Mycologia, 95(2), 2003, pp. 285–293.

Molecular identification and phylogeny of *Armillaria* isolates from South America and Indo-Malaysia

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Abstract: Armillaria root rot is a serious disease, chiefly of woody plants, caused by many species of *Armillaria* that occur in temperate, tropical and subtropical 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* species, and their phylogenetic relationship with species from the Southern Hemisphere was considered. ITS sequence data for *Armillaria* also were compared with those available at GenBank. 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*.

Key words: *Armillaria limonea*, *Armillaria luteobubalina*, *Armillaria novae-zelandiae*, IGS-1, ITS, phylogeny, systematics

Accepted for publication August 31, 2002.
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INTRODUCTION

Armillaria root rot is a serious disease mainly of woody plants, caused by species of *Armillaria* (Fr. : Fr.) Staude. *Armillaria* species 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 species are known in many parts of the world and can be found on infected plants in temperate, subtropical 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 also has 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* species 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 also have been reported in 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* species have increased substantially since the first publication on the phylogeny of *Armillaria* in 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 at 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 centers. 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* species. 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 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 I and II) 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 2 wk 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 2 h 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 mix-

TABLE I. *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 II. *Armillaria* isolates from Australia and New Zealand used in this study

Species	Culture no.	Alternative number	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
	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. novaezelandiae	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	—

tures 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 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 out 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* species showing high sequence homology to them was determined in a phylogenetic analysis based on distances and parsimony using PAUP* version 4 (Swofford 1998). Neighbor-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 gap-mode 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 (*g*1) (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 II) 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 also were 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 II) were used to determine the phylogenetic relationships among the isolates. *Armillaria hinnulea* Kile & Watling (CMW4983 and CMW4990) (TABLE II), a South-

TABLE III. Statistics for ITS data set with indels treated differently

Treatment	Nc[a]	Npic[b]	Nt[c]	TI[d]	CI[e]	RI[f]	*g*1
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.

ern 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 parsimony analyses (TABLE III). 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.

Neighbor-joining and MP trees generated in this

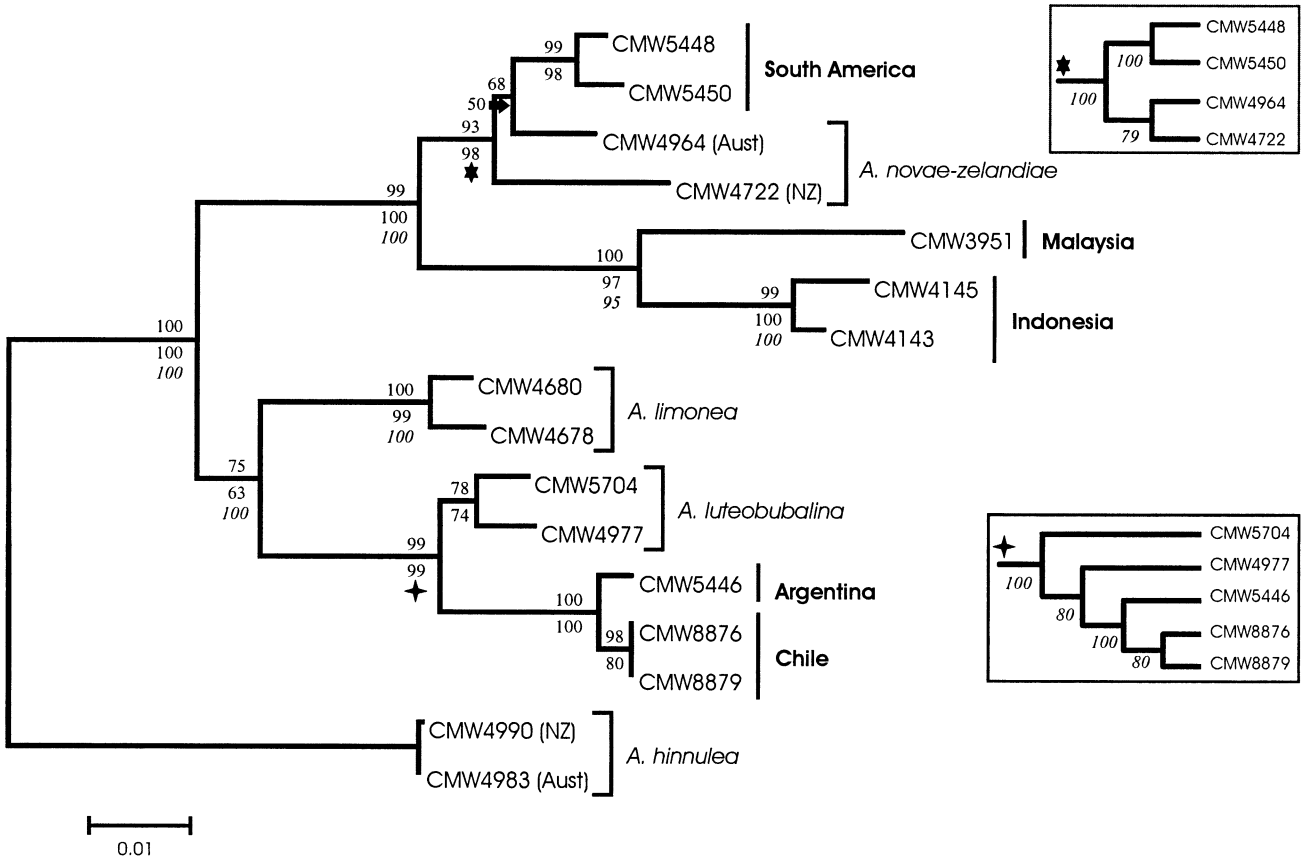

FIG. 1. Phylogenetic tree generated after distance and parsimony analyses of the ITS sequence data. Bootstrap values (1000 replicates) are indicated above the branches for the Neighbor-joining tree. Values below the branches are bootstrap-support values for branching points obtained for trees generated 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 Neighbor-joining analysis.

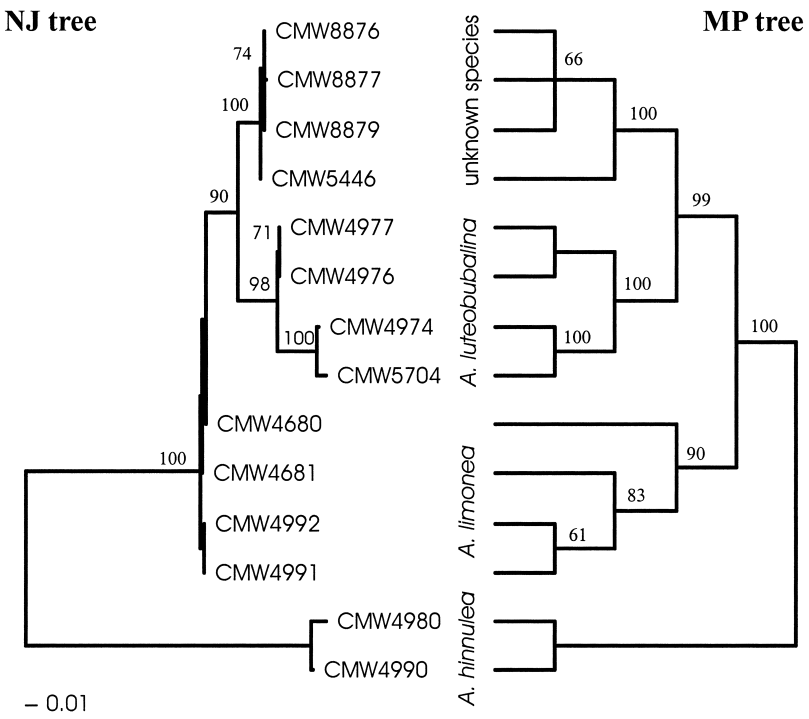

FIG. 2. Neighbor-joining and one of three MP trees generated from IGS-1 sequences with indels included and gaps treated as missing. Values above the branches are bootstrap-support values (1000 replicates) for the branching nodes. Number of parsimony-informative characters = 176, length of tree = 213,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

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 America 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 distribution of *Armillaria* species 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* has 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-New Caledonia to southern South America when these landmasses were part of the supercontinent 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 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 in this study, it previously had been shown that the Southern Hemisphere *Armillaria* species 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* species do not occur in India. The close phylogenetic relationship among 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* species, 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 in New Zealand as *Armillariella limonea* G.Stev. (Stevenson 1964) but also was 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 repre-

sent *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 by 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 previously have not 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 later might be regarded as independent taxa.

ACKNOWLEDGMENTS

This study would not have been possible without cultures that were obtained with some difficulty from inaccessible areas. We thus thank Rodrigo Ahumada, Lee Su See and Paul Clegg for assistance in collecting isolates. We thank members of the Tree Pathology Co-operative Programme (TPCP), the National Research Foundation (NRF), the THRIP programme of the Department of Trade and Industry, South Africa, and the Mellon Foundation for their financial support.

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