemically Competent *E. coli* cells following the manufacturer's directions. Positive inserts were verified by amplifying the IGS-1 directly from transformed *E. coli* cells. The PCR mixture included dNTPs (250 µM each), *Taq* Polymerase (2.5 U) (Roche Diagnostics, Mannheim, Germany), PCR buffer with MgCl2 (supplied by the manufacturer) and primers P-1 and O-1 (0.1 µM each). The final volume of the PCR reaction mix was brought to 50 µL with water. PCR conditions were as follows: 1 cycle at 95 °C for 1 min (denaturation), 35 cycles of 60 °C for 30 s (primer annealing), 70 °C for 30 s (elongation) and 95 °C for 30 s (denaturation). A final elongation step was allowed at 70 °C for 7 min. PCR products were visualized under UV illumination on a 1% agarose gel stained with ethidium bromide. Two to three IGS-1 PCR products that had been successfully amplified from positively transformed cells were sequenced as described above.

### Sequence and phylogenetic analyses

The identity of Bhutanese isolates was further investigated by comparing the ITS and IGS-1 sequences from representative isolates with sequence data available on the NCBI (National Center for Biotechnology Information) databases using a nucleotide BLAST (Basic Local Alignment Search Tool) search. This was followed by phylogenetic analyses to determine the relationship between the Bhutanese isolates and the *Armillaria* species, with which they had a high sequence similarity. ITS and IGS-1 DNA sequences for representative isolates from Bhutan were aligned with sequences of various *Armillaria* spp. available on GenBank. Alignment was done with Clustal X (Thompson *et al.* 1997) and manually corrected. Phylogenetic analysis was based on parsimony methods using PAUP* version 4.10 (Swofford 1998). Indels larger than two base pairs were coded using a multistate character system as outlined by Coetzee *et al.* (2001). Missing, parsimony-uninformative and ambiguously aligned regions were excluded from the data sets before analyses. Gaps were treated as a fifth character, "newstate". Most parsimonious trees were generated by heuristic searches with random addition of sequences (100 replicates), TBR (tree bisection reconnection) branch swapping and MULPARS active. MaxTrees was set to auto-increase after 100 MP trees were generated and

branches collapsed if negative branch lengths were obtained. Most parsimonious trees obtained were optimized by applying successive weighting of parsimony-informative characters according to their mean consistency index. Confidence in branching points was determined by bootstrap analysis (1000 replicates) (Felsenstein 1985).

### Sexual compatibility tests

Diploid isolates belonging to RFLP GROUP 2, were paired with haploid tester strains of *A. calvescens*, *A. cepistipes*, *A. gallica*, *A. gemina*, *A. mellea* and *A. sinapina* (Table 2) to confirm the results from DNA-based identifications. Sexual compatibility tests were conducted on MEA (1.5% Difco malt extract, 1.5% Difco agar) medium. Small (2 mm diam) plugs from diploid Bhutanese cultures and haploid tester strains were placed 5 mm apart on the medium and incubated at 24 °C in the dark. Mating reactions were evaluated after 4 and again after 6 weeks. Sexual compatibility tests were conducted at both FABI (all tester strains) and IFFF (only for *A. cepistipes* and *A. gallica*).

## RESULTS

### RFLP analysis

All isolates from Bhutan resided in one of two groups based on their RFLP profiles (Fig. 2). These are, hereafter, referred to as RFLP GROUP 1 and RFLP GROUP 2 isolates. RFLP GROUP 1 isolates had a profile with fragment sizes of 376 (374-379) and 166 (165-167) bp. This profile corresponded most closely to that of *A. mellea* subsp. *nipponica* from Japan (Terashima *et al.* 1998).

The fragment sizes for isolates in RFLP GROUP 2 were 309 (305-316), 195 (189-199) and 139 (137-141) bp. Some variation was, however, observed amongst banding patterns for these isolates. Isolate CMW10578 (Phob6), from the Phobjikha valley, had a profile slightly different to those of the other isolates. RFLP fragment sizes for this isolate were 417, 313, 198 and 138 bp. A species name could not be assigned to isolates residing in RFLP GROUP 2 because the banding patters were similar to those of *A. borealis*, *A. cepistipes*, *A. gemina* and *A. ostoyae* (Harrington and Wingfield 1995, Pérez Sierra *et al.* 1999, Kim *et al.* 2001).

## Sequence analyses

### *RFLP GROUP 1 isolates*

IGS-1 DNA sequences for isolates CMW8082 and CMW8202 from Yusipang residing in RFLP GROUP 1, were most similar to those of *A. mellea* from Japan (AF163610) and South Korea (AF163613, AF163612 and AF163611) and *A. mellea* subsp. *nipponica* (D89922) (98%). The highest blast score (932 bits) was obtained with *A. mellea* (AF163610) from Japan. The highest ITS sequence identity for these Bhutanese isolates was with *A. mellea* (98%) from South Korea (AF163592, AF163593 and AF163591).

Phylogenetic trees generated from IGS-1 sequences (Fig. 3) placed isolates CMW8082 and CMW8202 in a strongly supported monophyletic group that included *A. mellea* s. str. from Japan (AF163610) and South Korea (AF163611, AF162613) as well as *A. mellea* subsp. *nipponica* (100% bootstrap support). Most parsimonious trees obtained from ITS sequences (Fig. 4) placed the two isolates in a strongly supported monophyletic group (100% bootstrap support) that included isolates representing *A. mellea* s. str. from Japan (AF163594) and South Korea (AF163592 and AF163593).

### *RFLP GROUP 2 isolates*

The IGS-1 amplicons for representative isolates residing in RFLP GROUP 2 could not be sequenced directly and the fragments were subsequently cloned. Sequence heterogeneity within the IGS-1 repeat region of the rDNA was observed when comparing cloned IGS-1 amplicons from the same individual. IGS-1 sequence comparisons indicated the presence of one 4 bp. indel and eleven nucleotide substitution sites with five of these sites being unique to CMW10578 (Fig. 5).

The highest IGS-1 sequence similarity for isolate CMW10583 from the Phobjikha valley, was with *A. cepistipes* (AF243069 and D89919), *A. sinapina* (D89925), *A. jezoensis* (D89921) and NABS X (AF243062). Although IGS-1 sequences of these species were all 97% similar to those of the isolate from Bhutan, the highest blast score was obtained with *A. cepistipes* and NABS X (888 bits). ITS sequences for isolate CMW10583 had the highest identity with ITS sequences for *A. cepistipes* (AJ250053) (99%, 1501 bits).

Parsimony trees generated from the IGS-1 region grouped representative isolates (CMW8095, CMW10578, CMW10581 and CMW10583) from RFLP GROUP 2 in a strongly supported clade (Fig. 6). Isolate CMW10578 from Phobjika valley, which had a different RFLP pattern, grouped within this clade with a 95% bootstrap support. RFLP GROUP 2 isolates formed a sister group to *A. cepistipes* (D89919), *A. sinapina* (D89925), *A. jezoensis* (D89921) and *A. singula* (D89926) from Japan, but this relationship had only a 50% bootstrap support. Most parsimonious trees generated from the ITS data set placed isolates CMW10583, CMW10581, CMW8095 and CMW8096 from Bhutan in a clade that included *A. cepistipes* (U54811, U54810 and AJ250053) and *A. gallica* (U54814, U54814 and AJ250054) with a 55% bootstrap support (Fig. 4). *Armillaria sinapina* (AF169646) formed a sister taxon to this clade with a 74% bootstrap support.

### Sexual compatibility tests

Haploid tester strains representing *A. calvescens, A. cepistipes, A. gallica*, and *A. sinapina* were used for sexual compatibility tests because of their phylogenetic relationships with RFLP GROUP 2 isolates. Tester strains of *A. mellea* and *A. gemina*, two species distantly related to the Bhutanese isolates, were included as negative controls. The haploid tester strains of these species retained their fluffy, white aerial mycelium when crossed with diploid isolates in RFLP GROUP 2 (Fig. 7). Demarcation lines were also observed where mycelial growth from the different inocula interacted. These results indicate that the RFLP GROUP 2 isolates from Bhutan are sexually incompatible with the tester strains included in this study.

## DISCUSSION

This study represents a first attempt to identify a reasonably large collection of *Armillaria* isolates from Bhutan. The isolates were from a variety of locations and hosts at different altitudes in Bhutan and we, therefore, anticipated finding a variety of *Armillaria* spp. RFLP analyses, however, showed that all isolates resided in one of two distinct groups that could easily be recognised.

RFLP profiles of Bhutanese isolates from *P. wallichiana* at Yusipang (RFLP GROUP 1) were similar to those previously published by Terashima *et al.* (1998) for the homothallic *A. mellea*

subsp. *nipponica* from Japan. It was, therefore, suspected that RFLP GROUP 1 isolates from Bhutan represent this subspecies of *A. mellea*. Phylogenetic analyses based on parsimony that incorporated IGS-1 and ITS sequence data were subsequently used to confirm this finding. Parsimony trees generated in this study grouped the RFLP GROUP 1 isolates in a strongly supported monophyletic Asian *A. mellea* subclade, comprised of isolates from Japan and Korea. This subclade included *A. mellea* subsp. *nipponica* in cladograms generated from IGS-1 sequence data. The strongly supported grouping of this subspecies of *A. mellea* within the Asian subclade suggests that other isolates included in this clade also represent *A. mellea* subsp. *nipponica*. Based on these findings we believe that the Bhutanese RFLP GROUP 1 isolates belong to *A. mellea* subsp. *nipponica*.

Direct sequencing of the IGS-1 PCR products for representative isolates residing in RFLP GROUP 2 was difficult, despite various attempts using different reaction conditions. The IGS-1 region forms part of the tandemly repeated rDNA multigene family (Long and Dawid 1980). Sequences from a limited number of cloned IGS-1 fragments showed sequence heterogeneity among multi-copies of this region; indicating intragenomic IGS-1 sequence variation within individuals. Our limited data further indicated that the IGS-1 could be separated into two non-orthologous (homologs that are not the result of speciation) intragenomic types based on the presence or absence of a four base pair indel.

It was not possible to fully resolve the identity of isolates residing in RFLP GROUP 2. This was firstly because their RFLP profiles resembled those of more than one *Armillaria* sp. Furthermore, there was poor statistical support for groupings based on phylogenetic analyses of ITS and IGS-1 sequences. However, it was clear that RFLP GROUP2 isolates are closely related to *A. cepistipes*, *A. sinapina* and *A. gallica*. The isolates are, therefore, considered to be part of the "*A. gallica* cluster" that includes *A. cepistipes*, *A. gallica*, *A. sinapina* and various other *Armillaria* spp. from the Northern Hemisphere (Korhonen 1995). Species residing in this group are similar in having basidiocarps with a delicate annulus and a bulbouse stipe-base, thin cylindrical monopodially branching rhizomorphs, and saprophytic or weakly parasitic life cycles (Korhonen 1995).

Isolates from Chimitankha, Changaphug and all but one of the isolates from Phobjika valley had the same RFLP profiles and most likely represent a single taxon. Isolate CMW10578 from *P. spinulosa* in Phobjika valley was, however, the exception in having a RFLP profile slightly

different to the rest of the RFLP GROUP 2 isolates. IGS-1 sequence data obtained for this isolate showed that a number of unique base substitutions were present, thus explaining the anomalous RFLP results. Phylogenetic analyses, however, placed this isolate in a strongly supported clade that included representative isolates from the same region and host. Despite RFLP and IGS-1 sequence variation, this isolate (CMW10578) is, therefore, thought to represent the same species as others in RFLP GROUP 2.

Representative isolates in RFLP GROUP 2 could not be identified based on mating tests. These isolates were clearly intersterile with those species (*A. calvescens*, *A. cepistipes*, *A. gallica* and *A. sinapina*) phylogenetically most closely related to them. Isolates of RFLP GROUP 2, therefore, either represent an undescribed taxon or one of the Indian (Himalayan) *Armillaria* spp. (Chandra and Watling 1981) for which neither tester strains for matings, reference cultures, nor molecular data are available. Until basidiocarps linked to this group of isolates can be found and collected, their exact identity cannot be resolved. For the present, we will refer to them as Bhutanese Phylogenetic Species I (BPS I).

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**TABLE 1:** *Armillaria* isolates from Bhutan included in this study.

| Isolate number | Alternative number | Location in Bhutan | Host tree |
|---|---|---|---|
| CMW8081 | Yus1 | Yusipang | *Pinus wallichiana* |
| CMW8082 | Yus2 | " | " |
| CMW8084 | Yus3 | " | " |
| CMW8202 | Yus4 | " | " |
| CMW8095 | Cha1 | Changaphug | *Abies densa* |
| CMW8096 | Cha2 | " | " |
| CMW10583 | Phob2 | Phobjikha valley | *Tsuga dumosa* |
| CMW10576 | Phob3 | " | *Picea spinulosa* |
| CMW10577 | Phob4 | " | " |
| CMW10578 | Phob6 | " | " |
| CMW10579 | Phob7 | " | " |
| CMW10581 | Phob9 | " | " |
| CMW10582 | Chim2 | Chimithankha | *T. dumosa* |

TABLE 2: *Armillaria* isolates used as testers in the sexual compatibility tests.

| Species | Isolate number | Other numbers | Origin | Collector | Host |
|---|---|---|---|---|---|
| *A. calvescens* | CMW6893 | PR-2, ss-2 | USA | Banik MT | *Acer rubrum* |
| *A. cepistipes* | CMW6909 | 82-14-14 | Canada | Morrison DJ | unknown |
| " | CMW6912 | HHB-14868, ss-2 | USA | Banik MT | *Alnus rubra* |
| " | CMW11262 | IFFF 416, 92165 | Finland | Korhonen K. | *Salix caprea* |
| " | CMW11263 | IFFF 417, 93288 | Poland | Zólciak A. | unknown |
| " | CMW11269 | IFFF 441 | Unknown | Unknown | unknown |
| *A. gallica* | CMW3169 | B500, ATCC52114 | USA | Anderson JB | unknown |
| " | CMW11272 | IFFF 451 | unknown | unknown | unknown |
| *A. gemina* | CMW3166 | B735, AMP4B | USA | Worrall JJ | unknown |
| " | CMW3181 | B485, ATCC52102 | USA | Anderson JB | unknown |
| " | CMW6889 | TJV 94-47, ss-2 | USA | Banik MT | *Quercus velutina* |
| *A. sinapina* | CMW6894 | HHB-14911, ss-9 | USA | Banik MT | *Tsuga heterophylla* |
| *A. mellea* | CMW6901 | IL-7, ss-3 | USA | Banik MT | *Ulmus* sp. |
| " | CMW11271 | IFFF 448 | Unknown | unknown | unknown |

**Figure 1.** Map of Bhutan, showing the four collection sites.

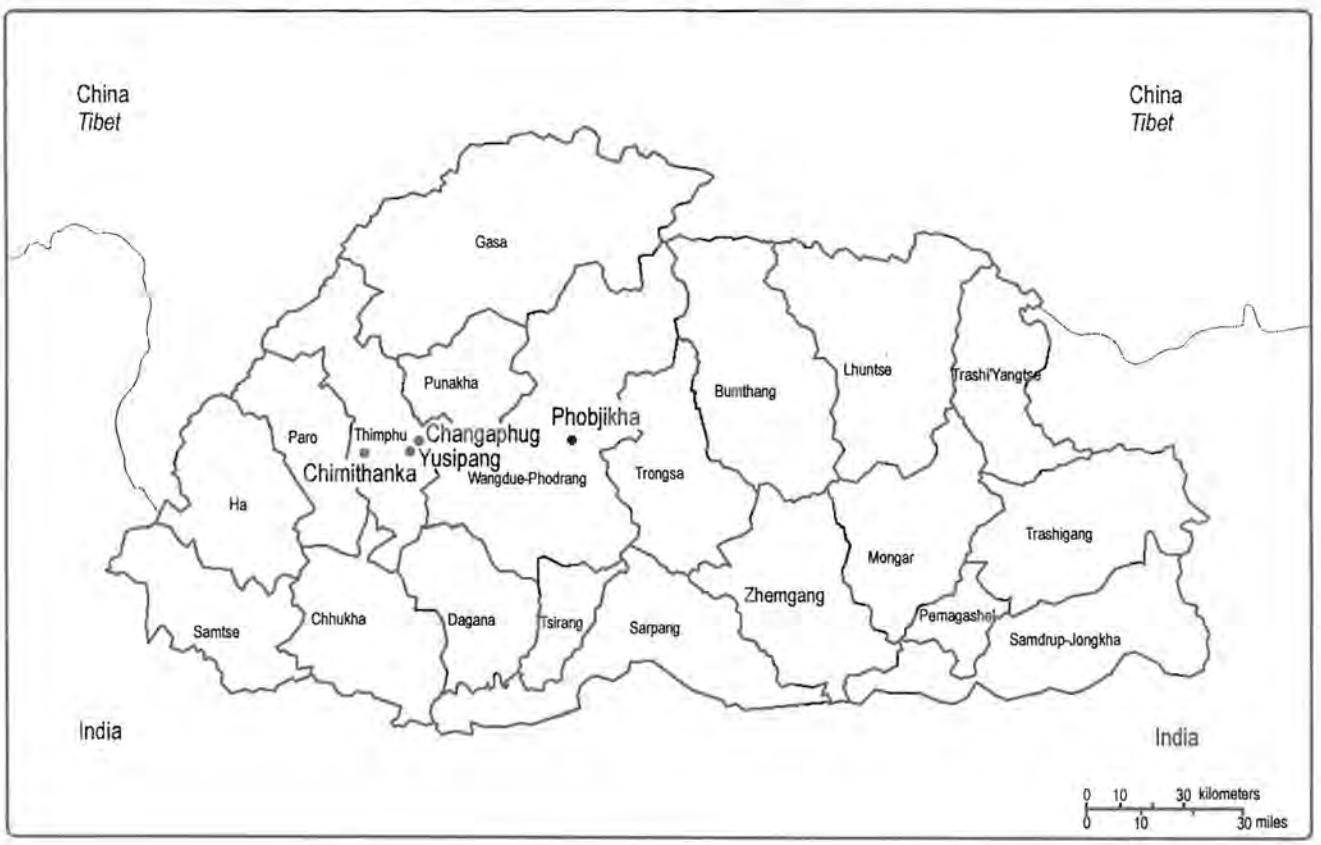
China
Tibet
China
Tibet
Gasa
Punakha
Phobjikha
Bumthang
Lhuntse
Trashi Yangtse
Paro
Thimphu
Changaphug
Yusipang
Chimithanka
Wangdue-Phodrang
Trongsa
Ha
Trashigang
Mongar
Zhemgang
Pemagashel
Samdrup-Jongkha
Samtse
Chhukha
Dagana
Tsirang
Sarpang
India
India
0 10 30 kilometers
0 10 30 miles

**Figure 2.** A 3% agarose gel stained with ethidium bromide showing *Alu*I restriction fragments for isolates of *Armillaria* from Bhutan. Lanes labeled M show a 100 bp. molecular marker (band sizes indicated in base pairs).

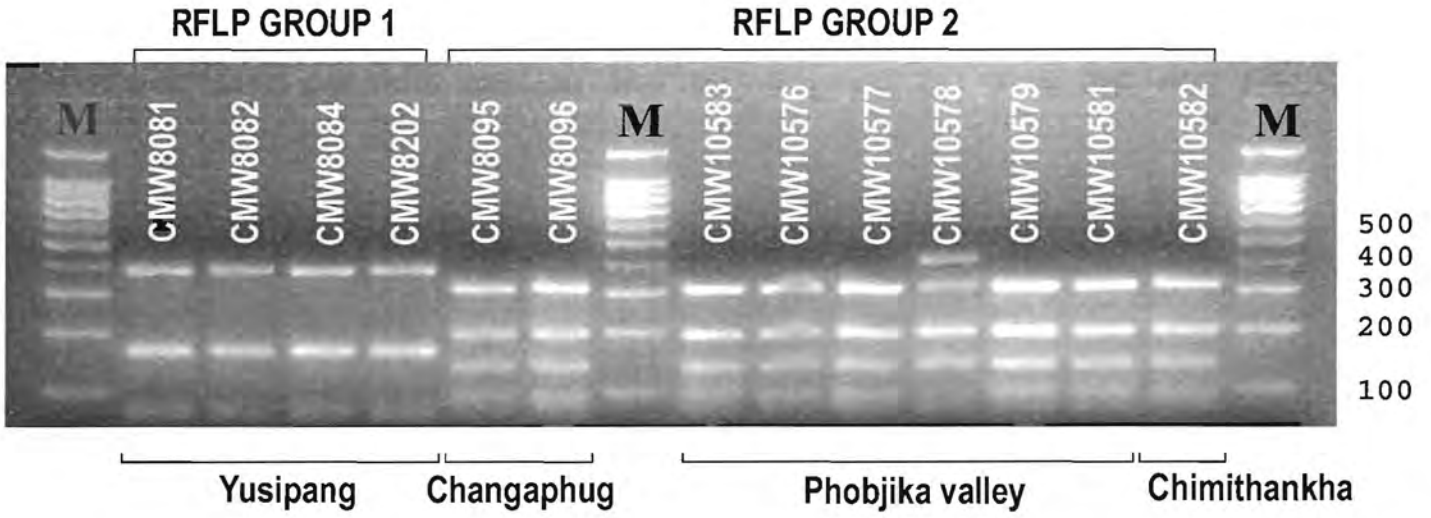

**Figure 3.** The single most parsimonious trees generated after a heuristic search in PAUP* using IGS-1 sequence data (782 characters, 180 parsimony informative characters) from RFLP GROUP 1. Tree length = 203 steps, *CI* = 0.929 and *RI* =0.958. Numbers above and below the tree branches indicate the branch length and the bootstrap support values for the branching nodes, respectively. The tree is rooted to *A. ostoyae*.

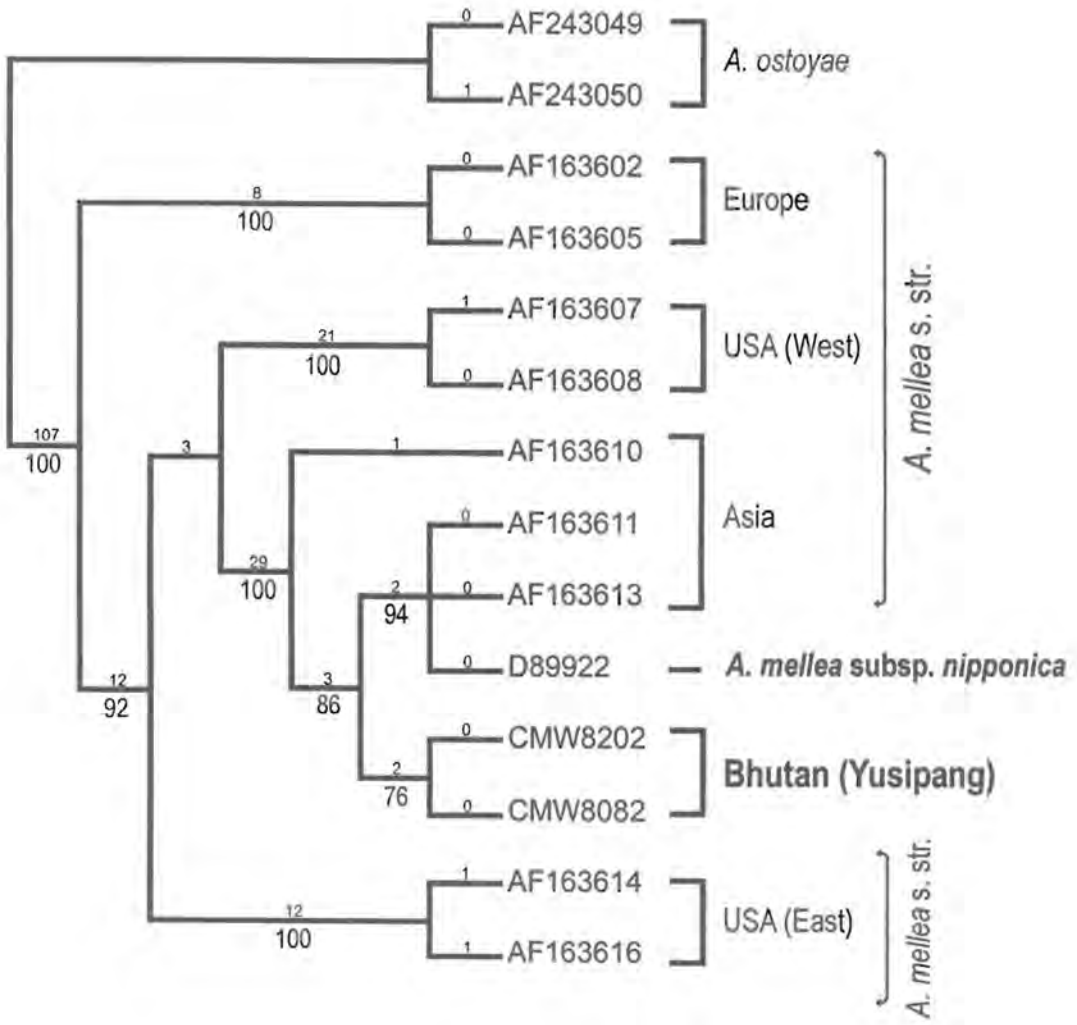
AF243049
AF243050
A. ostoyae
AF163602
AF163605
Europe
AF163607
AF163608
USA (West)
AF163610
AF163611
AF163613
Asia
A. mellea s. str.
D89922
A. mellea subsp. nipponica
CMW8202
CMW8082
Bhutan (Yusipang)
AF163614
AF163616
USA (East)
A. mellea s. str.

**Figure 4.** One of 18 most parsimonious trees based on ITS sequence data (1033 characters, 266 parsimony informative characters) for RFLP GROUP 1 and 2 from Bhutan generated after a heuristic search in PAUP*. Tree length = 433 steps, *CI* = 0.849 and *RI* = 0.916. *Armillaria fuscipes* is used as outgroup.

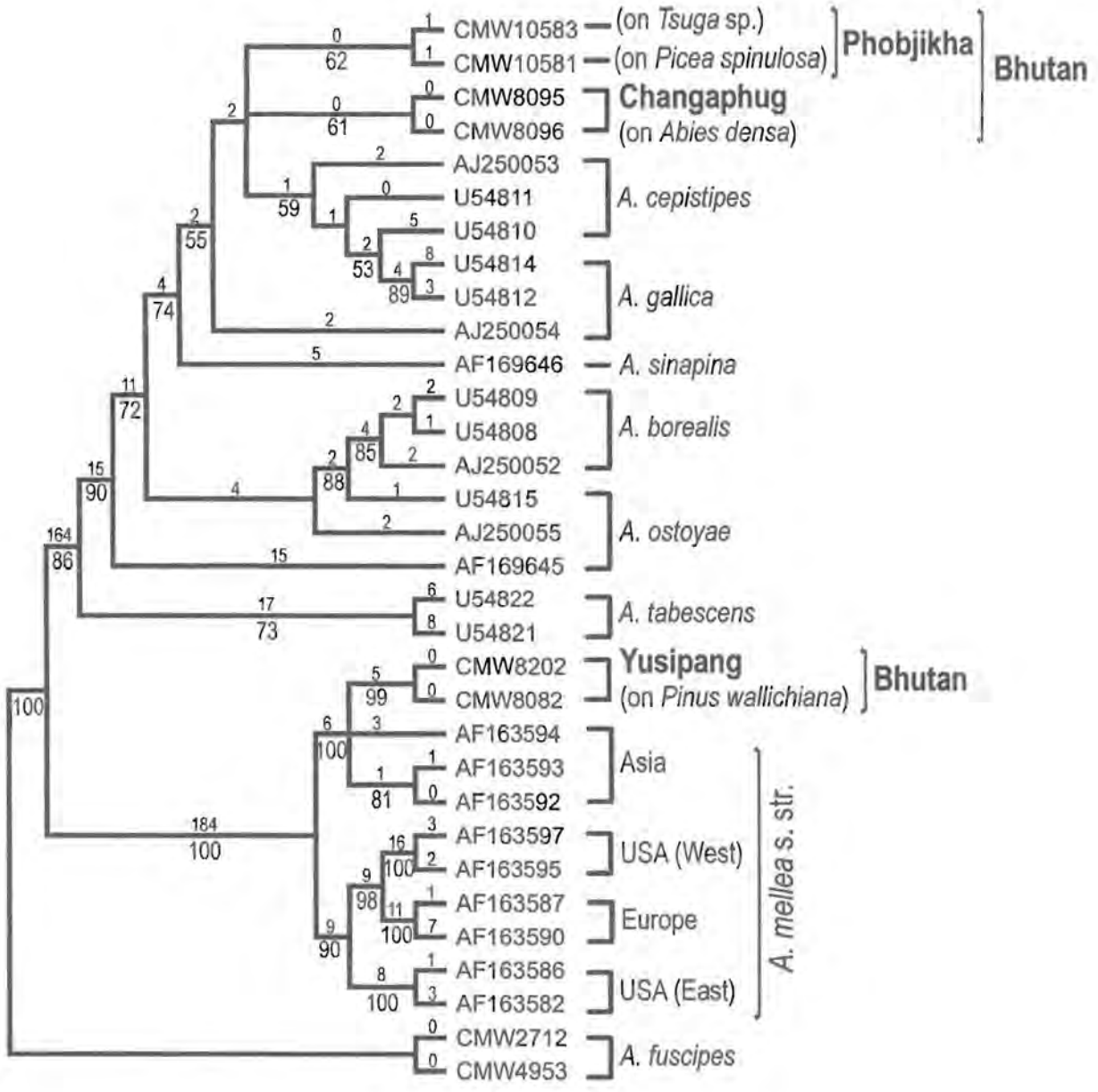

**Figure 5.** Alignment of DNA sequences from cloned IGS-1 fragments. Numbers (C) following the isolate number refer to the clone number. Dashes and stars below the sequences indicate homogeneous and heterogenous regions, respectively. Blocks in gray indicate sites with substitution unique for CMW10578 from Phobjikha valley that had a different RFLP pattern to the rest of the isolates from the same area as well as those from Chimithankha and Changaphug.

```
                    10        20        30        40        50        60
                    ....|....|....|....|....|....|....|....|....|....|....|....|
CMW8095c3           CGATCCACTGAGGTTAAGCCCTTGTTCTAAAGATTTGTTCAACTTTGTTGGACTTTCTCT
CMW10583c1          CGATCCACTGAGGTTAAGCCCTTGTTCTAAAGATTTGTTCAACTTTGTTGGACTTTCTCT
CMW10583c2          CGATCCACTGAGGTTAAGCCCTTGTTCTAAAGATTTGTTCAACTTTGTTGGACTTTCTCT
CMW10578c1          CGATCCACTGAGGTTAAGCCCTTGTTCTAAAGATTTGTTCAACTTTGTTGGACTTTCTCT
CMW10578c2          CGATCCGCTGAGGTTAAGCCCTTGTTCTAAAGATTTGTTCAACTTTGTTGGACTTTCTCT
CMW10578c5          CGATCCACTGAGGTTAAGCTCTTGTTCTAAAGATTTGTTCAACTTTGTTGGACTTTCTCT
CMW10581c1          CGATCCACTGAGGTTAAGCCCTTGTTCTAAAGATTTGTTCAACTTTGTTGGACTTTCTCT
CMW10581c2          CGATCCACTGAGGTTAAGCCCTTGTTCTAAAGATTTGTTCAACTTTGTTGGACTTTCTCT
CMW10582c4          CGATCCACTGAGGTTAAGCCCTTGTTCTAAAGATTTGTTCAACTTTGTTGGACTTTCTCT
                    ------------------*-----------------------------------------

                              70        80        90       100       110       120
                    ....|....|....|....|....|....|....|....|....|....|....|....|
CMW8095c3           TTTCTTTTTACATGCTGAGACCTTGAGGGCCGGGATAGTATCCTTTGTGCACTCGCGACA
CMW10583c1          TTTCTTTTTACATGCTGAGACCTTGAGGGCCGGGATAGTATCCTTTGTGCACTCGCGACA
CMW10583c2          TTTCTTTCTACATGCTGAGACCTTGAGGGCCGGGATAGTATCCTTTGTGCACTCGCGACA
CMW10578c1          TTTCTTTCTACATGCTGAGACCTTGAGGGCCGGAATAGTATCCTTTGTGCACTCGCGACA
CMW10578c2          TTTCTTTCTACATGCTGAGACCTTGAGGGCCGGAATAGTATCCTTTGTGCACTCGCGACA
CMW10578c5          TTTCTTTCTACATGCTGAGACCTTGAGGGCCGGAATAGTATCCTTTGTGCACTCGCGACA
CMW10581c1          TTTCTTTCTACATGCTGAGGCCTTGAGGGCCGGGATAGTATCCTTTGTGCACTCGCGACA
CMW10581c2          TTTCTTTCTACATGCTGAGACCTTGAGGGCCGGGATAGTATCCTTTGTGCACTCGCGACA
CMW10582c4          TTTCTTTCTACATGCTGAGACCTTGAGGGCCGGGATAGTATCcTTTGTGCACTCGCGACA
                    -------*---------------------------*---------------------------

                             130       140       150       160       170       180
                    ....|....|....|....|....|....|....|....|....|....|....|....|
CMW8095c3           GCATGTTACTTAGATTCGAAAGGGTAAGCTAACAACAACGCCTTAGTGTTTTGTT----A
CMW10583c1          GCATGTTACTTAGATTCGAAAGGGTAAGCTAACAACAACGCCTTAGTGTTTTGTT----A
CMW10583c2          GCATGTTACTTAGATTCGAAAGGGTAAGCTAACAACAACGCCTTAGTGTTTTGTTTGTTA
CMW10578c1          GCATGTTACTTAGATTCGAAAGGGTAAGTTAACAACAATGCCTTAGTGTTTTGTTCGTTA
CMW10578c2          GCATGTTACTTAGATTCGAAAGGGTAAGTTAACAACAATGCCTTAGTGTTTTGTTTGTTA
CMW10578c5          GCATGTTACTTAGATTCGAAAGGGTAAGTTAACAACAATGCCTTAGTGTTTTGTTTGTTA
CMW10581c1          GCATGTTACTTAGATTCGAAAGGGTAAGCTAACAACAACGCCTTAGTGTTTTGTTTGTTA
CMW10581c2          GCATGTTACTTAGATTCGAAAGGGTAAGCTAACAACAACGCCTTAGTGTTTTGTTTGTTA
CMW10582c4          GCATGTTACTTAGATTCGAAAGGGTAAGCTAACAACAACGCCTTAGTGTTTTGTTTGTTA
                    ------------------------------*---------*-----------------****-

                             190       200       210       220       230       240
                    ....|....|....|....|....|....|....|....|....|....|....|....|
CMW8095c3           CCTTTCTCCTTTGAATCACGAGTTATTATGAGCCTTGAAGGCTTATAAGGCACTTAGTTA
CMW10583c1          CCTTTCTCCTTTGAATCACGAGTTATTATGAGCCTTGAAGACTTATAAGGCACTTAGTTA
CMW10583c2          CCTTTCTCCTTTGAATCACGAGTTATTATGAGCCTTGaAgACTTATAAGGCACTTAGTTA
CMW10578c1          CCTTTCTCCTTTGAATCACGAGTTATTATGAGCCTTGAAGACTTATAAGGCACTTAGTTA
CMW10578c2          CCTTTCTCCTTTGAATCACGAGTTATTATGAGCCTTGAAGACTTATAAGGCACTTAGTTA
CMW10578c5          CCTTTCTCCTTTGAATCACGAGTTATTATGAGCCTTGAAGACTTATAAGGCACTTAGTTA
CMW10581c1          CCTTTCTCCTTTGAATCACGAGTTATTATGAGCCTTGAAGACTTATAAGGCACTTAGTTA
CMW10581c2          CCTTTCTCCTTTGAATCACGAGTTATTATGAGCCTTGAAGACTTATAAGGCACTTAGTTA
CMW10582c4          CCTTTCTCCTTTGAATCACGAGTCATTATGAGCCTTGAAGACTTATAAGGCACTTAGTTA
                    ------------------------------------------------------------
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                    250        260        270        280        290        300
                    ....|....|....|....|....|....|....|....|....|....|....|....|
CMW8095c3           GCAAGCTCTAACCGCGCGCTGACTTGGAACGGTCTTTACCTTGTACTTGATATCGACTTT
CMW10583c1          GCAAGCTCTAACCGCGCGCTGACTTGGAACGCTCTTTACCTTGTACTTGATATCGACTTT
CMW10583c2          GCAAGCTCTAACCGCGCGCTGACTTGGAACGGTCTTTACCTTGTACTTGATATCGACTTT
CMW10578c1          GCAAGCTCTAACCGCGCGCTGACTTGGAACGGTCTTTACCTTGTACTTGATATCGACTTT
CMW10578c2          GCAAGCTCTAACCGCGCGCTGACTTGGAACGGTCTTTACCTTGTACTTGATATCGACTTT
CMW10578c5          GCAAGCTCTAACCGCGCGCTGACTTGGAACGGTCTTTACCTTGTACTTGATATCGACTTT
CMW10581c1          GCAAGCTCTAACCGCGCGCTGACTTGGAACGGTCTTTACCTTGTACTTGATATCGACTTT
CMW10581c2          GCAAGCTCTAACCGCGCGCTGACTTGGAACGGTCTTTACCTTGTACTTGATATCGACTTT
CMW10582c4          GCAAGCTCTAACCGCGCGCTGACTTGGAACGGTCTTTACCTTGTACTTGATATCGACTTT
                    ------------------------------------------------------------

                    310        320        330        340        350        360
                    ....|....|....|....|....|....|....|....|....|....|....|....|
CMW8095c3           ATGGCCGATATCCCGTATATGGTATAGCCAAGATCCTTGAAAGGGCAAGTCAACGACTGA
CMW10583c1          ATGGCCGATATCCCGTATATGGTATAGCCAAGATCCTTGAAAGGGCAAGTCAACGACTGA
CMW10583c2          ATGGCCGATATCCCGTATATGGTATAGCCAAGATCCTTGAAAGGGCAAGTCAACGACTGA
CMW10578c1          ATGGCCGATATCCCATATATGGTATAGCCAAGATCCTTGAAAGGGCAAGTCAACGACTGA
CMW10578c2          ATGGCCGATATCCCATATATGGTATAGCCAAGATCCTTGAAAGGGCAAGTCAACGACTGA
CMW10578c5          ATGGCCGATATCCCATATATGGTATAGCCAAGATCCTTGAAAGGGCAAGTCAACGACTGA
CMW10581c1          ATGGCCGATATCCCGTATATGGTATAGCCAAGATCCTTGAAAGGGCAAGTCAACGACTGA
CMW10581c2          ATGGCCGATATCCCGTATATGGTATAGCCAAGATCCTTGAAAGGGCAAGTCAACGACTGA
CMW10582c4          ATGGCCGATATCCCGTATATGGTATAGCCAAGATCCTTGAAAGGGCAAGTCAACGACTGA
                    ------------------------------------------------------------

                    370        380        390        400        410        420
                    ....|....|....|....|....|....|....|....|....|....|....|....|
CMW8095c3           TTTTCTGGATCGTTAGTGAGCTTGAGGGTCTGCCCTAAGGTTGCCATGATTGAAAAGGCC
CMW10583c1          TTTTCTGGATCGTTAGTGAGCTTGAGGGTCTGCCCTAAGGTTGCCATGATTGAAAAGGAC
CMW10583c2          TTTTCTGGATCGTTAGTGAGCTTGAGGGTCTGCCCTAAGGTTGCCATGATTGAAAAGGCC
CMW10578c1          TTTTCTGGATCGTTAGTGAGCTTGAGGGTCTGCCCTAAGGTTGCCATGATTGAAAAGGCC
CMW10578c2          TTTTCTGGATCGTTAGTGAGCTTGAGGGTCTGCCCTAAGGTTGCCATGATTGAAAAGGCC
CMW10578c5          TTTTCTGGATCGTTAGTGAGCTTGAGGGTCTGCCCTAAGGTTGCCATGATTGAAAAGGCC
CMW10581c1          TTTTCTGGATCGTTAGTGAGCTTGAGGGTCTGCCCTAAGGTTGCCATGATTGAAAAGGCC
CMW10581c2          TTTTCTGGATCGTTAGTGAGCTTGAGGGTCTGCCCTAAGGTTGCCATGATTGAAAAGGCC
CMW10582c4          TTTTCTGGATCGTTAGTGAGCTTGAGGGTCTGCCCTAAGGTTGCCATGATTGAAAAGGCC
                    ------------------------------------------------------------

                    430        440        450        460        470        480
                    ....|....|....|....|....|....|....|....|....|....|....|....|
CMW8095c3           TTAGAAGCTAAGTAAGTTAAGCTACGGTTACCTTTTTAACCGTTTCAACCGTTTACTTAG
CMW10583c1          TTAGAAGCTAAGTAAGTTAAGCTACGGTTACCTTTTTAACCGTTTCAACCGTTTACTTAG
CMW10583c2          TTAGAAGCTAAGTAAGTTAAGCTACGGTCACTTTTTTAACCGTTTCAACTGTTTACTTAG
CMW10578c1          TTAGAAGCTAAGTAAGTTAAGCTACGGTTACCTTTTTAACCGTTTCAACCGTTTACTTAG
CMW10578c2          TTAGAAGCTAAGTAAGTTAAGCTACGGTTACCTTTTTAACCGTTTCAACCGTTTACTTAG
CMW10578c5          TTAGAAGCTAAGTAAGTTAAGCTACGGTTACCTTTTTAACCGTTTCAACCGTTTACTTAG
CMW10581c1          TTAGAAGCTAAGTAAGTTAAGCTATGGTTACTTTTTTAACCGTTTCAACTGTTTACTTAG
CMW10581c2          TTAGAAGCTAAGTAAGTTAAGCTACGGTTACTTTTTTAACCGTTTCAACTGTTTACTTAG
CMW10582c4          TTAGAAGCTAAGTAAGTTAAGCTACGGTTACTTTTTTAACCGTTTCAACTGTTTACTTAG
                    -------------------------*---*--*-----------------*----------
```

```
                490       500       510       520       530       540
           ....|....|....|....|....|....|....|....|....|....|....|....|
CMW8095c3  CTTTCGAGGGCTACGTTCAAAATTTGAACGGCAACTGGTTCTGAAACGAAAGGTTTGCTA
CMW10583c1 CTTTCGAGGGCTACGTTCAAAATTTGAACGGCAACTGGTTCTGAAACGAAAGGTTTGCTA
CMW10583c2 CTTTCGAGGGCTACGTTCAAAATTTGAACGGCAACTGGTTCTGAAACGAAAGGTTTGCTA
CMW10578c1 CTTTCGAGGGCTAGGTTCAAAATTTGAACGGCAACTTGTTCTGAAACGAAAGGTTTGCTA
CMW10578c2 CTTTCGAGGGCTAGGTTCAAAATTTGAACGGCAACTTGTTCTGAAACGAAAGGTTTGCTA
CMW10578c5 CTTTCGAGGGCTAGGTTCAAAATTTGAACGGCAACTTGTTCTGAAACGAAAGGTTTGCTA
CMW10581c1 CTTTCGAGGGCTACGTTCAAAATTTGAACGGCAACTGGTTCTGAAACGAAAGGTTTGCTA
CMW10581c2 CTTTCGAGGGCTACGTTCAAAATTTGAACGGCAACTGGTTCTGAAACGAAAGGTTTGCTA
CMW10582c4 CTTTCGAGGGCTACGTTCAAAATTTGAACGGCAACTGGTTCTGAAACGAAAGGTTTGCTA
           ----------*----------*----------

                550       560       570       580       590
           ....|....|....|....|....|....|....|....|....|....|....
CMW8095c3  AGTAAACCATTGGTCAAGACCGGTTTGCAACAATTTTGGTGGCTGTAGGGTGAG
CMW10583c1 AGTAAACCATTGGTCAAGACCGGTTTGCAACAATTTTGGTGGCTGTAGGGTGAG
CMW10583c2 AGTAAACCATTGGTCAAGACCGGTTTGCAACAATTTTGGTGGCTGTAGGGTGAG
CMW10578c1 AGTAAACCATTGGTCAAGACCGGTTTGCAACAATTTTGGTGGCTGTAGGGTGAG
CMW10578c2 AGTAAACCATTGGTCAAGACCGGTTTGCAACAATTTTGGTGGCTGTAGGGTGAG
CMW10578c5 AGTAAACCATTGGTCAAGACCGGTTTGCAACAATTTTGGTGGCTGTAGGGTGAG
CMW10581c1 AGTAAACCATTGGTCAAGACCGGTTTGCAACAATTTTGGTGGCTGTAGGGTGAG
CMW10581c2 AGTAAACCATTGGTCAAGACCGGTTTGCAACAATTTTGGTGGCTGTAGGGTGAG
CMW10582c4 AGTAAACCATTGGTCAAGACCGGTTTGCAACAATTTTGGTGGCTGTAGGGTGAG
           ----------
```

**Figure 6.** One of five most parsimonious trees based on IGS-1 sequence data (531 characters, 125 parsimony informative characters) for isolates from RFLP GROUP 2 after a heuristic search in PAUP*. Tree length = 162 steps, *CI* = 0.878 and *RI* = 0.949. C-numbers indicate the clone number for a specific isolate. Numbers above and below the tree branches indicate the branch length and the bootstrap support values for the branching nodes, respectively. The outgroup taxon for this tree is *A. hinnulea*.

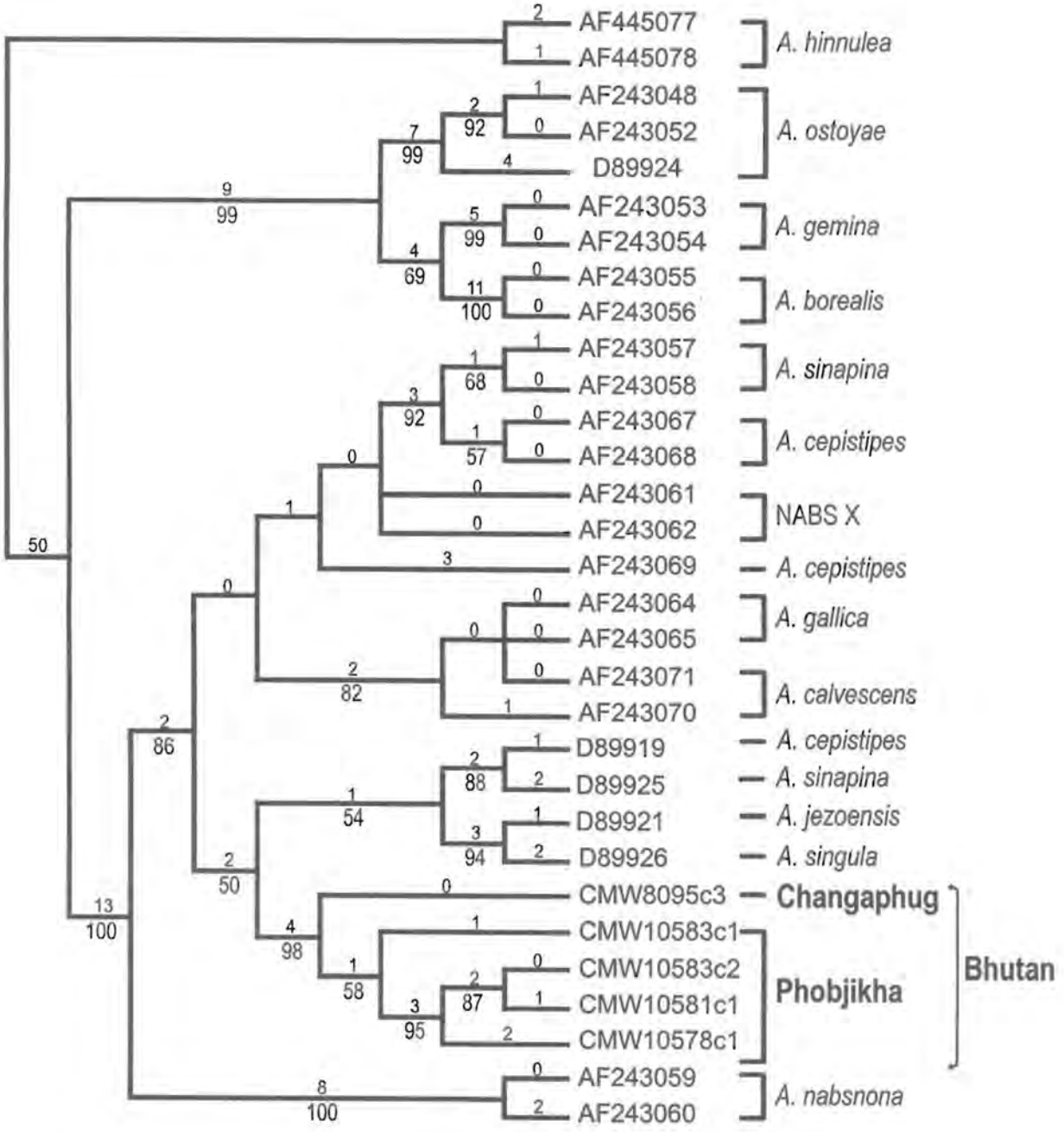
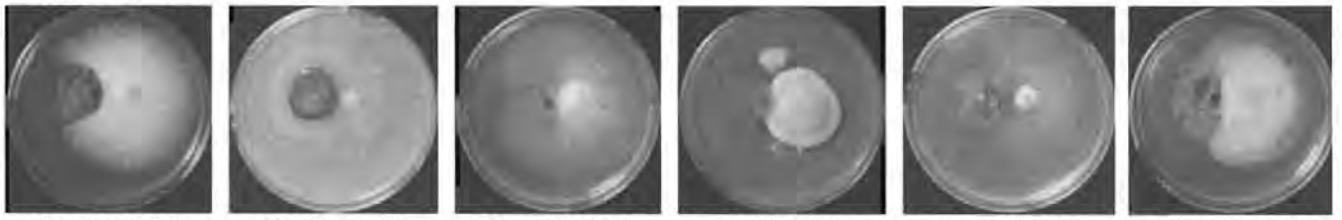


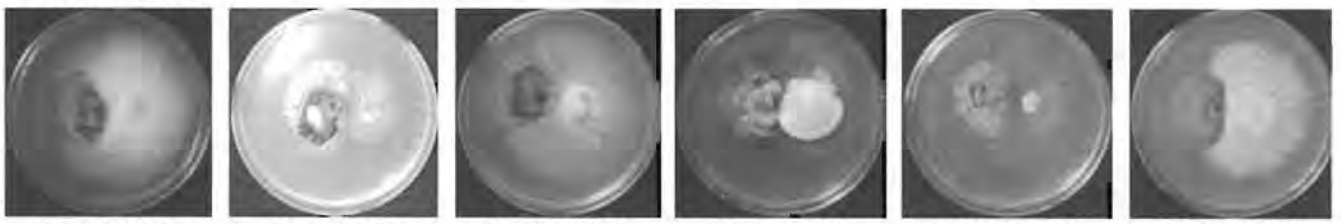
**Figure 7.** Two examples of results obtained after sexual compatibility tests between diploid RFLP GROUP 2 isolates from Bhutan (left inoculum) and haploid tester strains (right inoculum).

**CMW9585**



CMW6909 *A. cepistipes* | CMW3169 *A. gallica* | CMW6894 *A. sinapina* | CMW3181 *A. gemina* | CMW6893 *A. calvescens* | CMW11271 *A. mellea*

**CMW9588**



CMW6909 *A. cepistipes* | CMW3169 *A. gallica* | CMW6894 *A. sinapina* | CMW3181 *A. gemina* | CMW6893 *A. calvescens* | CMW6901 *A. mellea*

# CHAPTER EIGHT

# RFLP IDENTIFICATION TOOL FOR *ARMILLARIA* SPECIES

# RFLP IDENTIFICATION TOOL FOR *ARMILLARIA* SPECIES

## ABSTRACT

*Armillaria* spp. cause an important disease known as Armillaria root rot on woody plants throughout the world. Strategies to monitor and control this disease require correct and efficient identification of species. Identification of *Armillaria* spp. is typically based on basidiocarp morphology, which is complicated by the fact that these structures are rare and ephemeral. Sexual compatibility tests between isolates are also used for identification, but these are time consuming and often yield ambiguous results. Recently, restriction fragment length polymorphisms of the ITS and IGS-1 rRNDA regions have been employed and are now standard procedure for rapid and effective identification of *Armillaria* spp. The extensive use of this method has yielded a large number RFLP profiles for different species, which are available from a substantial and rapidly expanding suite of publications. Identification following this approach consequently requires a large number of comparisons between RFLP profiles of unknown isolates with those that have been published. This is a procedure that is becoming increasingly cumbersome. We have, therefore, developed an electronic database of published profiles and an automated search algorithm for rapid identification of *Armillaria* isolates. At present this application is "stand-alone" and includes RFLP profiles only from the ITS and IGS-1 rDNA regions of *Armillaria* spp. In future it will be converted to a WEB-based application and expanded to include profiles from other gene regions and genera.

***Key words:*** RFLP, IGS, ITS, taxonomy.

## INTRODUCTION

*Armillaria* (Basidiomycetes, Agaricales, Tricholomataceae) comprises a group of fungi causing the important disease known as Armillaria root rot. This disease is well known to plant pathologists due to the substantial losses that it can cause in natural forests, commercial forest plantations, horticultural crops and in agriculture where specifically cash crop plantations are damaged (Hood *et al.* 1991, Kile *et al.* 1991). The impact of Armillaria root rot is exacerbated by its cosmopolitan distribution (Hood *et al.* 1991). It thus poses a potential threat to industries based on woody crops and needs to be continually monitored and correctly managed.

Strategies for monitoring and managing Armillaria root rot disease require correct and efficient identification of the *Armillaria* spp. involved in the various disease syndromes. Historically, these fungi have been classified based on their basidiocarp morphology, but this poses several problems. These structures are seasonal and often unavailable when field surveys are conducted. They are also ephemeral and disappear within a relatively short period after sporulation. Furthermore, some of the *Armillaria* species have similar basidiocarp morphology and are difficult to distinguish from one another. It was largely due to these problems that the biological species concept was adopted for species recognition (Korhonen 1978, Anderson and Ullrich 1979, Guillaumin and Berthelay 1981). Identification based on recognition of biological species involves sexual compatibility tests between known haploid tester strains and cultures made from field samples. These tests are routinely employed in some laboratories but they are time consuming and often yield ambiguous results. In recent years, identification using DNA-based data has become increasingly common. This approach is relatively simple and time efficient. Thus, although a reasonable repertoire of methods is available to identify *Armillaria* spp., those based on DNA data are considered to be the most robust.

DNA-based data for *Armillaria* spp. identification are currently generated from DNA sequences and PCR-RFLPs (restriction fragment length polymorphisms) from the ITS and IGS-1 regions of the rRNA operon (e.g. Anderson and Stasovski 1992, Harrington and Wingfield 1995, Chillali *et al.* 1998, Coetzee *et al.* 2000b). Identification based on DNA sequences is hampered by the fact that generating and comparing sequence data is slow and expensive when large numbers of samples are to be processed. In contrast, PCR-RFLPs represent a relatively inexpensive and

rapid approach that does not require highly specialised services. These advantages lend impetus to the application of PCR-RFLP analysis as standard procedure for identifying *Armillaria* spp.

Extensive application of PCR-RFLPs by several research laboratories has yielded large numbers of RFLP profiles associated with various *Armillaria* spp. (Tables 1 and 2). These profiles are available from a large and rapidly expanding suite of publications. Identification involves obtaining the information from all relevant publications and comparing RFLP profiles from isolates of unknown identity with those that have previously been produced. Due to the large number of comparisons that must be made, this procedure is becoming increasingly cumbersome and difficult to achieve manually.

The time and effort required to make RFLP-based identifications would be substantially reduced if all available information were collated in a single, organised body of data, and if a rapid technique were devised for comparing the numerous profiles. Computer technology presents an appropriate tool for achieving both these goals. The aim of this study was, therefore, to develop an electronic database and automated search algorithm based on PCR-RFLP profiles to facilitate the identification of *Armillaria* isolates.

## COMPUTER SOFTWARE DESIGN

### Specific requirements

In order to be effective, a computerised RFLP-based identification tool must meet a number of criteria. It has to:

- Be compatible with different Microsoft® Windows® operating systems.
- Enable the user to store, change, extract and present data in the database.
- Compare RFLP data for an isolate entered by the user with those in the database.
- Take into account the fact that the user profile might not match any of the profiles in the database exactly; the closest match must, therefore, be returned as its probable identity.

### Database design

The database was developed in Microsoft® Access. Data for the database were obtained from all previous publications containing RFLP profiles for *Armillaria* spp. (Tables 1 and 2). The design of the database and relationships among components and sub-components within the database are depicted in Fig. 1.

### Application design

Code for this application was written in Microsoft® Visual Basic and has interactions with Microsoft® Access, Macromedia Flash and Microsoft® Word. Interaction between Microsoft® Visual Basic and Microsoft® Access takes place when data are being written to or extracted from the database. Macromedia Flash provides animation to the interface when the user is presented with options from menus within the application. Reports are generated through an interaction between Microsoft® Visual Basic and Microsoft® Word after data has been extracted from the database. The architecture of this application is depicted in Figs. 2 - 4.

### Search algorithms for analyses

*Algorithm 1 (Sum of differences - default):* This algorithm calculate the summed squared deviation ($S$) between the user profile ($I$) and every profile in the database ($D$) that has the same number of fragments as the user profile. The summed deviation is calculated by squaring the difference between each fragment length in the user profile and the corresponding fragment length in the profile with which is being compared, and then taking the square root of the sum of these squared differences. Hence, the summed squared deviation between the user profile and profile $i$ in the database is given by:

$$S_i = \sqrt{\sum_{j=1}^{n_i} (I_j - D_{ij})^2} \qquad (1)$$

where $I_j$ is the length of Fragment $j$ in the user profile, $D_{ij}$ is the length of the corresponding fragment for Profile $i$ in the database, and $n_i$ is the number of fragments in each profile.

The database profile that yields the smallest value for $S_i$ is then returned as the best match for the user profile.

*Example:* User profile = 350, 172 and 125 bp. (base pairs).

| | | Fragment number ($j$) | | | |
|---|---|---|---|---|---|
| | | **1** | **2** | **3** | $S_i$ |
| | $D_1$ | 350 | 180 | 119 | $[(350\text{-}350)^2 + (172\text{-}180)^2 + (125\text{-}119)^2]^{1/2} = 10$ |
| ☺ | $D_2$ | 348 | 175 | 120 | $[(350\text{-}348)^2 + (172\text{-}175)^2 + (125\text{-}120)^2]^{1/2} = 6.16$ |
| | $D_3$ | 345 | 172 | 130 | $[(350\text{-}345)^2 + (172\text{-}170)^2 + (125\text{-}130)^2]^{1/2} = 7.07$ |
| | *I* | *350* | *172* | *125* | |

Thus, database profile $D_2$ ($S_2 = 6.16$) is the best much for the user profile.

*Algorithm 2 (Normal distribution error):* Algorithm 1, which was described above, only draws comparisons with those profiles in the database that have the same number of RFLP fragments as the user profile. If two similar fragment lengths are mistakenly entered as a single fragment in the user profile, Algorithm 1 would compare this profile with the wrong subset of profiles in the database, yielding incorrect results. A second algorithm was, therefore, developed. This algorithm rounds user and database RFLP fragment sizes to the nearest 5 bp. Fragment sizes are then distributed over a probability matrix with increments of 5 bp. $S_i$ (Eqn 1) is then calculated, with the smallest value being the closest match.

*Example:* User profile = 454, 448 and 254 bp.

Initial dataset

| | | **Fragment number (*j*)** | | |
|---|---|---|---|---|
| | | **1** | **2** | **3** |
| (*i*) | $D_1$ | 448 | 249 | |
| | $D_2$ | 451 | 302 | 247 |
| | $D_3$ | 299 | 248 | |
| | *I* | *454* | *448* | *254* |

Is then converted to

| | | **Fragment number (*j*)** | | |
|---|---|---|---|---|
| | | **1** | **2** | **3** |
| (*i*) | $D_1$ | 450 | 250 | |
| | $D_2$ | 450 | 300 | 245 |
| | $D_3$ | 300 | 250 | |
| | *I* | *455* | *450* | *255* |

Probability matrix

| | **Fragmenth lengths** | | | | | | | | | | | | | | | | | | | $S_i$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ... | 460 | 455 | 450 | 445 | 440 | ... | 310 | 305 | 300 | 295 | 290 | ... | 260 | 255 | 250 | 245 | 240 | ... | |
| $D_1$ | 0 | 0 | 0.5 | 1 | 0.5 | 0 | ... | 0 | 0 | 0 | 0 | 0 | ... | 0 | 0.5 | 1 | 0.5 | 0 | ... | 1 |
| $D_2$ | 0 | 0 | 0.5 | 1 | 0.5 | 0 | ... | 0 | 0.5 | 1 | 0.5 | 0 | ... | 0 | 0.5 | 1 | 0.5 | 0 | ... | 1.58 |
| $D_3$ | 0 | 0 | 0 | 0 | 0 | 0 | ... | 0 | 0.5 | 1 | 0.5 | 0 | ... | 0 | 0.5 | 1 | 0.5 | 0 | ... | 2 |
| *I* | 0 | 0 | 0.5 | 1 | 0.5 | 0 | ... | 0 | 0 | 0 | 0 | 0 | ... | 0.5 | 1 | 0.5 | 0 | 0 | ... | |

Thus, database profile $D_1$ ($S_1 = 1$) in the above example is the best match.

## DISCUSSION

In this study we have developed an electronic database and search algorithm for rapid identification of *Armillaria* spp. using previously published RFLP profiles. This application allows the user to search, add, and update data in the database. Identification of unknown isolates using this application is achieved through search algorithms comparing user and database profiles to determine the closest match.

The computer program presented in this study is currently a stand-alone application for Microsoft® Windows®. All classes created in Microsoft® Visual Basic and animations developed in Macromedia Flash can be converted to function in a web-based environment. A future aim is thus to convert the application to function in a web-environment and to place it on a server at the Campus of the University of Pretoria (RSA) for use and update through the World Wide Web.

The application "RFLP Identification Tool for *Armillaria* species" was developed for identification of *Armillaria* species based only on ITS and IGS-1 PCR RFLP data. This application will, however, in future be expanded to incorporate RFLPs from other genes. It will also be made more informative regarding the species within the database by including information about species, descriptions, illustrations etc. At the present time, this application is restricted to identification and RFLP profiles pertaining to *Armillaria* spp., but it could be easily augmented in future to accommodate RFLP data for other genera of fungi.

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TABLE 1: Published IGS-1 PCR-RFLP profiles (published RFLP fragment size ranges are indicated in brackets but are not included in the database).

| Species | Reference | RFLP Profile (bp) |
|---|---|---|
| **IGS-1 digested with *Alu*I** | | |
| *Armillaria* sp. | Otieno *et al.* (2003) | 310, 220, 135 |
| *A. borealis* | Peréz Sierra *et al.* (1999) | 305, 200, 100 |
| *A. borealis* | " | 305, 200, 135 |
| *A. borealis* | Harrington and Wingfield (1995) | 310, 200, 104 |
| *A. borealis* | " | 310, 200, 135 |
| *A. calvescens* | " | 582, 240 |
| *A. calvescens* | Kim *et al.* (2000) | 401 (6), 239 (4), 184 (2) |
| *A. cepistipes* | Peréz Sierra *et al.* (1999) | 305, 200, 135 |
| *A. cepistipes* | Kim *et al.* (2001) | 309, 199, 137 |
| *A. cepistipes* | Harrington and Wingfield (1995) | 310, 200, 135 |
| *A. cepistipes* | " | 399, 200, 183 |
| *A. cepestipes* | Peréz Sierra *et al.* (1999) | 400, 200, 190 |
| *A. fuscipes* | Mwenje *et al.* (2003) | 380, 255, 130 |
| *A. fuscipes** | Otieno *et al.* (2003) | 380, 245, 135 |
| *A. fuscipes* | Coetzee *et al.* (2000a) | 365, 245, 135 |
| *A. gallica* | Terashima *et al.* (1998) | 317, 209, 135 |
| *A. gallica* | Peréz Sierra *et al.* (1999) | 390, 230, 190 |
| *A. gallica* | Kim *et al.* (2000) | 398 (2), 249 (5), 236 (2), 180 (3) |
| *A. gallica* | Harrington and Wingfield (1995) | 399, 240, 183 |
| *A. gallica* | Banik *et al.* (1996) | 400, 235, 175 |
| *A. gallica* | White *et al.* (1998) | 400, 235, 190 |
| *A. gallica* | Peréz Sierra *et al.* (1999) | 400, 240, 190 |
| *A. gallica* | White *et al.* (1998) | 400, 245, 190 |
| *A. gallica* | Peréz Sierra *et al.* (1999) | 400, 250, 240, 190 |
| *A. gallica* | Harrington and Wingfield (1995) | 582, 240 |
| *A. gallica* | Kim *et al.* (2000) | 584 (8), 234 (4) |
| *A. gallica* | " | 584 (8), 398 (2), 235 (3), 180 (2) |
| *A. gemina* | " | 308 (3), 196 (2), 138 (1), 93 (3) |
| *A. gemina* | " | 308 (3), 196 (2), 168 (2), 138(1), 93 (3) |

TABLE 1 (continued)

| Species | Reference | RFLP Profile (bp) |
|---|---|---|
| *A. gemina* | Harrington and Wingfield (1995) | 310, 200, 135 |
| *A. heimii* | Mwenje *et al.* (2003) | 480, 255, 175 |
| *A. heimii* | " | 480, 230, 175 |
| *A. heimii* | Coetzee *et al.* (2000a) | 520, 220, 175 |
| *A. jezoensis* | Terashima *et al.* (1998) | 312, 250, 185 |
| *A. jezoensis* | " | 413, 308, 249, 185 |
| *A. jezoensis* | " | 417, 252, 187 |
| *A. mellea* | Otieno *et al.* (2003) | 310, 170 |
| *A. mellea* | Peréz Sierra *et al.* (1999) | 320, 155 |
| *A. mellea* | Harrington and Wingfield (1995) | 320, 155 |
| *A. mellea* | Peréz Sierra *et al.* (1999) | 320, 180, 155 |
| *A. mellea* | Kim *et al.* (2000) | 472 (6), 186 (2), 175 (1), 153 (1) |
| *A. mellea* | " | 473 (7), 175 (2) |
| *A. mellea* | Harrington and Wingfield (1995) | 490, 180 |
| *A. mellea* subsp. *nipponica* | Terashima *et al.* (1998) | 371, 162 |
| *A. nabsnona* | Volk *et al.* (1996) | 306 (299-314), 230 (223-237), 196 (191-202) |
| *A. nabsnona* | Kim *et al.* (2000) | 308 (4), 229 (3), 196 (2) |
| *A. nabsnona* | White *et al.* (1998) | 310, 225, 200 |
| *A. nabsnona* | Harrington and Wingfield (1995) | 534, 200 |
| *A. nabsnona* | White et al. (1998) | 535, 200 |
| *A. nabsnona* | Kim *et al.* (2000) | 541 (7), 197 (1) |
| *A. nabsnona* | " | 541 (7), 308 (4), 229 (3), 196 (2) |
| *A. nabsnona* | Banik *et al.* (1996) | 553 (490-615), 210 |
| *A. nabsnona* | " | 556 (513-598), 314 (302-327), 233 (221-246), 203(191-216) |
| *A. nabsnona* | Volk *et al.* (1996) | 560 (541-581), 321 (311-332), 237 (229-245), 203 (197-210) |
| *A. nabsnona* | " | 563 (552-575), 200 (144-206) |
| *A. ostoyae* | Peréz Sierra *et al.* (1999) | 305, 200, 135 |
| *A. ostoyae* | Kim *et al.* (2000) | 308 (3), 196 (2), 138 (1) |

TABLE 1 (continued)

| Species | Reference | RFLP Profile (bp) |
|---|---|---|
| *A. ostoyae* | Kim *et al.* (2000) | 308 (3), 196 (2), 138 (1), 93 (3) |
| *A. ostoyae* | Harrington and Wingfield (1995) | 310, 200, 135 |
| *A. ostoyae* | White *et al.* (1998) | 310, 200, 135 |
| *A. ostoyae* | Terashima *et al.* (1998) | 312, 210, 137 |
| *A. ostoyae* | Banik *et al.* (1996) | 314 (309-319), 207 (203-211), 141(137-145) |
| *A. sinapina* | Harrington and Wingfield (1995) | 399, 200, 135 |
| *A. sinapina* | Kim *et al.* (2001) | 401, 241, 186 |
| *A. sinapina* | White *et al.* (1998) | 400, 200, 135 |
| *A. sinapina* | " | 400, 200, 190 |
| *A. sinapina* | " | 400, 200, 190, 135 |
| *A. sinapina* | " | 400, 235, 190 |
| *A. sinapina* | " | 400, 235, 200, 190, 135 |
| *A. sinapina* | Kim *et al.* (2000) | 401 (4), 239 (4), 196 (2), 184 (2), 139 (1) |
| *A. sinapina* | " | 401 (6), 239 (4), 184 (2) |
| *A. sinapina* | Banik *et al.* (1996) | 401 (391-410), 237 (299-245), 184 (177-191) |
| *A. sinapina* | Kim *et al.* (2000) | 402 (7), 196 (2), 184 (2), 139 (1) |
| *A. sinapina* | Terashima *et al.* (1998) | 423, 258, 190 |
| *A. singular* | " | 410, 207, 184 |
| *A. singular* | " | 417, 266, 186 |
| *A. tabescens* | Harrington and Wingfield (1995) | 320, 240, 100 |
| *A. tabescens* | Peréz Sierra *et al.* (1999) | 430, 240 |
| *A. tabescens* | Harrington and Wingfield (1995) | 430, 240 |
| NABS X | " | 399, 183, 142 |
| NABS X | Kim *et al.* (2001) | 401, 186, 144 |
| NABS X | " | 401 (3), 184 (1), 145 (1) |
| NABS XI | " | 401 (3), 197 (1), 184 (1) |
| NABS XI | " | 401, 197, 186 |
| NABS XI | Banik *et al.* (1996) | 413 (389-436), 203 (198-207), 185 |

TABLE 1 (continued)

| Species | Reference | RFLP Profile (bp) |
|---|---|---|
| **IGS-1 digested with *Dde* I** | | |
| *A. gallica* | Terashima *et al.* (1998) | 237, 211, 148 |
| *A. jezoensis* | " | 235, 222, 147, 112 |
| *A. ostoyae* | " | 214, 179, 120 |
| *A. sinapina* | " | 235, 218, 148, 111 |
| *A. singular* | " | 234, 150, 113 |
| **IGS-1 digested with *Bsm*I** | | |
| *A. ostoyae* | Peréz Sierra *et al.* (1999) | 600, 300 |
| *A. ostoyae* | Harrington and Wingfield (1995) | 620, 300 |
| **IGS-1 digested with *Nde*I** | | |
| *A. borealis* | Harrington and Wingfield (1995) | 550, 370 |
| *A. borealis* | Peréz Sierra *et al.* (1999) | 565, 380 |
| *A. gemina* | Kim *et al.* (2000) | 913, 552, 461, 372 |
| *A. ostoyae* | Harrington and Wingfield (1995) | 550, 370 |
| *A. ostoyae* | Peréz Sierra *et al.* (1999) | 565, 380 |
| *A. ostoyae* | Kim *et al.* (2000) | 552, 372 |
| **IGS-1 digested with *Hind*II** | | |
| *A. cepistipes* | Harrington and Wingfield (1995) | 580, 340 |

* As *A. heimii*

TABLE 2: Published ITS PCR-RFLP profiles.

| Species | Reference | RFLP Profile (bp) |
|---|---|---|
| **ITS digested with *Alu*I** | | |
| *A. fuscipes** | Otieno *et al.* (2003) | 480, 160, 85 |
| *A. heimii* | Chillali *et al.* (1997) | 530, 72 |
| *A. heimii* | " | 530, 72 |
| *A. mellea* | Otieno *et al.* (2003) | 320, 235, 190, 150 |
| *A. mellea* subsp *africana* | Chillali *et al.* (1997) | 390, 271, 150, 72 |
| *Armillaria* SIG III | Chillali *et al.* (1997) | 540, 234, 72 |
| *Armillaria* sp. | Otieno *et al.* (2003) | 510, 225, 95 |
| **ITS digested with *Cfo* I** | | |
| *A. borealis* | Chillali *et al.* (1998) | 400, 350, 92 |
| *A. cepistipes* | " | 400, 350 |
| *A. ectypa* | " | 500, 350 |
| *A. gallica* | " | 400, 350 |
| *A. ostoyae* | " | 400, 350 |
| *A. tabescens* | " | 500, 350 |
| **ITS digested with *EcoR* I** | | |
| *A. borealis* | Chillali *et al.* (1998) | 510, 330 |
| *A. cepistipes* | " | 510, 330 |
| *A. ectypa* | " | 500, 330 |
| *A. gallica* | " | 510, 330 |
| *A. heimii* | Chillali *et al.* (1997) | 315 |
| *A. heimii* | " | 315 |
| *A. mellea* subsp *africana* | " | 500, 360 |
| *A. ostoyae* | Chillali *et al.* (1998) | 510, 330 |
| *A. tabescens* | " | 510, 330 |
| *Armillaria* SIG III | Chillali *et al.* (1997) | 500, 360 |

TABLE 2 (continued)

| | | |
|---|---|---|
| **ITS digested with *Hin*fI** | | |
| *A. borealis* | Chillali *et al.* (1998) | 310, 234, 170, 110 |
| *A. cepistipes* | " | 310, 234, 130, 110 |
| *A. fuscipes** | Otieno *et al.* (2003) | 220, 190, 170, 72 |
| *A. gallica* | Chillali *et al.* (1998) | 310, 234, 130, 110 |
| *A. gallica* | " | 310, 234, 118, 90 |
| *A. heimii* | Chillali *et al.* (1997) | 271, 234, 100 |
| *A. heimii* | " | 420, 234 |
| *A. mellea* | Otieno *et al.* (2003) | 280, 180, 170, 140, 100 |
| *A. mellea* subsp. *africana* | Chillali *et al.* (1997) | 400, 234, 200 |
| *A. ostoyae* | Chillali *et al.* (1998) | 310, 234, 170, 110 |
| *Armillaria* SIG III | Chillali *et al.* (1997) | 460, 281, 200 |
| *Armillaria* sp. | Otieno *et al.* (2003) | 360, 230, 150, 100 |
| **ITS digested with *Nde* II** | | |
| *A. fuscipes** | Otieno *et al.* (2003) | 390, 250 |
| *A. heimii* | Chillali *et al.* (1997) | 369, 271 |
| *A. heimii* | " | 369, 271 |
| *A. mellea* | Otieno et al. (2003) | 280, 240, 230, 150 |
| *A. mellea* subsp. *africana* | Chillali *et al.* (1997) | 281, 234, 230, 141 |
| *Armillaria* SIG III | " | 603, 230 |
| *Armillaria* sp. | Otieno *et al.* (2003) | 590, 270 |

* as *A. heimii*

**Figure 1.** Design of the RFLP database. Black boxes are the components and open boxes the sub-components of each component. Numbers and M (many) indicate the relationship (1:1 or 1:M) between two components or between a component and a sub-component.

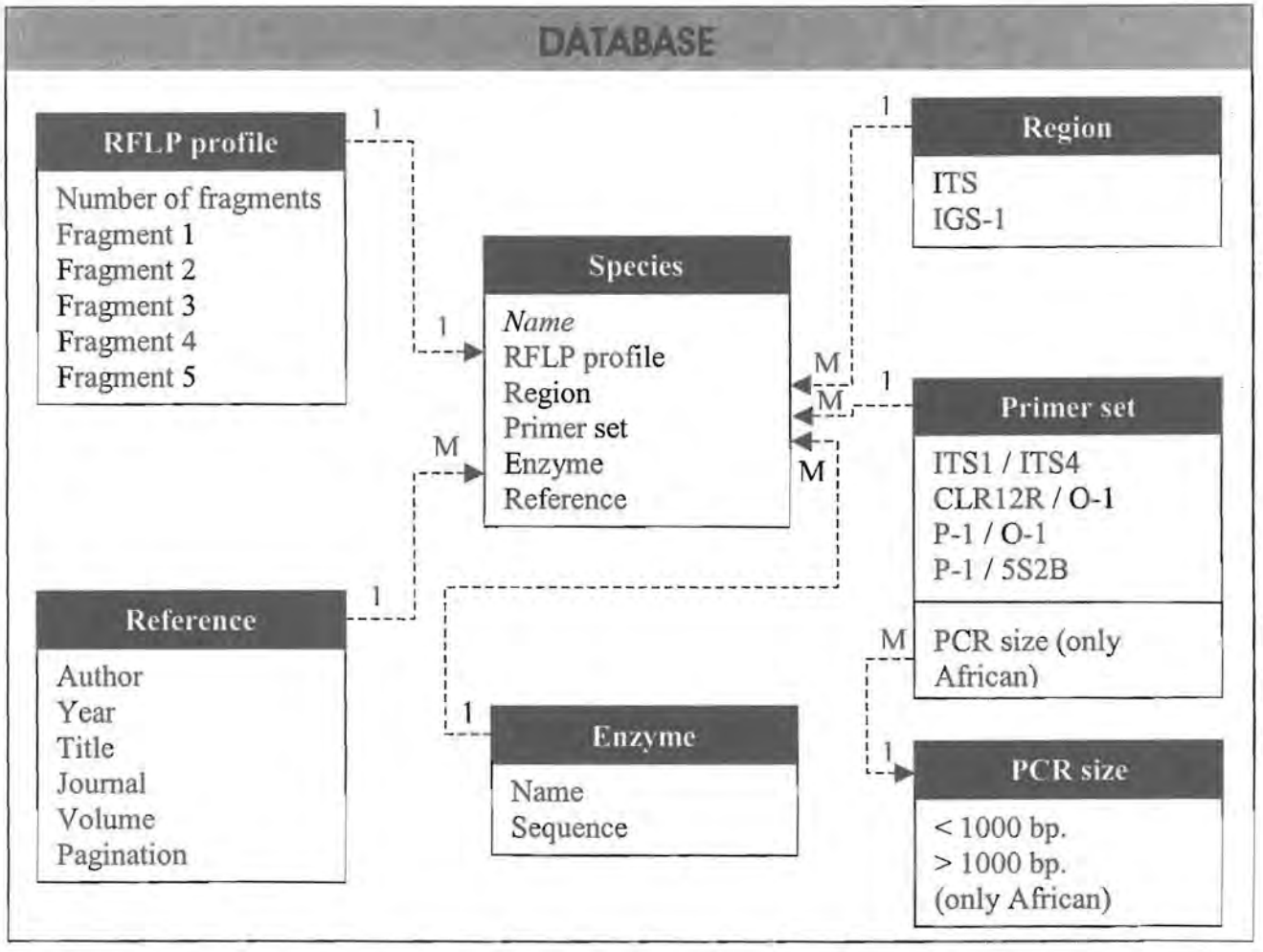

DATABASE
RFLP profile
Number of fragments
Fragment 1
Fragment 2
Fragment 3
Fragment 4
Fragment 5
Species
Name
RFLP profile
Region
Primer set
Enzyme
Reference
Region
ITS
IGS-1
Primer set
ITS1 / ITS4
CLR12R / O-1
P-1 / O-1
P-1 / 5S2B
PCR size (only African)
Reference
Author
Year
Title
Journal
Volume
Pagination
Enzyme
Name
Sequence
PCR size
< 1000 bp.
> 1000 bp.
(only African)
1
M

**Figure 2.** Architecture of the "RFLP Identification Tool for *Armillaria* species" computer application. Down arrows (⇳) indicate drop-down-menus with data from the database. Black boxes show the entities in the data base from Fig. 1.

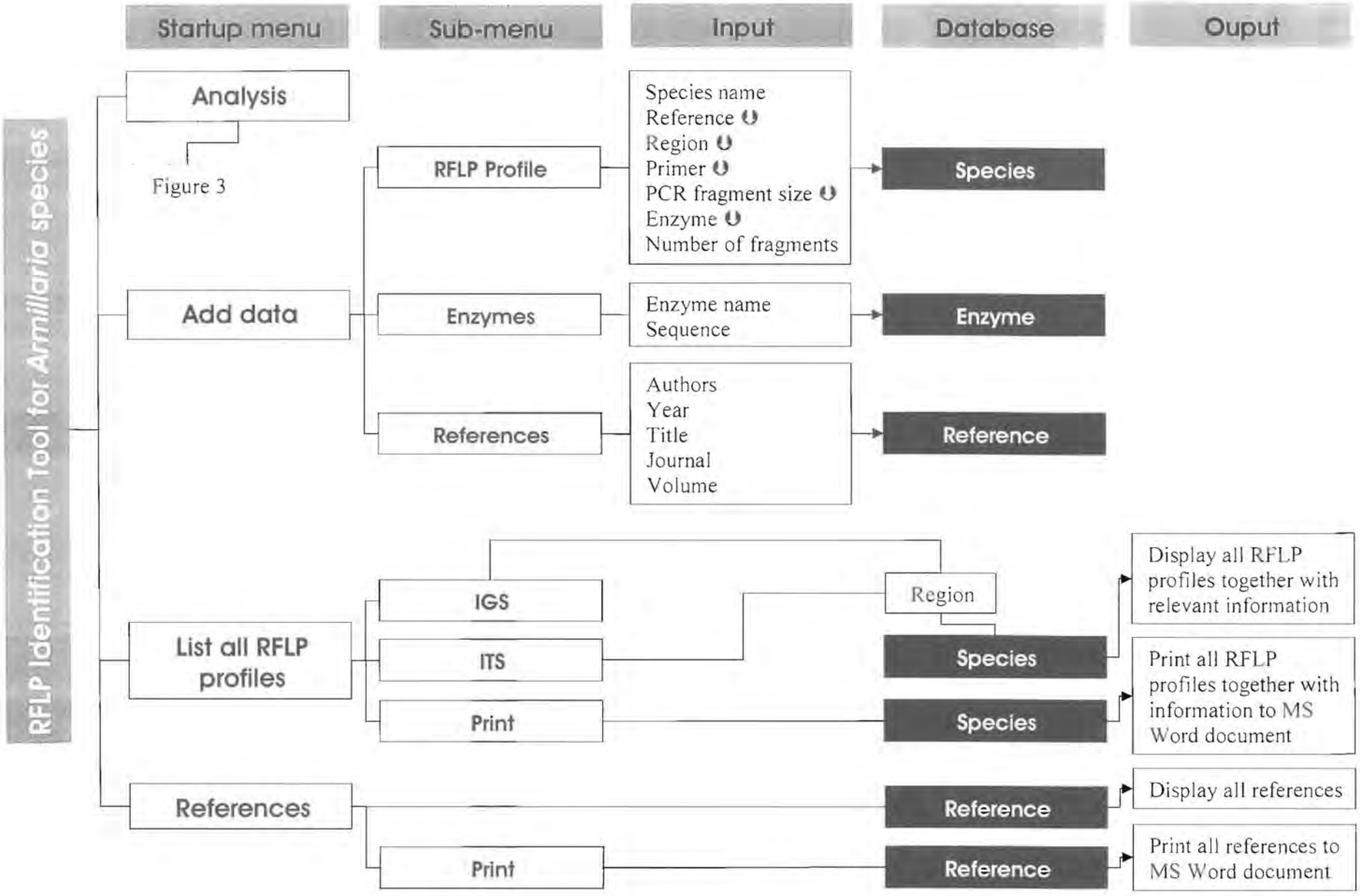


**Figure 3.** Analyses of user profiles. The user chooses one of the two algorithms to calculate the best much between the user-profile and the profiles selected from the procedure outlined in Fig. 4. The species name, RFLP profile and reference for the best as well as close matches are given as output.

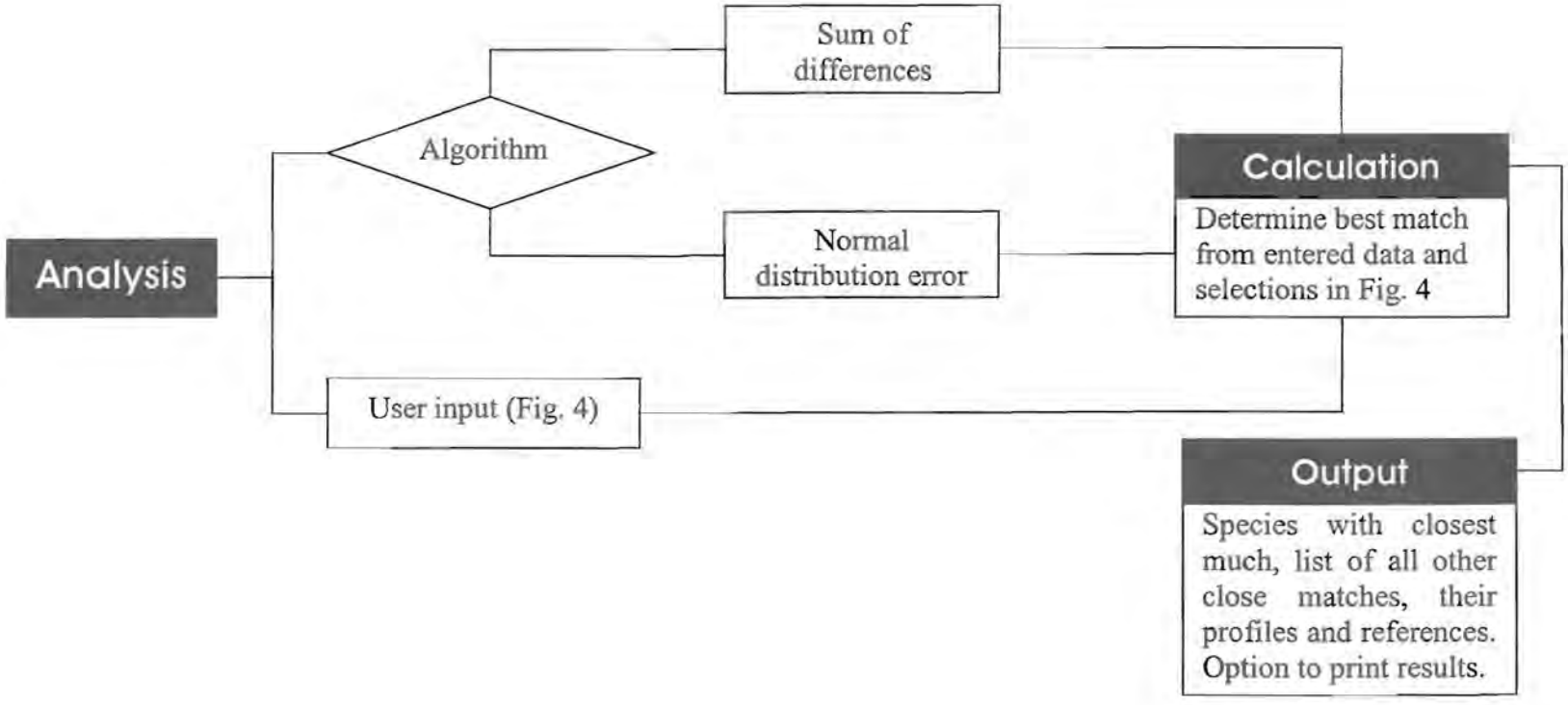
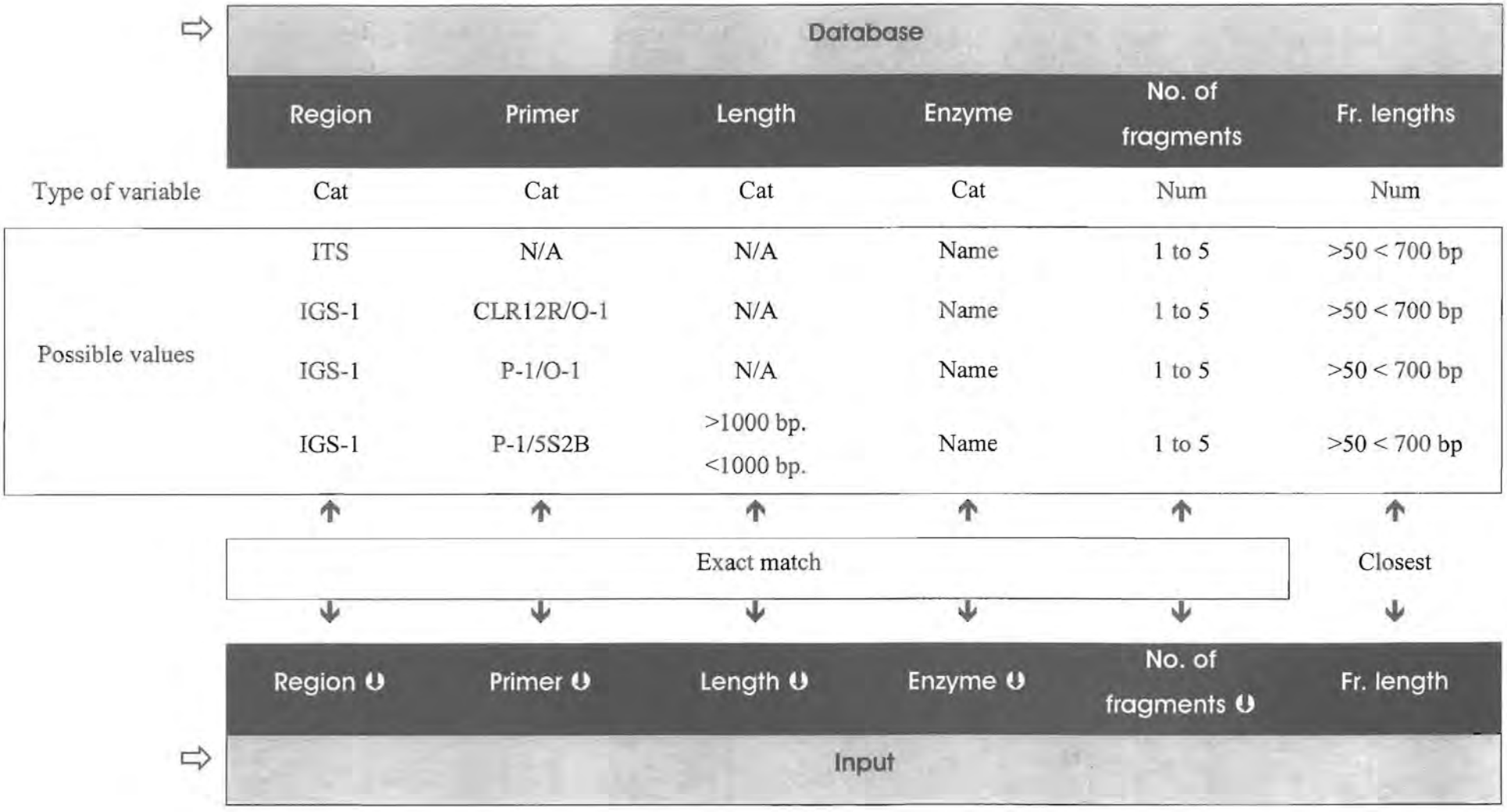


**Figure 4.** Interaction between database and application interface before calculating the best much between user and database profiles. The type of variables are either categorical (Cat), chosen via drop-down-menus or numerical (Num), provided by the user. Vertical arrows (⇨) indicate the directional sequence of events and encircled arrows (⓫), drop-down-menus with data from the database.



# DESCRIPTION OF THE USER GRAPHICAL INTERFACE

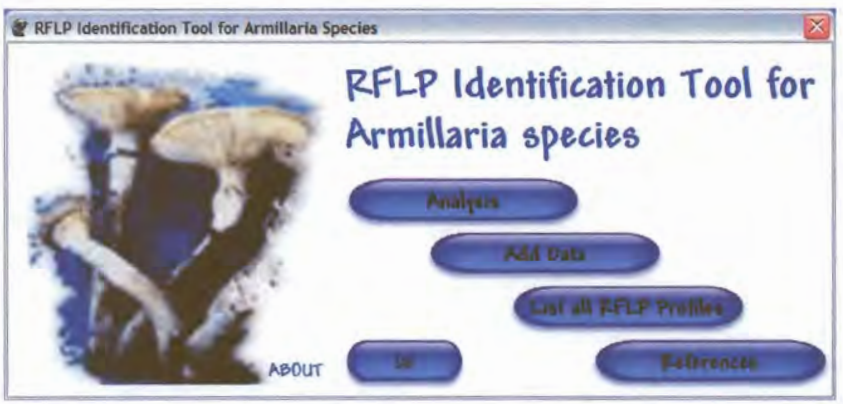
## REQUIREMENTS:

System: Windows® 95, Windows® 98, Windows® 2000, Windows® XP

Additional: Microsoft® Word

Best viewed at: 1152 x 864 pixels, 32 bit colour

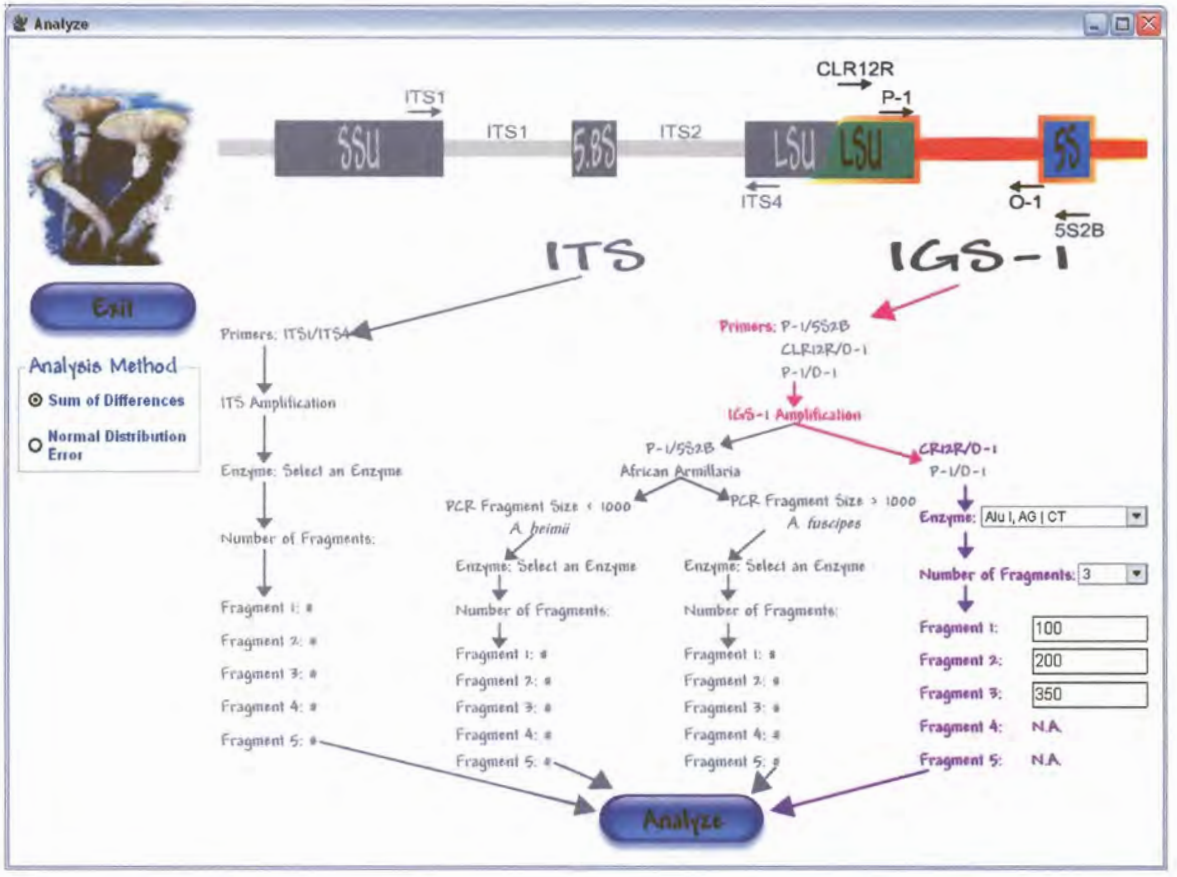
## GENERAL MENU:




The General (start) menu gives the five options:

- *Analysis:* RFLP profile analysis function of the application.
- *Add data*: RFLP profiles, enzymes or references can be added, edited or removed from the database.
- *List all RFLP profiles*: IGS-1 or ITS RFLP profiles are listed from the database and can be printed to a Microsoft® Word file.
- *References*: Lists all references from the database and exports references to a Microsoft® Word file if required.
- *Exit:* Exits the programme.

## ANALYSIS WINDOW

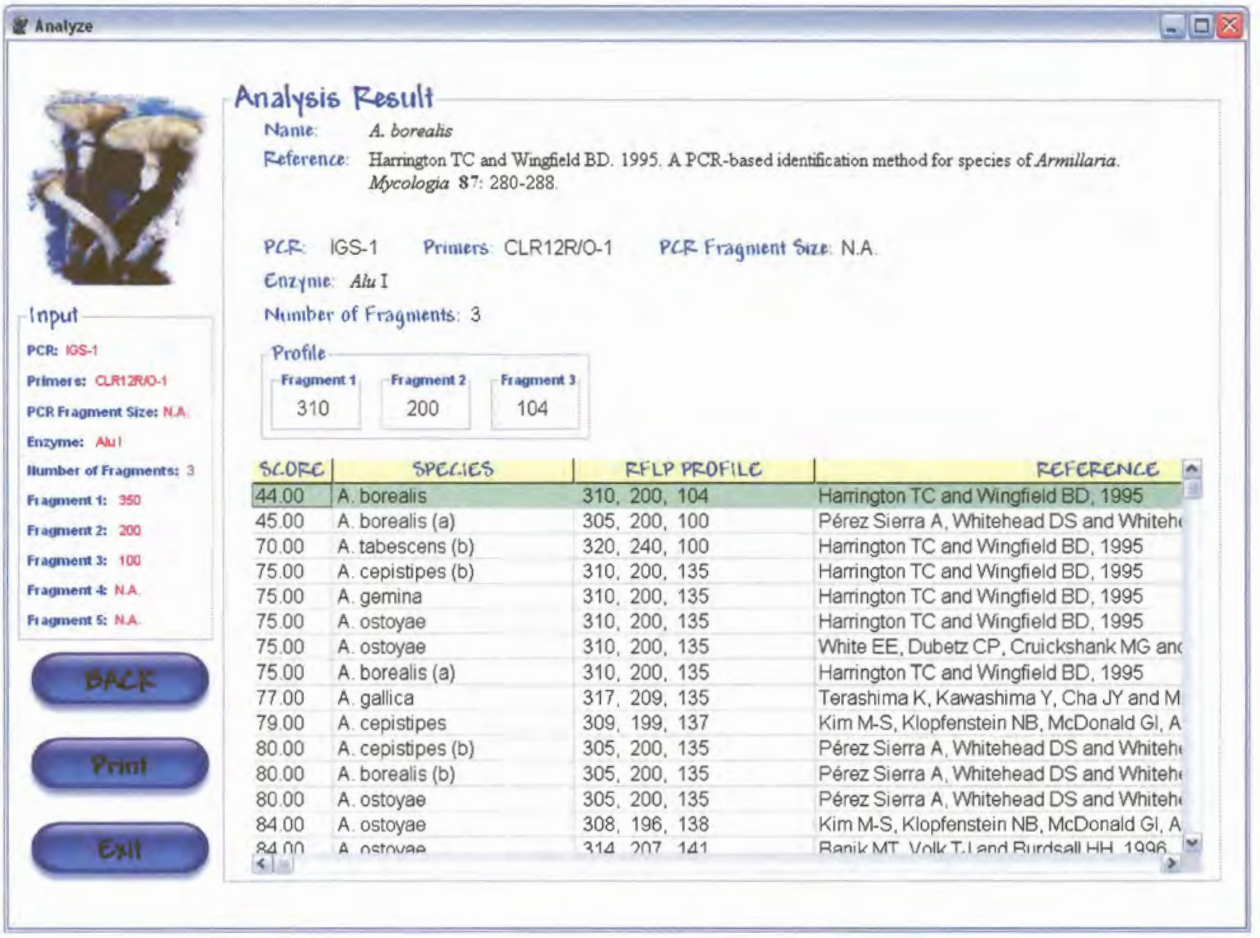



User RFLP profiles are analysed by following the steps outlined below:

1) ITS or IGS-1 rDNA region is selected by moving the mouse-pointer over the required region. The activated region change colours when selected.
2) A primer-pair is selected by moving the mouse-pointer over one of the primer-pairs; change in colour indicates the selected primer-pair.
3) Choosing primer pair P-1 / 5S2B will activate options for the PCR fragment size of the African *A. fuscipes* (> 1000 bp.) or *A. heimii* (< 1000 bp.).
4) An enzyme is selected from a drop-down menu that lists all enzymes and their restriction sequences from the database.
5) The number of fragments (between 1 and 5) to be entered are selected from a drop-down menu.
6) Boxes for the fragment sizes of the RFLP profile open after Step 4. Only fragment sizes between 50 and 700 bp. are accepted.
7) The Analyze button is pressed to execute the analysis.

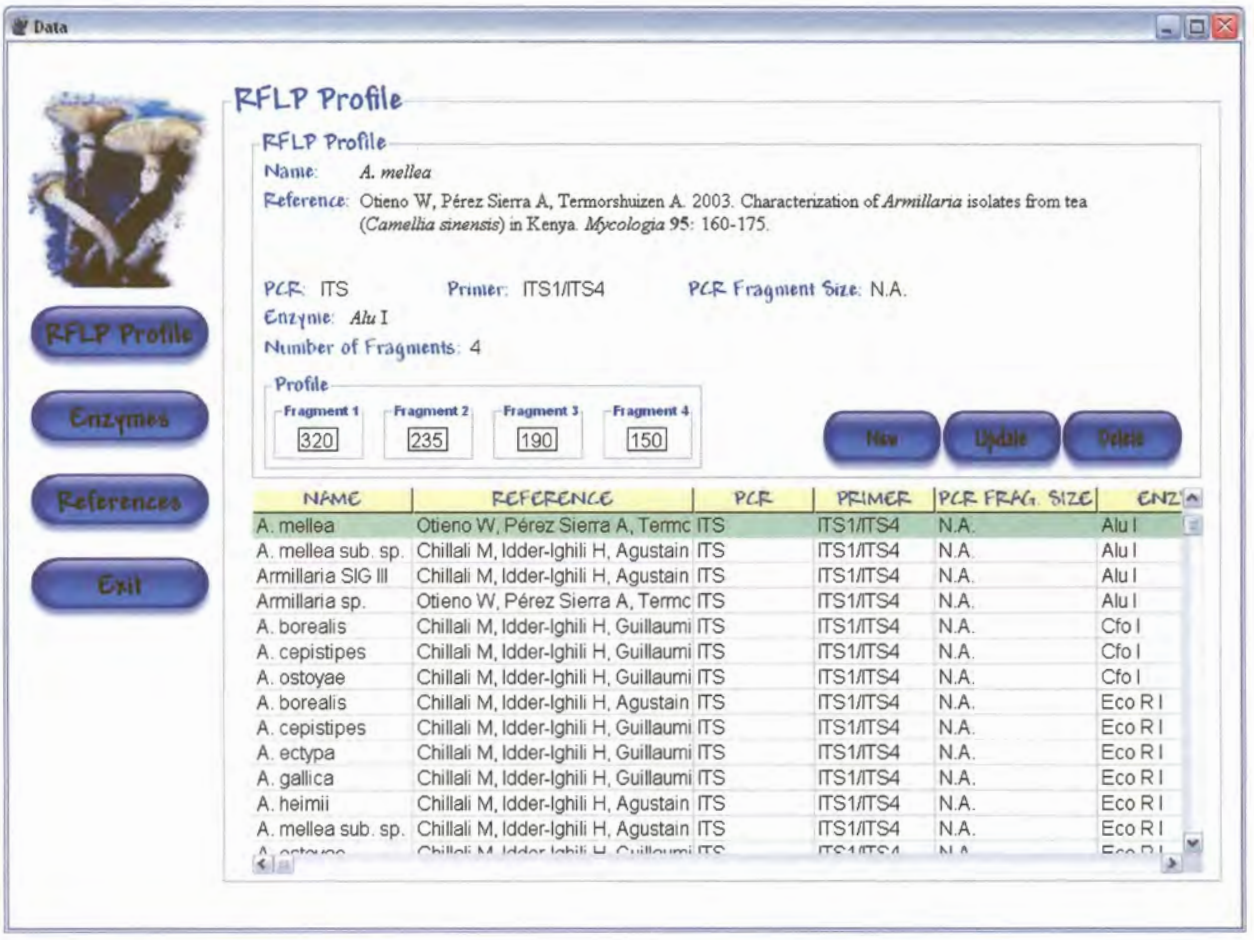
One of two analysis algorithms, sum of differences and normal distribution error, can be selected by the user for the analysis.

## ANALYSIS RESULT WINDOW



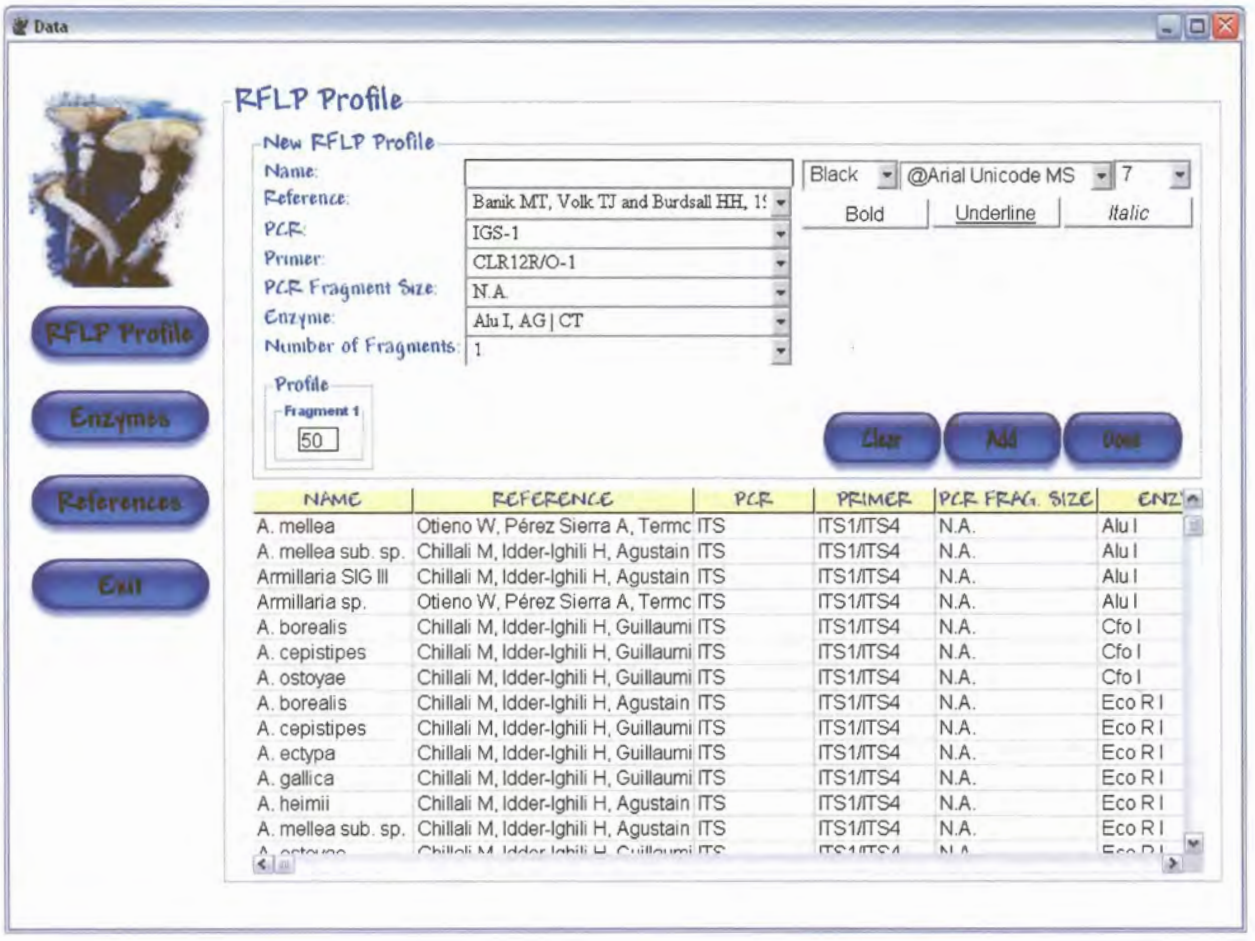

The Result window gives a list of species, in the order of the best to the worst match with the user RFLP profile, together with all their information. Detailed information pertaining to a specific species record (name, full reference, PCR region, primer pairs, PCR fragment sizes, enzyme used, number of fragments and fragment sizes) is displayed after selecting the record from the table. The table can also be exported to a Microsoft® Word file using the Print button. The Back button will take the user to the analysis window, which will still contain all the selected and entered data; these can be changed and the analysis repeated. The Exit button returns the user to the General window.

## ADD DATA – RFLP PROFILE WINDOW




*RFLP profile records can be added, updated or deleted within the Add Data window after pressing the RFLP Profile button. This procedure requires the following steps:*

1) A list of all records within the database is displayed in a table.
2) A specific record can be selected with the mouse-pointer by clicking on any of the items in the table; this will highlight the selection and show detailed information regarding the species.
3) The selected record can then be updated or deleted using the Update and Delete buttons.

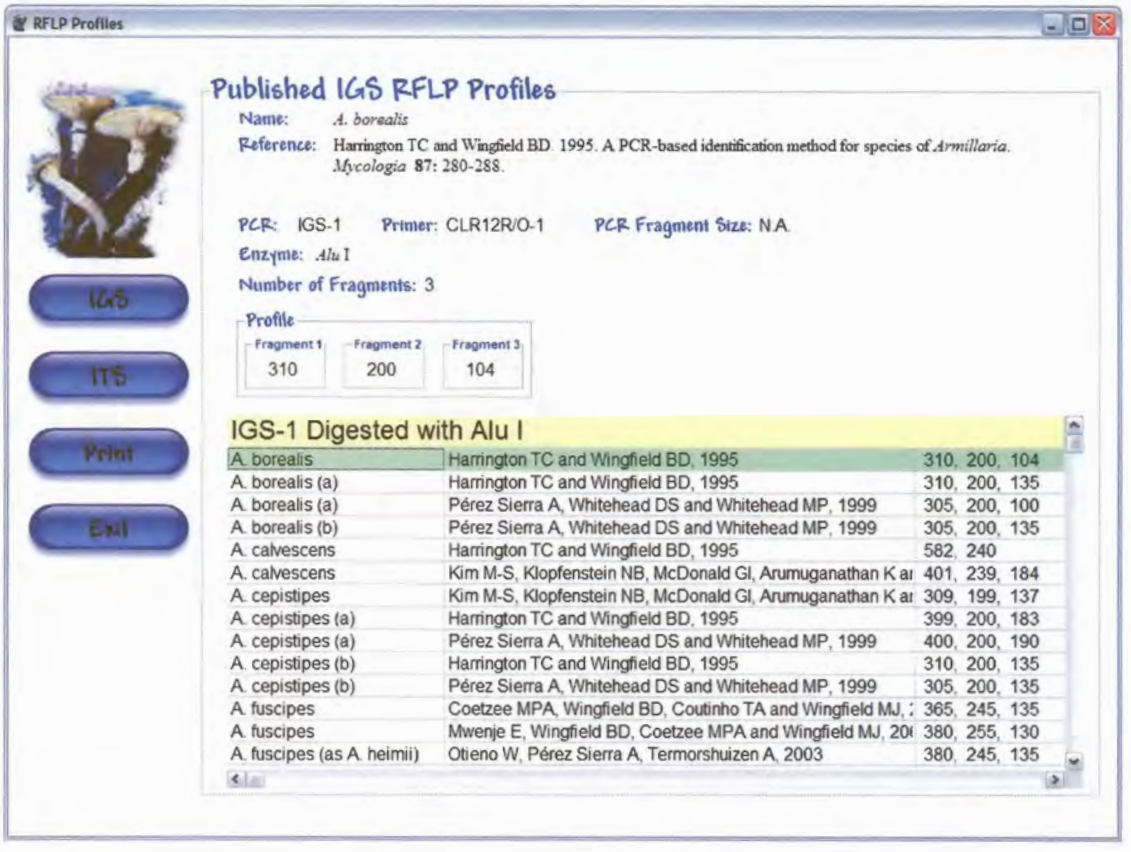



*New RFLP profiles are added to the database by pressing the New button (see screen on previous page).*

1) Data are entered by the user or selected from pull-down-menus.
2) The Add button will add the data to the database and the next record can then be entered.
3) The Done button will close the Add function and the new records are displayed in the table.

*The procedure outlined above is also applicable to adding, updating and removing enzymes and reference records.*

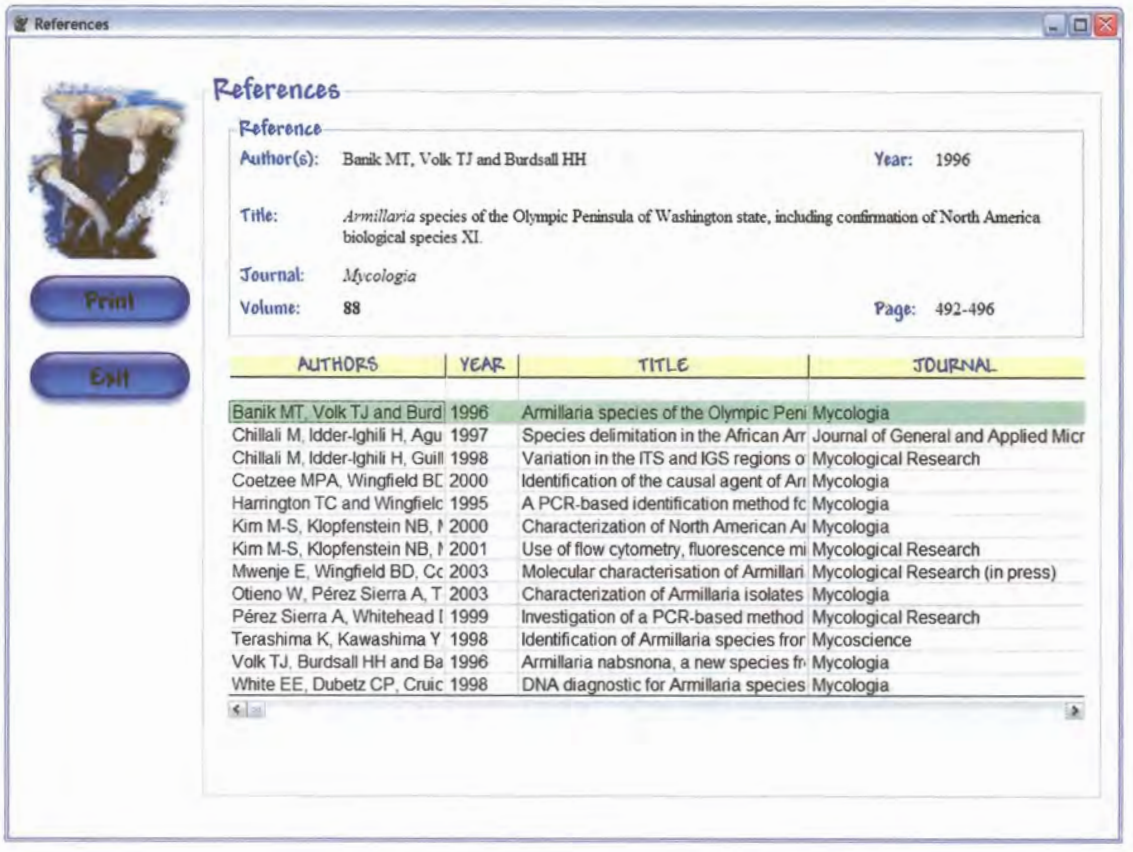
## LIST ALL PROFILES WINDOW




Selected subsets of RFLP profiles can be displayed following the steps outlined below:

1) *An rDNA region is selected using the IGS or ITS buttons.*
2) A table with all records in the database pertaining to that region is displayed.
3) A specific record can be selected from the database in the table.
4) The Selected record is highlighted and detailed information is displayed.
5) A list of all the records in the database pertaining to the selected rDNA region can be exported to Microsoft® Word using the Print button.
6) The Exit button returns the user to the General window.

## LIST ALL REFERENCES WINDOW




References stored in the database are viewed through the following steps:

1) A table with all records in the database is displayed.
2) A specific record can be selected from the database.
3) The selected record is highlighted and detailed information is displayed.
4) A list of all the reference records can be exported to Microsoft® Word using the Print button.
5) The Exit button returns the user to the General window.