# CHAPTER TWO

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

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

## ABSTRACT

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

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

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

## MATERIALS AND METHODS

### Fungal isolates

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

### DNA extraction

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

### PCR

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

### DNA sequencing

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

**Sequence analysis**

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

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

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

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

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

## RESULTS

### PCR

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

### Phylogenetic analysis

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

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

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

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

## DISCUSSION

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

TABLE 1 (continued)

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

TABLE 1 (continued)

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

**TABLE** 1 (continued)

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

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

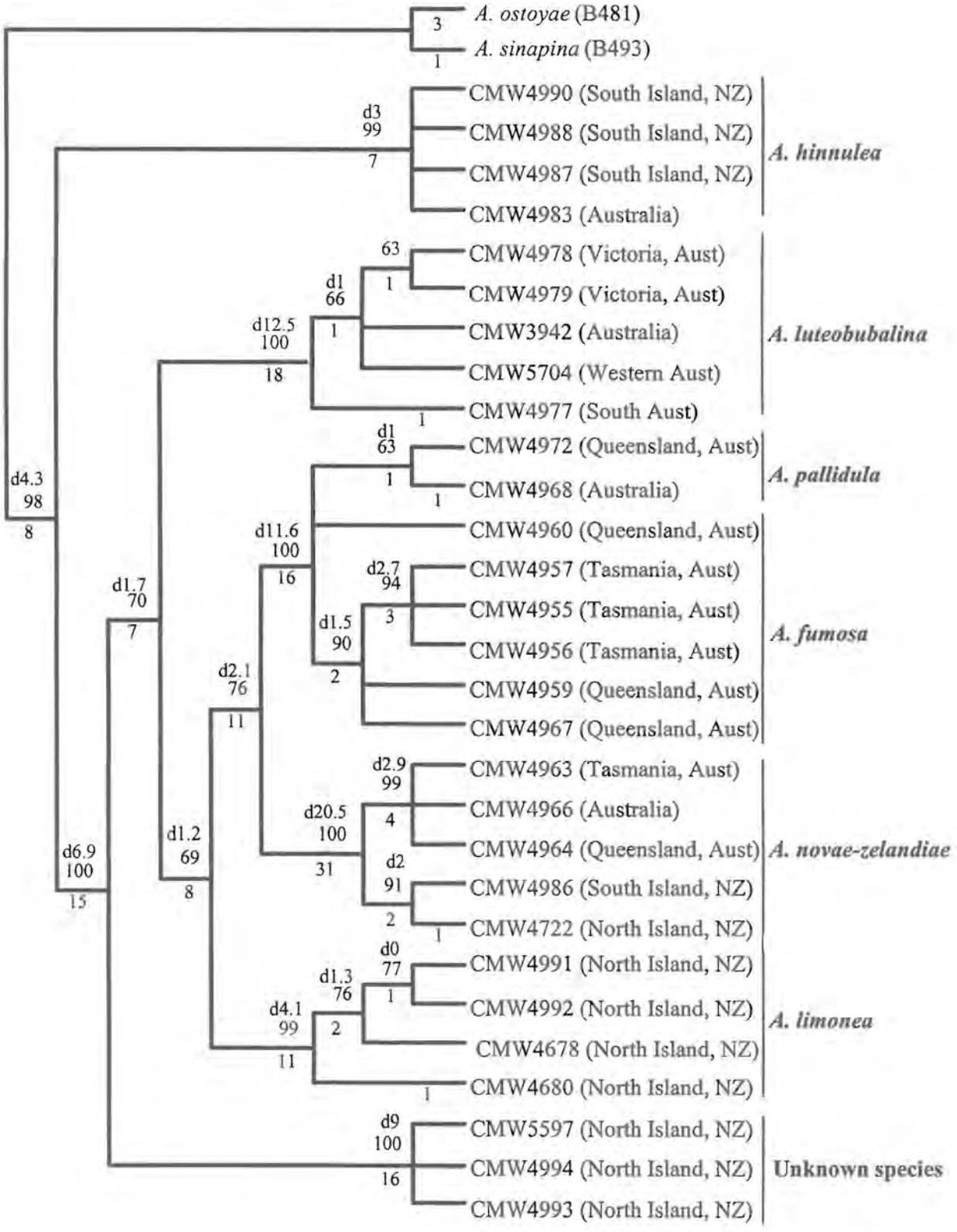
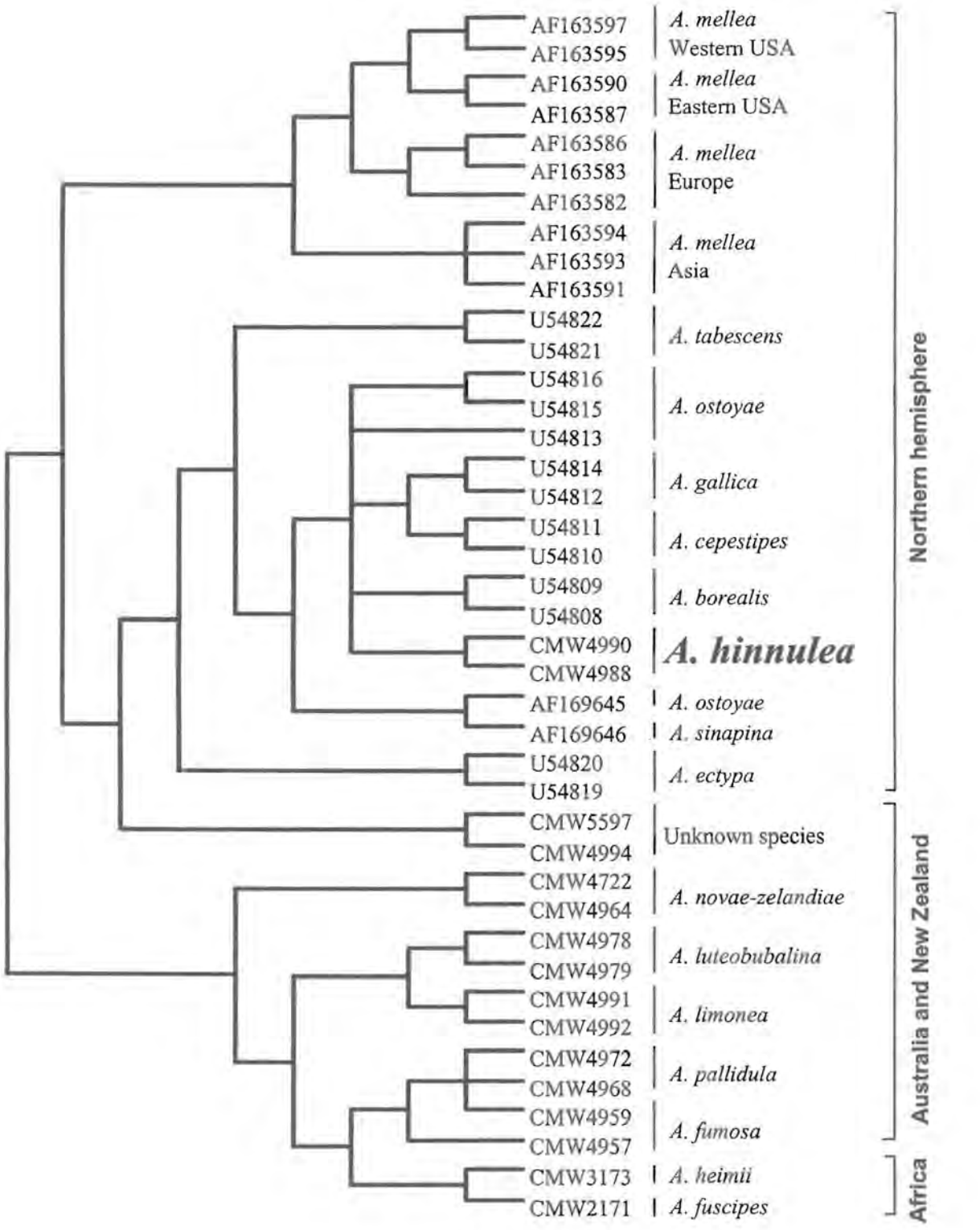


**Figure 2.** Strict consensus tree from 210 MP trees for *Armillaria* spp. from the Northern and the Southern Hemisphere. Number of parsimony informative characters = 339, length of tree = 485, CI = 0.786, RI =0.894. AF and U numbers refer to GenBank accession numbers.



# CHAPTER THREE

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

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

## ABSTRACT

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

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

## INTRODUCTION

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

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

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

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

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

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

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

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

## MATERIALS AND METHODS

### Fungal isolates

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

### DNA extraction

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

### PCR and sequencing

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

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

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

### Identification of unknown isolates

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

### Phylogenetic analyses

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

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

## RESULTS

### Identification of unknown isolates

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

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

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

### Phylogenetic analyses

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

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

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

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

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

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

## DISCUSSION

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

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

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

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

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

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

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

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

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

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

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

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

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

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

TABLE 2 (continued)

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

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

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

[a] Number of characters after alignment

[b] Number of parsimony informative characters

[c] Number of trees

[d] Tree length

[e] Consistency index

[f] Retention index

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

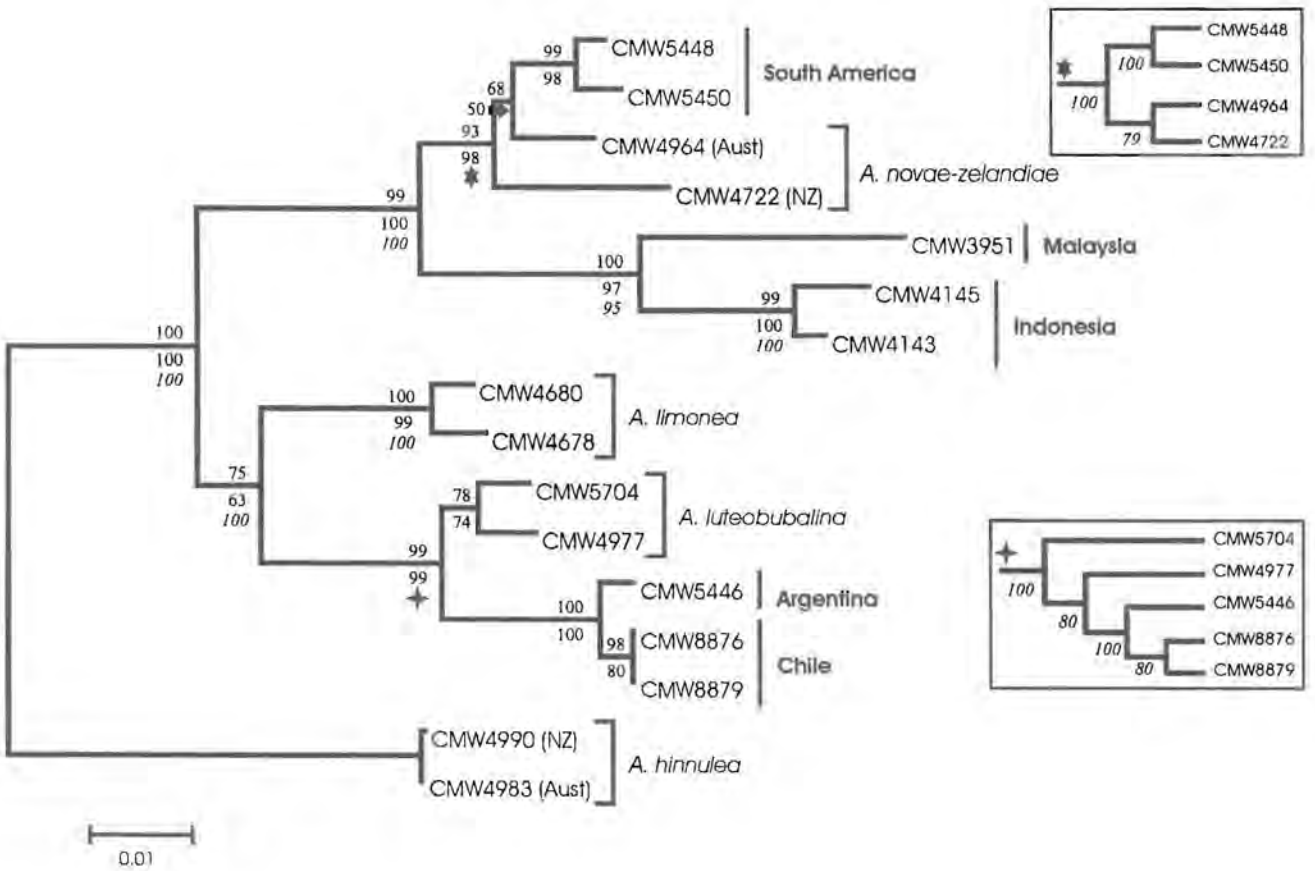
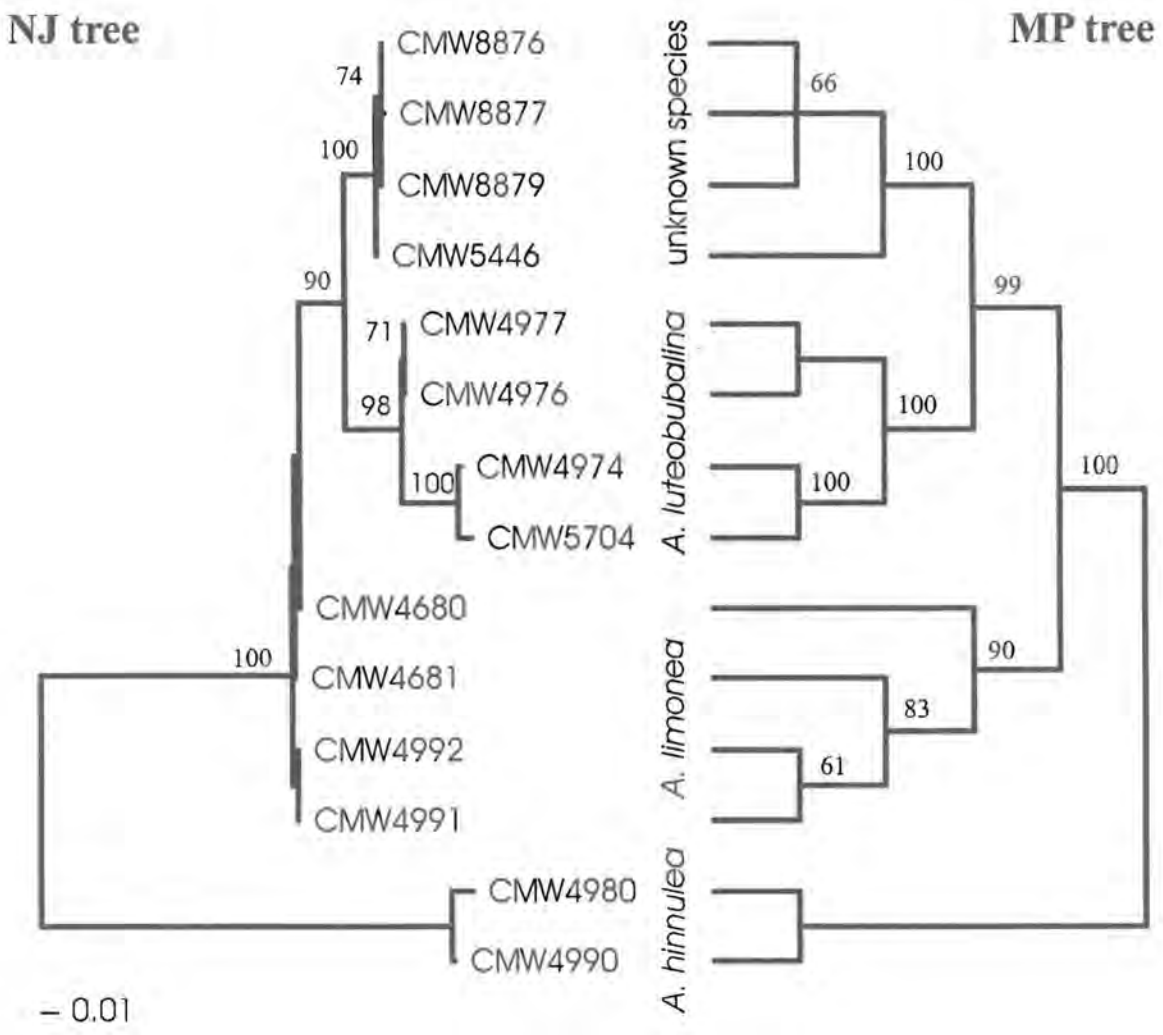

CMW5448
CMW5450
South America
CMW4964 (Aust)
CMW4722 (NZ)
A. novae-zelandiae
CMW3951
Malaysia
CMW4145
CMW4143
Indonesia
CMW4680
CMW4678
A. limonea
CMW5704
CMW4977
A. luteobubalina
CMW5446
Argentina
CMW8876
CMW8879
Chile
CMW4990 (NZ)
CMW4983 (Aust)
A. hinnulea
0.01
CMW5448
CMW5450
CMW4964
CMW4722
CMW5704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

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


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