CHAPTER FOUR

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

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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 χ^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.

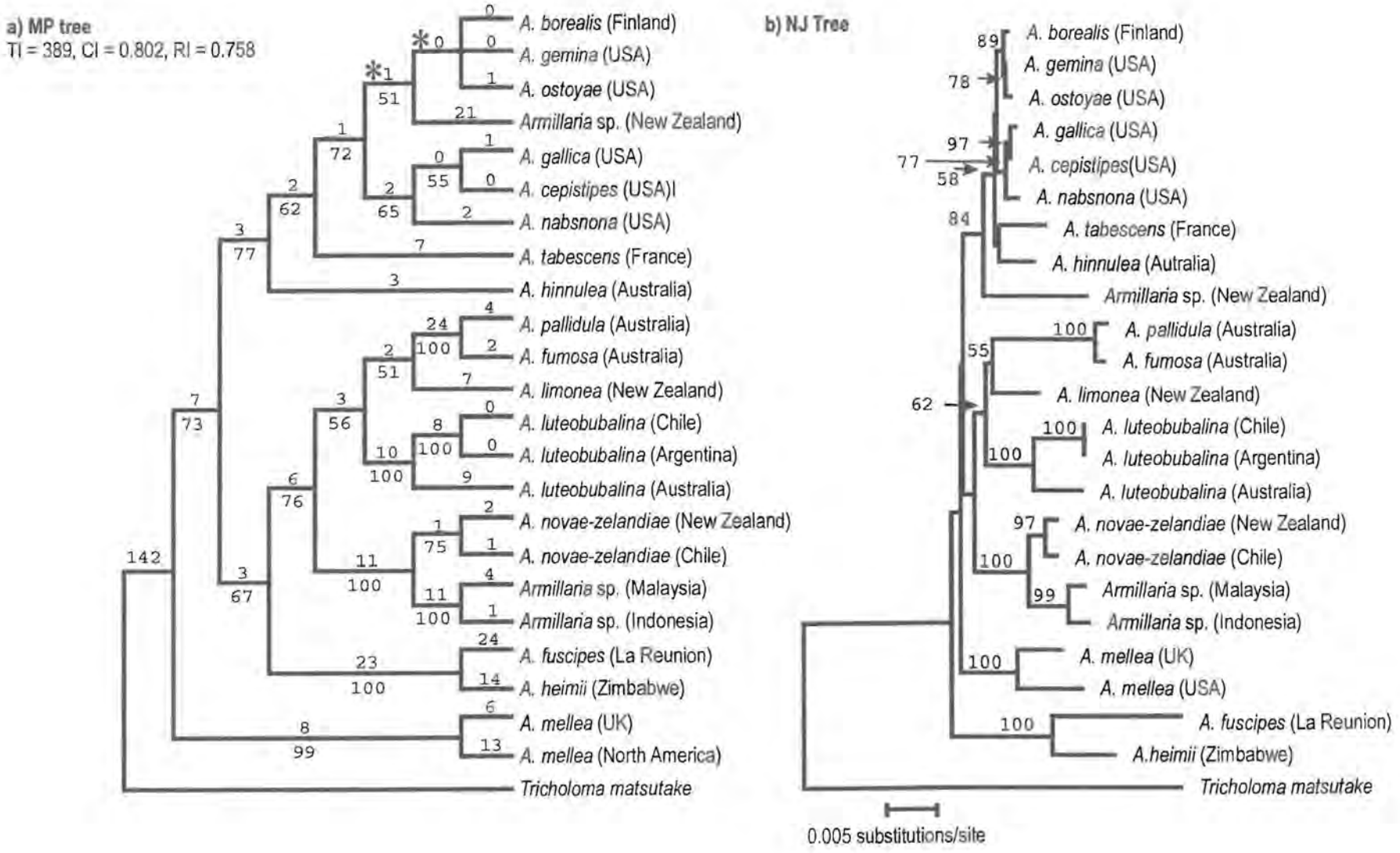


Figure 2. Phylogenetic trees obtained form D1 to D3 sequence data. a) Cladogram (MP), b) Neighbor-Joining tree. Numbers above tree branches in the cladogram indicate branch-length. Bootstrap values are indicated below the branches on the MP tree and above the branches on the NJ tree. Branches that collapsed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

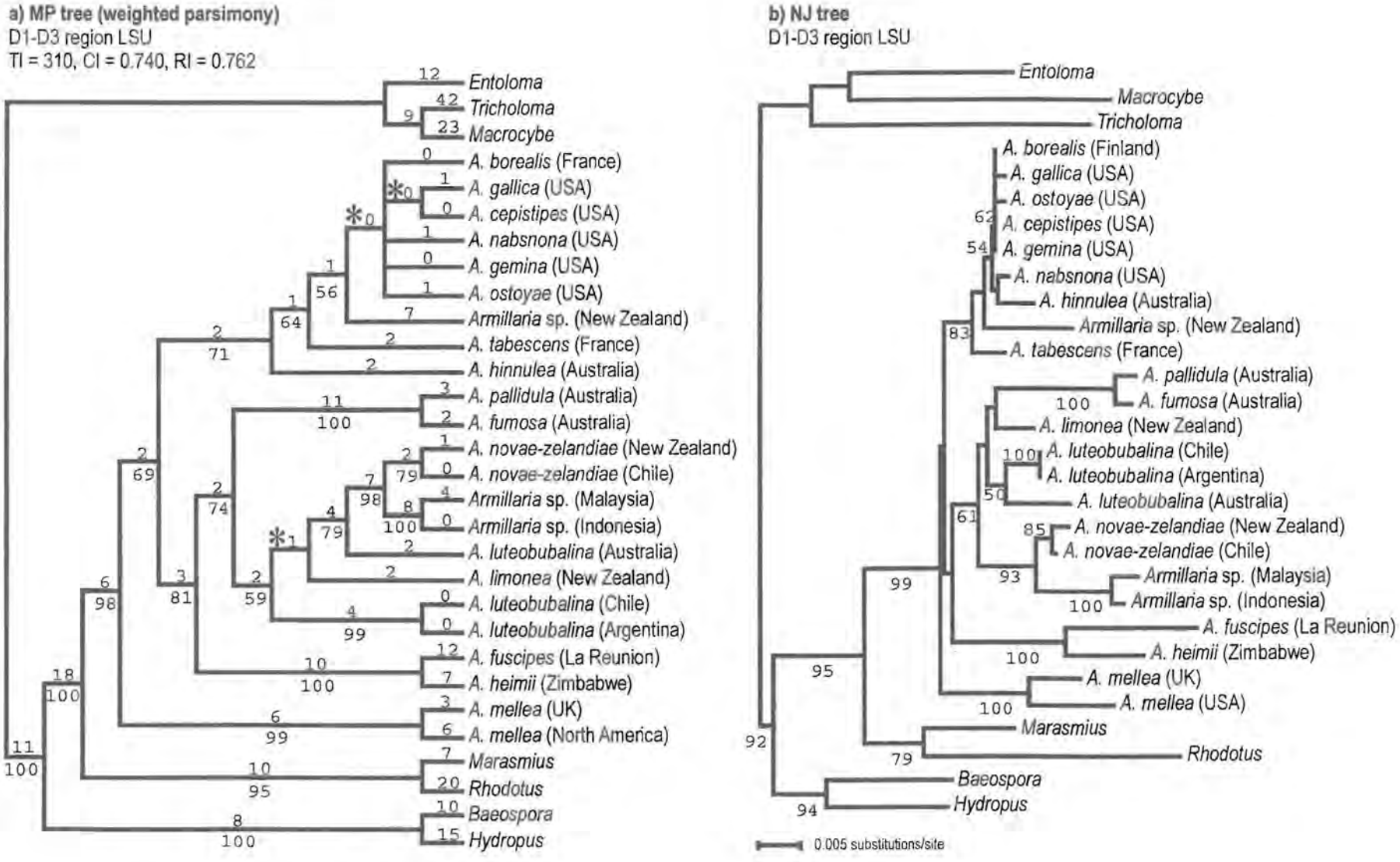


Figure 3. Branch-and-bound phylogenetic tree generated from D1 to D3 sequence data. Dashed lines indicate the position of *A .mellea* in the unconstrained branch-and-bound tree.

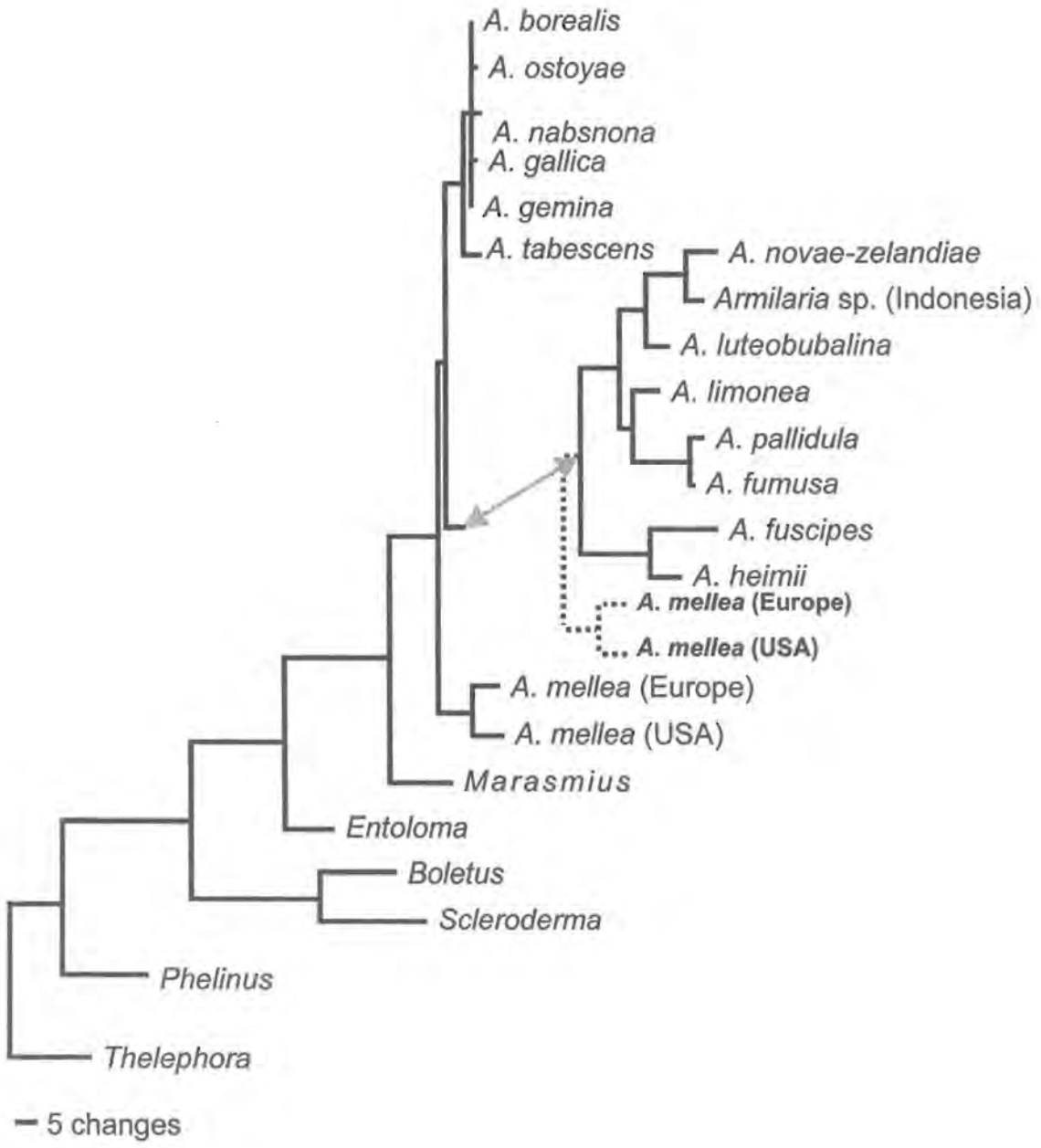

A. borealis
A. ostoyae
A. nabsnona
A. gallica
A. gemina
A. tabescens
A. novae-zelandiae
Armilaria sp. (Indonesia)
A. luteobubalina
A. limonea
A. pallidula
A. fumusa
A. fuscipes
A. heimii
A. mellea (Europe)
A. mellea (USA)
A. mellea (Europe)
A. mellea (USA)
Marasmius
Entoloma
Boletus
Scleroderma
Phelinus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

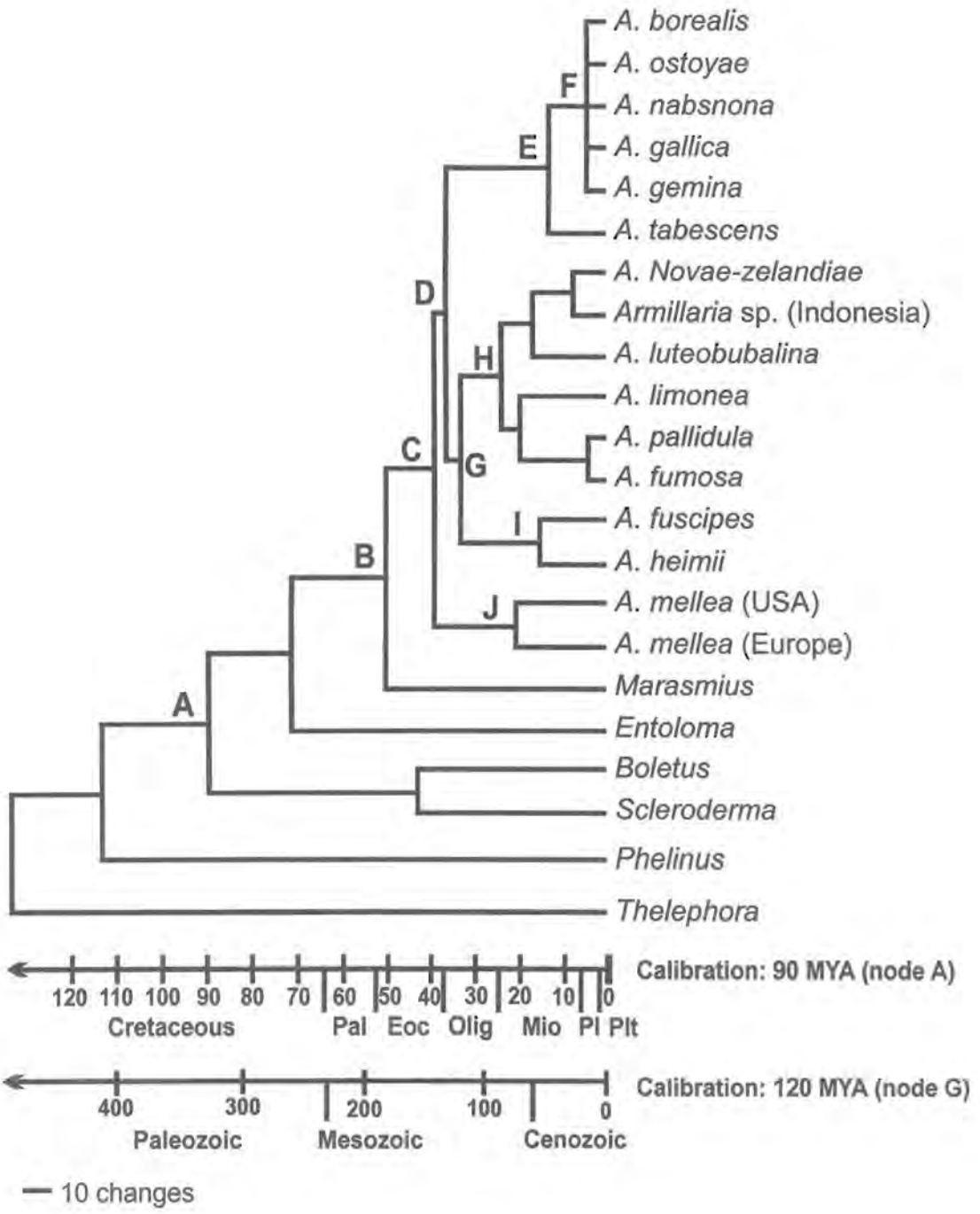
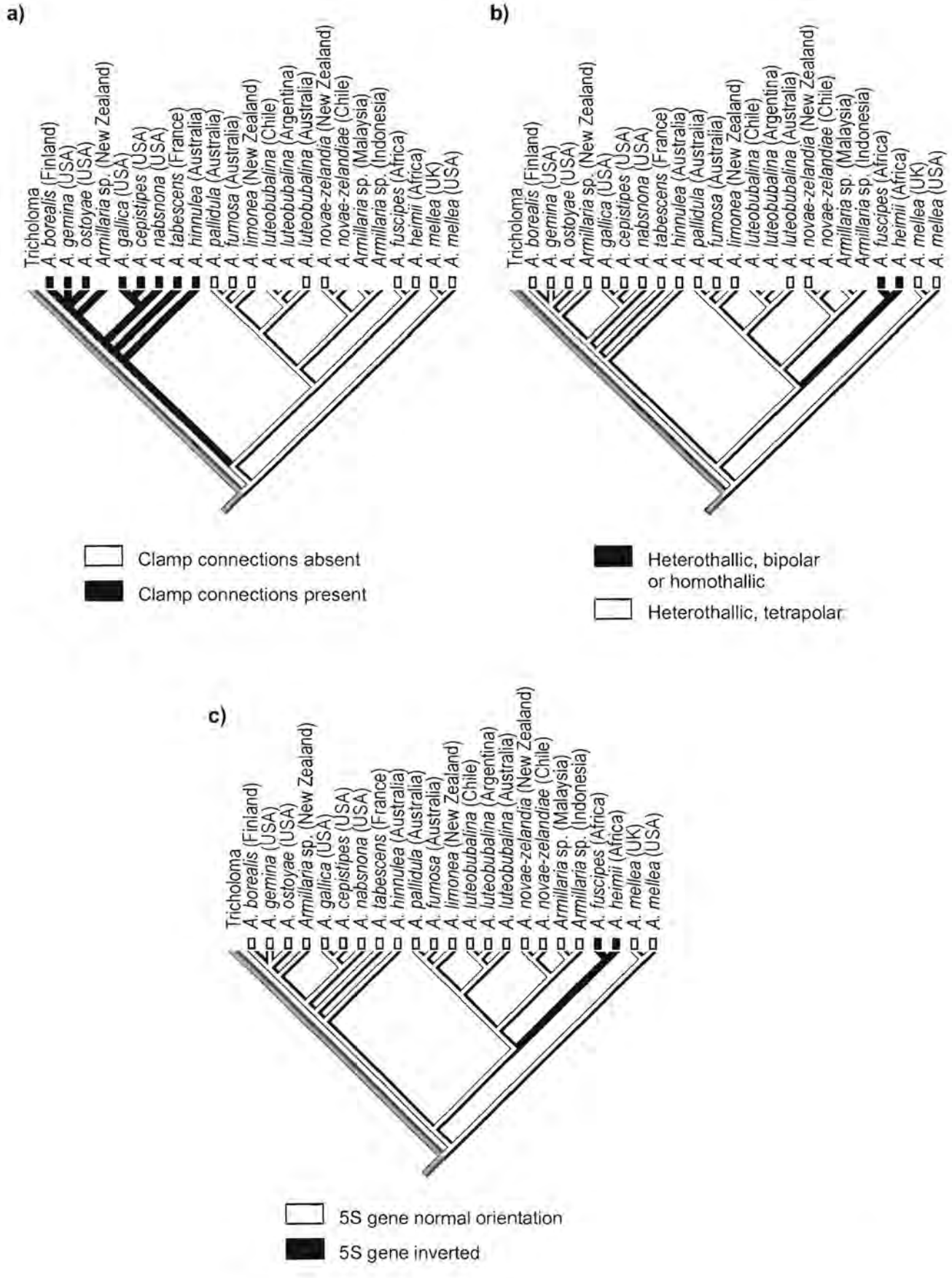

A. borealis
A. ostoyae
A. nabsnona
A. gallica
A. gemina
A. tabescens
A. Novae-zelandiae
Armillaria sp. (Indonesia)
A. luteobubalina
A. limonea
A. pallidula
A. fumosa
A. fuscipes
A. heimii
A. mellea (USA)
A. mellea (Europe)
Marasmius
Entoloma
Boletus
Scleroderma
Phelinus
Thelephora
A
B
C
D
E
F
G
H
I
J
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 (π) 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

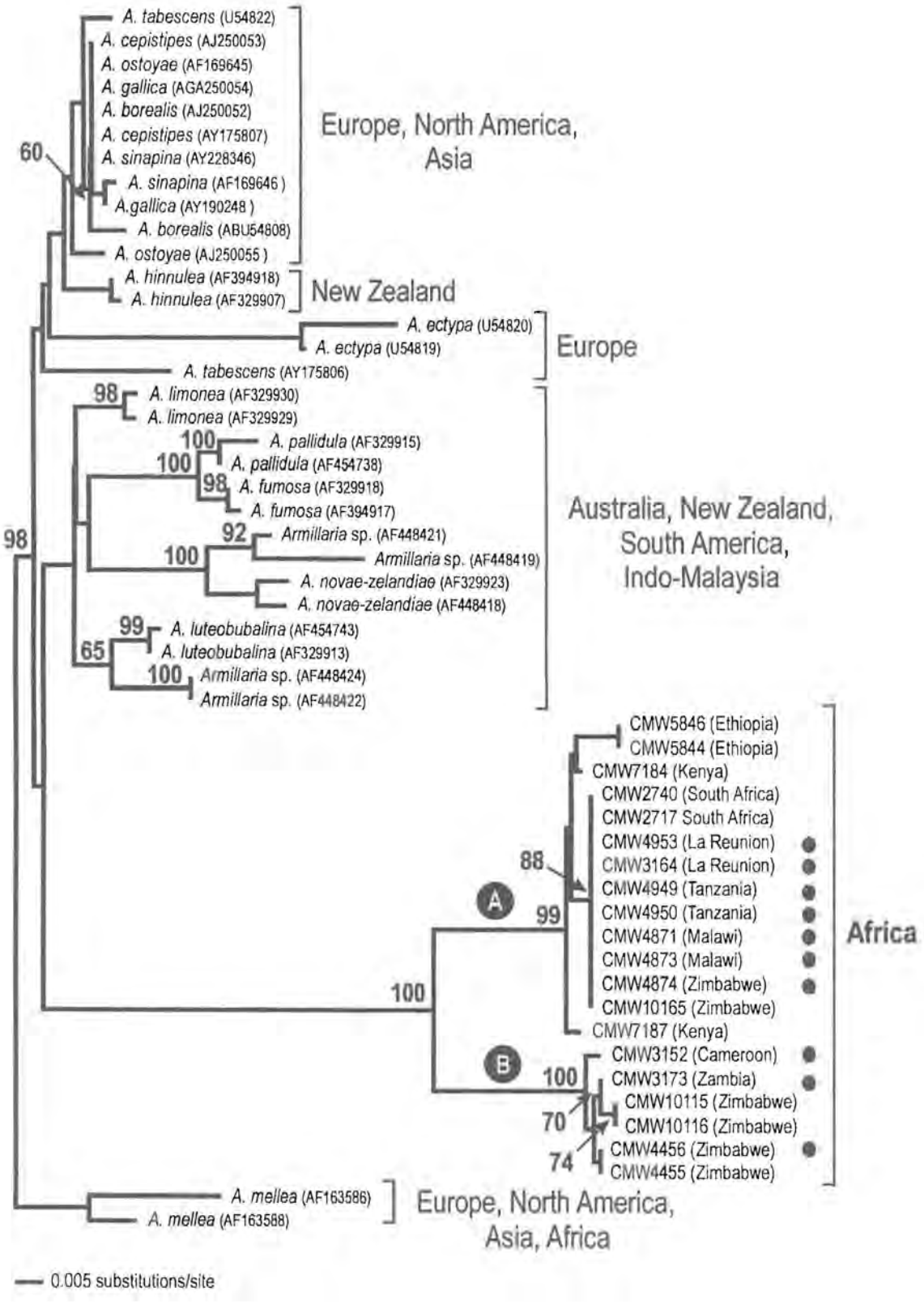

A. tabescens (U54822)
A. cepistipes (AJ250053)
A. ostoyae (AF169645)
A. gallica (AGA250054)
A. borealis (AJ250052)
A. cepistipes (AY175807)
A. sinapina (AY228346)
A. sinapina (AF169646)
A.gallica (AY190248)
A. borealis (ABU54808)
A. ostoyae (AJ250055)
Europe, North America, Asia
A. hinnulea (AF394918)
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).

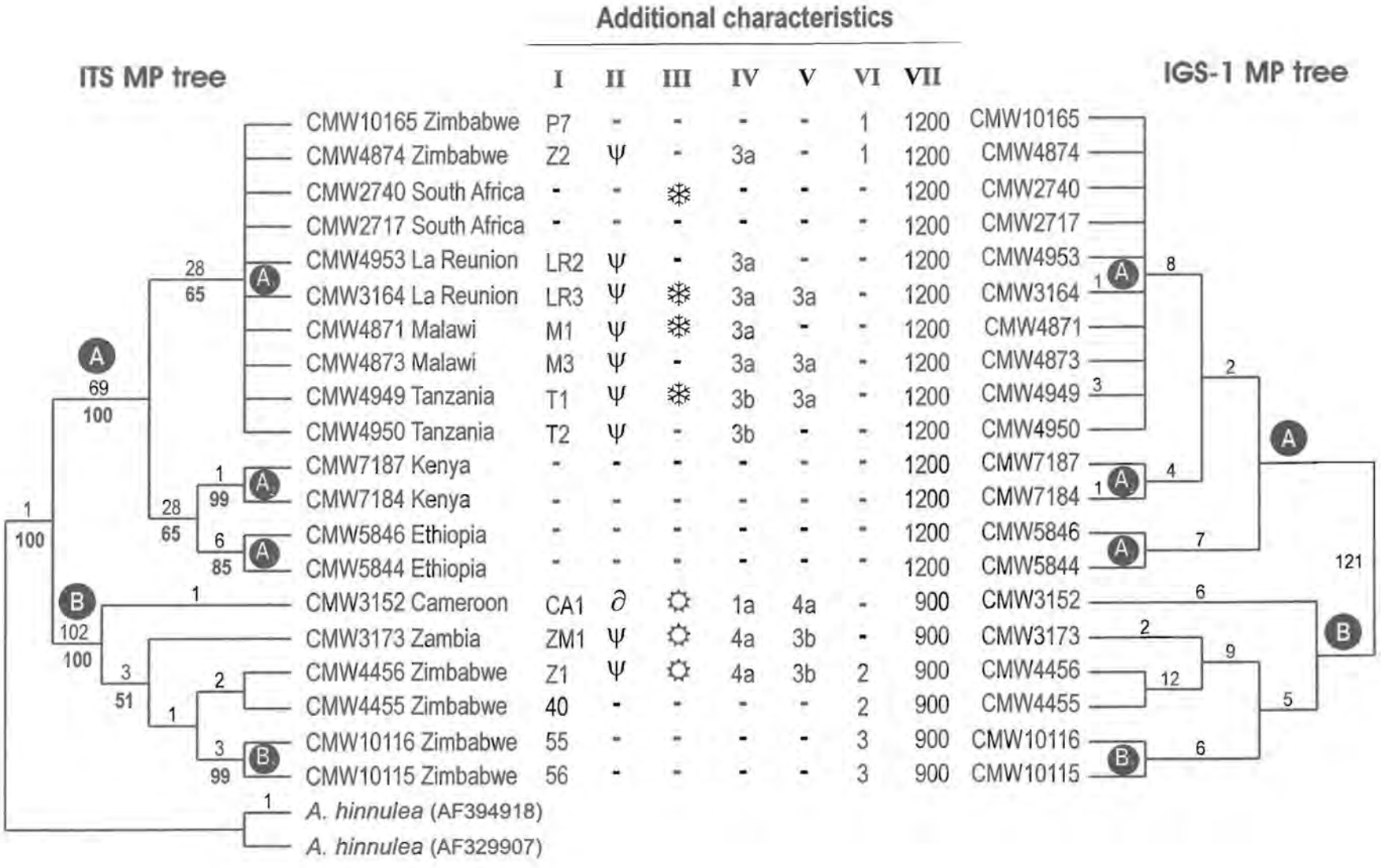


Figure 3. Neighbour-Joining trees generated from the African ITS and IGS-1 data matrixes. Bootstrap values are given below the tree branches.

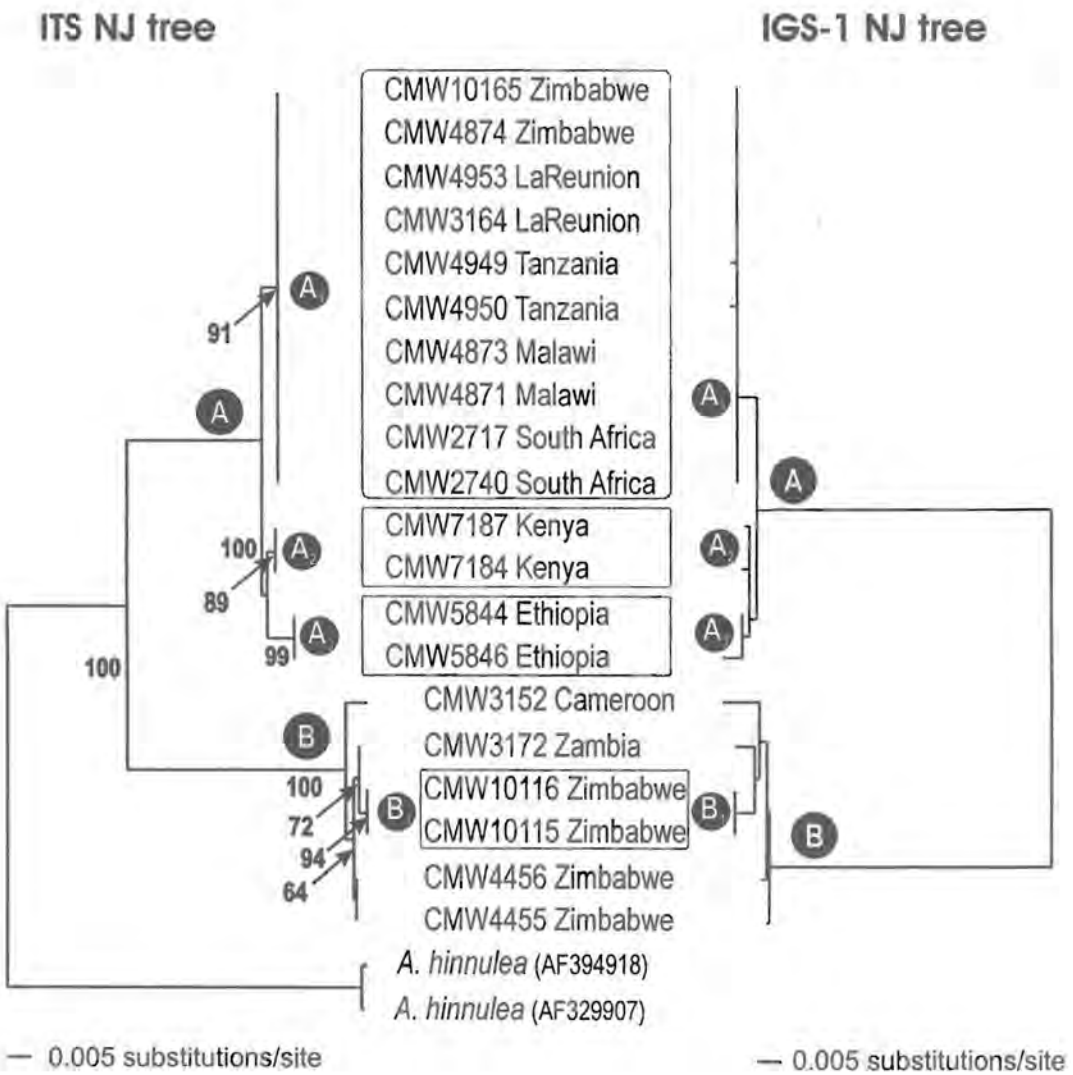
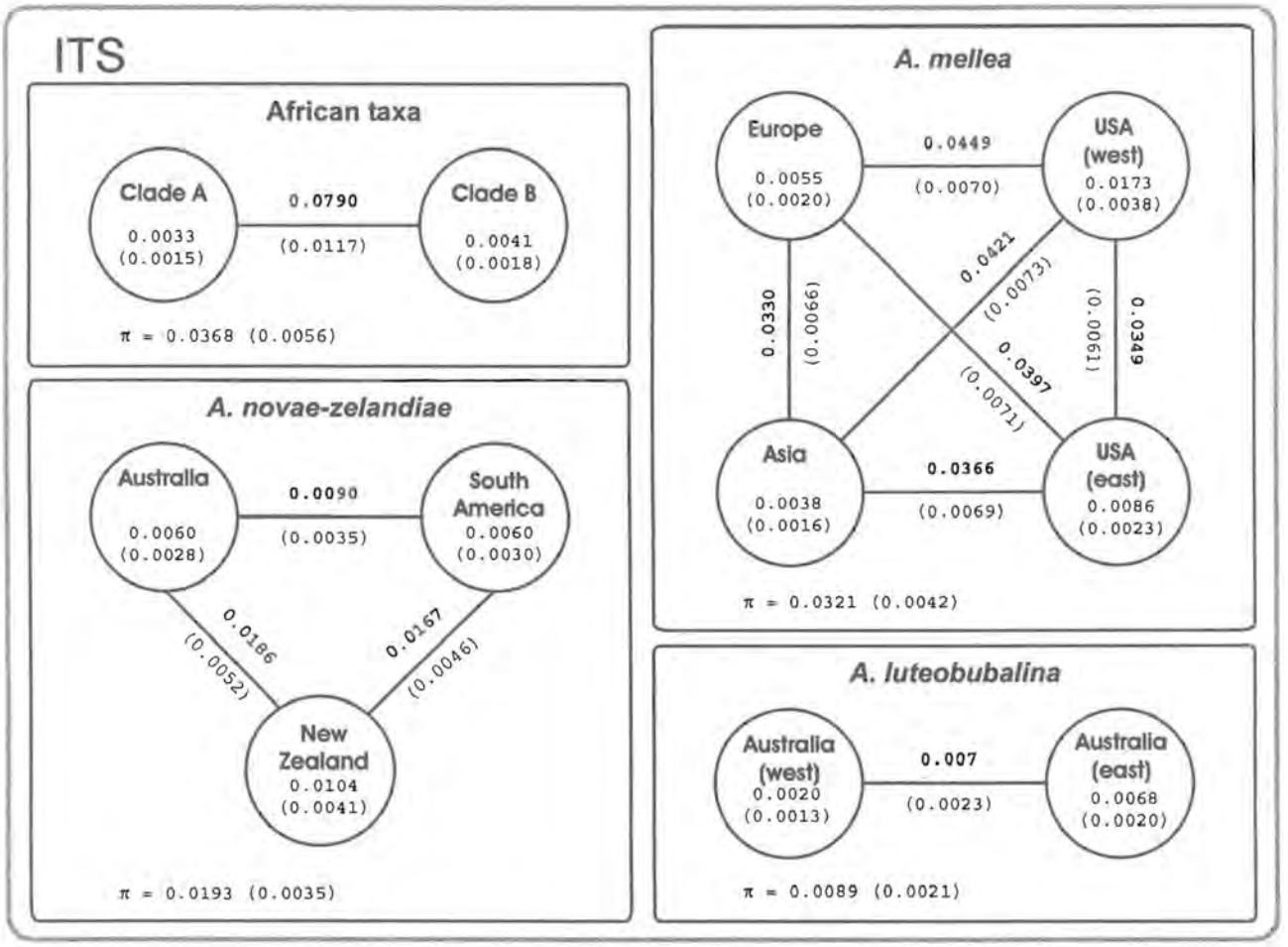
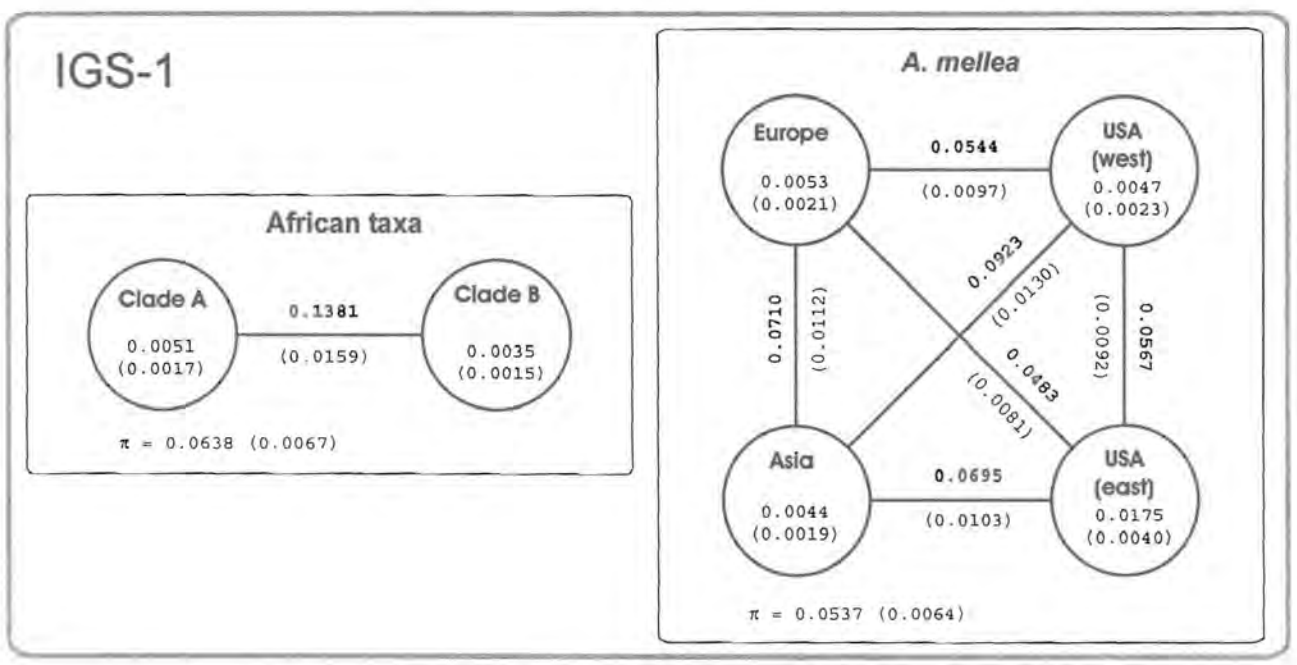

ITS NJ tree
IGS-1 NJ tree
CMW10165 Zimbabwe
CMW4874 Zimbabwe
CMW4953 LaReunion
CMW3164 LaReunion
CMW4949 Tanzania
CMW4950 Tanzania
CMW4873 Malawi
CMW4871 Malawi
CMW2717 South Africa
CMW2740 South Africa
CMW7187 Kenya
CMW7184 Kenya
CMW5844 Ethiopia
CMW5846 Ethiopia
CMW3152 Cameroon
CMW3172 Zambia
CMW10116 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 (π) 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).

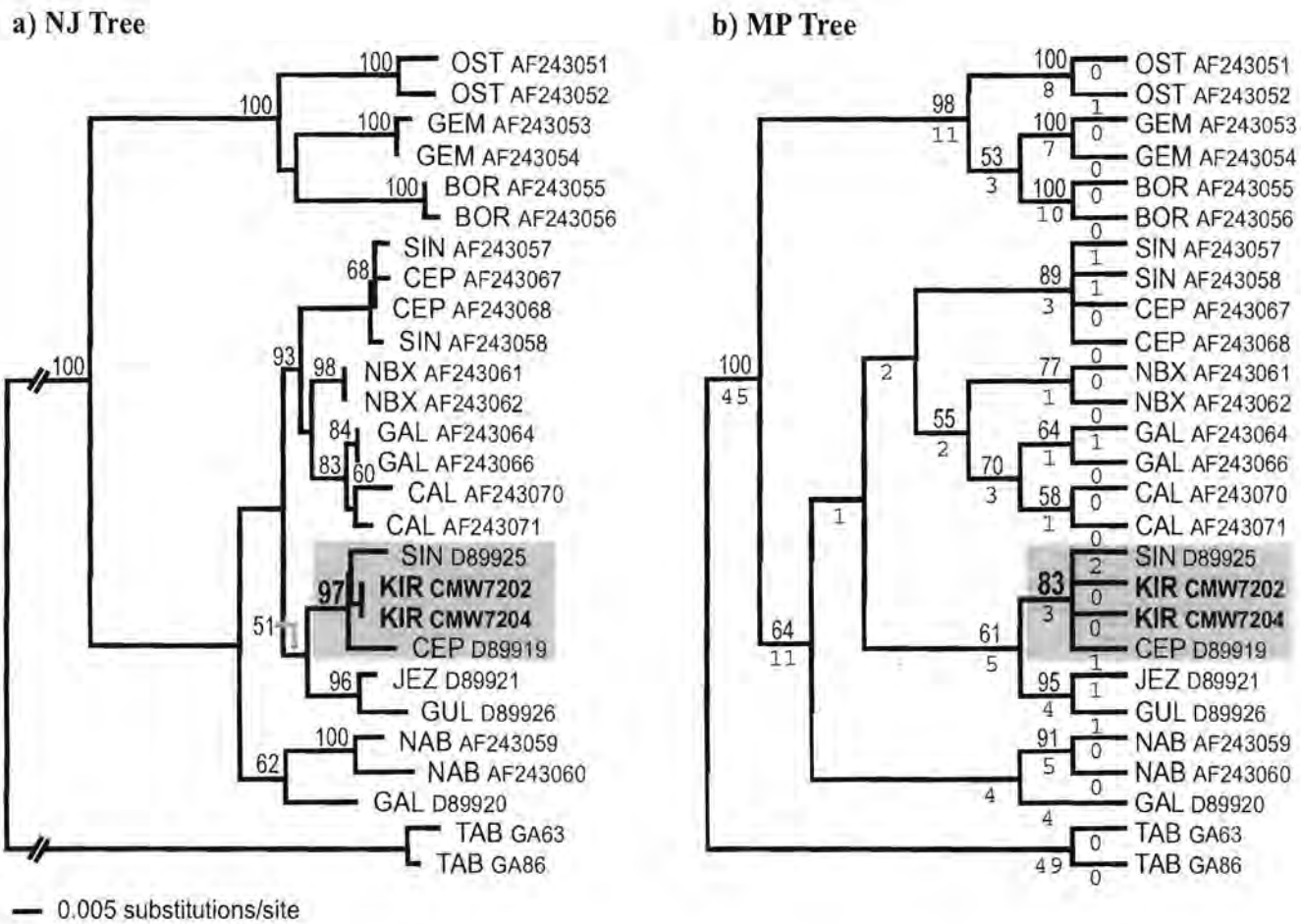
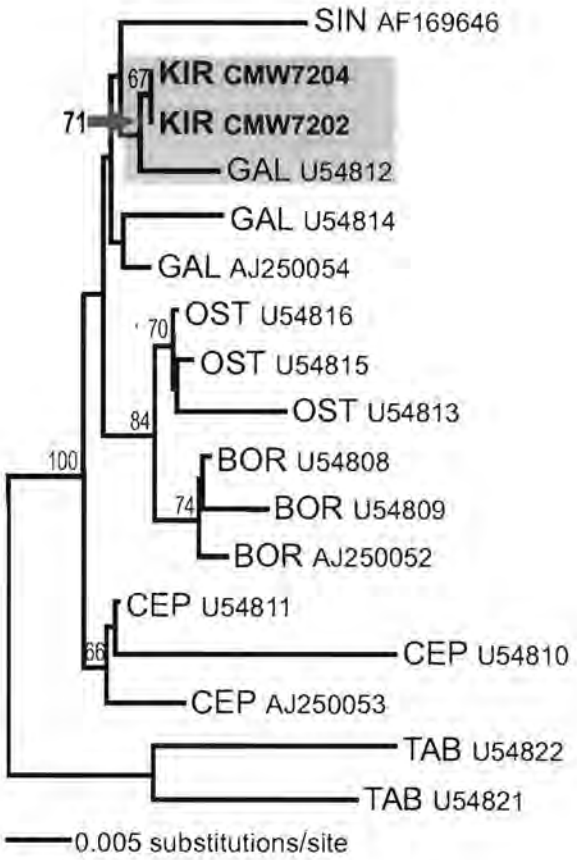

a) NJ Tree
OST AF243051
OST AF243052
GEM AF243053
GEM AF243054
BOR AF243055
BOR AF243056
SIN AF243057
CEP AF243067
CEP AF243068
SIN AF243058
NBX AF243061
NBX AF243062
GAL AF243064
GAL AF243066
CAL AF243070
CAL AF243071
SIN D89925
KIR CMW7202
KIR CMW7204
CEP D89919
JEZ D89921
GUL D89926
NAB AF243059
NAB 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

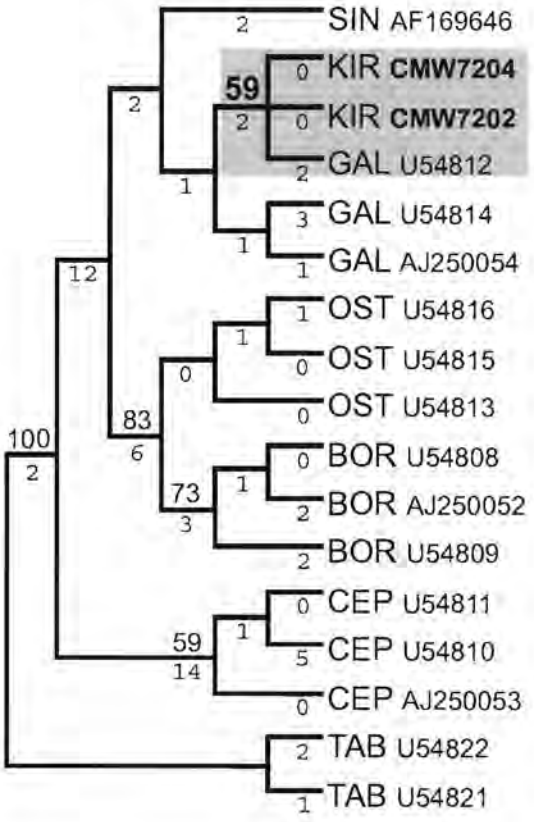
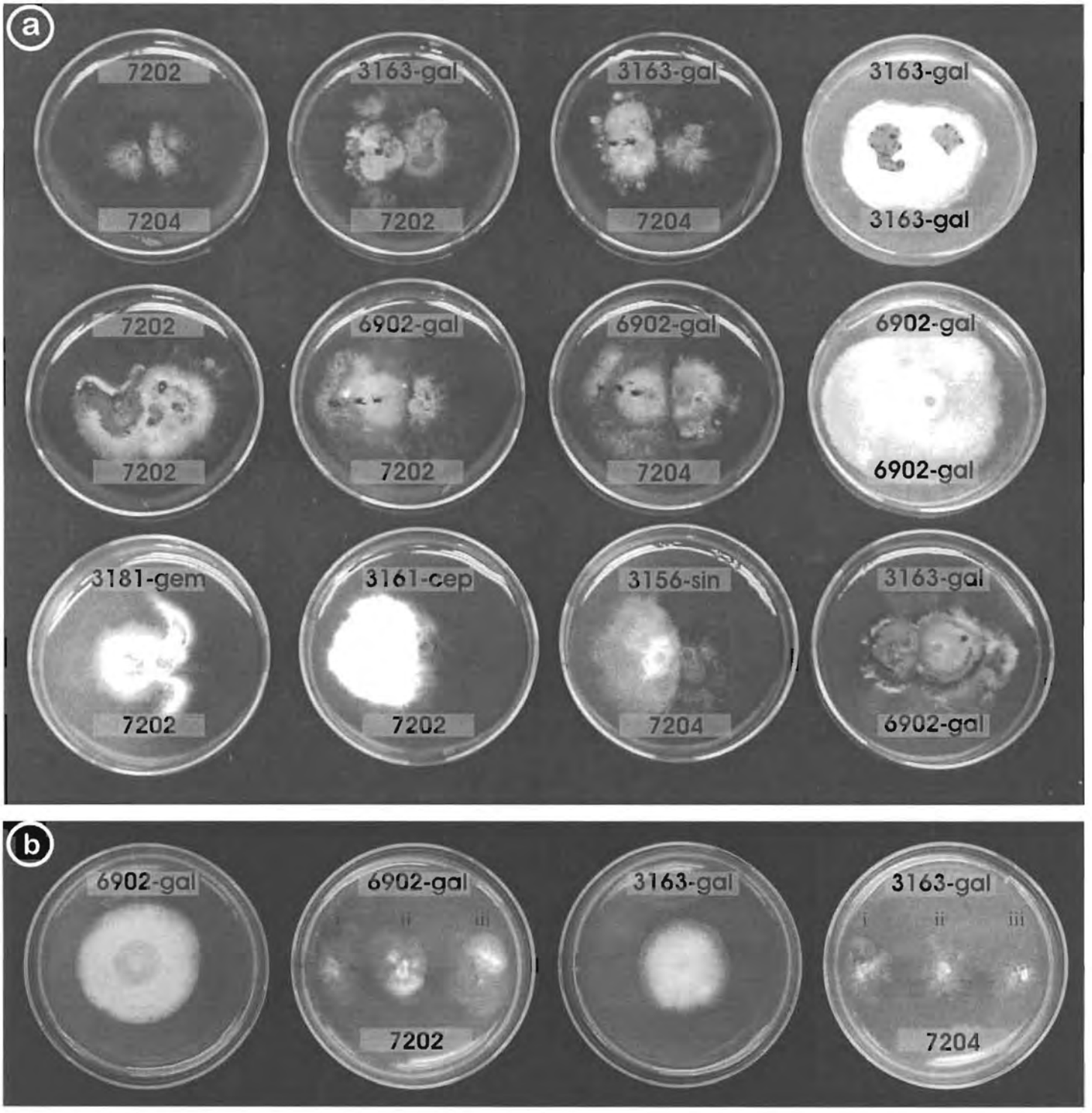


Figure 3. Sexual compatibility tests. a) Interaction between tester strains and isolates from Kirstenbosch. Numbers at the top and bottom of the Petri dish pertain to the inoculum at the left and right, respectively. b) Morphology of the secondary cultures made from the putative diploidized haploid tester 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