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ORIGINAL ARTICLE

Molecular phylogeny of *Armillaria* from the Patagonian Andes

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Abstract A number of species in the plant pathogen genus *Armillaria* are known from South America where they cause root rot disease on a wide variety of hosts. Knowledge pertaining to phylogenetic relationships of these species with those of other *Armillaria* species is almost non-existent. In addition, very few cultures representing these species are available, making DNA-based phylogenetic analyses impossible. The aim of this study was to characterise a collection of *Armillaria* isolates from the Patagonian Andes using DNA sequences and to determine their phylogenetic relationships with other *Armillaria* species. DNA sequences were obtained from the internal transcribed regions (ITS1, 5.8S and ITS4) and ribosomal large subunit (LSU) gene and used in phylogenetic analyses. Phylogenetic trees generated from the sequences separated the *Armillaria* isolates into four lineages. Lineages I and II represented *A. novae-zelandiae* and *A. luteobubalina*, respectively. Isolates belonging to *A. novae-zelandiae* from Malaysia, New Zealand, Australia and South America showed considerable intra-clade substructure. Lineages III and IV are probably distinct species and are most closely related to *A. hinnulea* and an unnamed species isolated from New Zealand and Kenya. This is the first comprehensive study of the phylogenetic relationships of *Armillaria* species from Patagonia and it provides a foundation for future research in this region.

Keywords *Armillaria luteobubalina* · *Armillaria novae-zelandiae* · *Armillaria hinnulea* · Patagonia · *Nothofagus*

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Introduction

The genus *Armillaria* (Fr.: Fr.) Straude includes species that are amongst the most common and important causes of root rot on woody plants, world-wide (Kile et al. 1991; Fox 2000). They survive as pathogens, saprobes or necrotrophs on a wide range of host plants (Hood et al. 1991; Kile et al. 1991). Species native to Argentina can act as saprophytes but may become aggressive pathogens when the native forest is replaced by introduced plantation tree species such as *Pinus* and *Eucalyptus*, as has been shown for Chile (Artigas 1984; Ramírez 1990; Ramírez et al. 1992), New Zealand and Australia (Hood et al. 1991). Although most *Armillaria* species have the potential to infect healthy and stressed trees, they differ in their pathogenicity to their hosts and under certain circumstances they will act as obligate saprophytes. It is, therefore, important to have a clear understanding of the species that are present in infected areas as this will determine the best disease management strategy to apply.

Conventional identification and taxonomy of basidiomycetes, and other fungi, has relied strongly on morphological characters taken from the basidiocarps. The use of these characters, however, poses various problems. In

Armillaria, minor differences and plasticity of the characters make it very difficult to distinguish species (Fox 2000). Basidiocarps are produced seasonally and not every year; they are, therefore, often not available during field work (Kile et al. 1991). Identification using morphological characters has to some extent been replaced by application of the biological species concept with species identification based on sexual compatibility tests (Korhonen 1978). These tests, however, are often complicated by the absence of known tester strains, lack of haploid strains, ambiguous mating interactions and degeneracy of cultures. For these reasons, DNA-based molecular techniques have been applied in *Armillaria* taxonomy, either complementing other methods or on their own. The techniques utilised for the taxonomy of *Armillaria* species include comparisons of restriction fragment length polymorphisms (RFLPs) (Harrington and Wingfield 1995) amplified fragment length polymorphisms (AFLPs) (Pérez-Sierra et al. 2004), and the use of sequences from the internal transcribed spacer regions (ITS), intergenic spacer region one (IGS-1) and partial elongation factor one alpha (EF1-α) gene in phylogenetic studies (Coetzee et al. 2000, 2001; Maphosa et al. 2006; Kim et al. 2006).

The taxonomy of *Armillaria* in South America has received little attention since the work of Singer and others (Singer 1956; Garrido 1988; Horak 1979). A number of *Armillaria* species have been reported from this continent but virtually nothing is known regarding their distribution or phylogenetic relationships with other species in the genus. Species described from southern South America include *A. montagnei* (Singer) Herink, *A. sparrei* (Singer) Herink, *A. griseomellea* (Singer) Kile & Watling, and the varieties *Armilariella sparrei* Singer var. *elaeodes* Singer and *Armilariella montagnei* Singer var. *umbrinobrunnea* Singer (Singer 1969, 1970) that are restricted to that region. *Armillaria novae-zelandiae* (G. Stev.) Herink, *A. luteobubalina* Watling & Kile and *A. limonea* Stev., which are also found in Australasia (Volk and Burdsall 1995), have also been reported or described from the area (Horak 1979; Garrido 1988; Coetzee et al. 2003).

Morphological descriptions of *Armillaria* species from southern Argentina and Chile have been published by Singer (1956, 1969), Horak (1979) and Garrido (1988). Coetzee et al. (2003) identified field samples of *Armillaria* from Argentina and Chile based on DNA sequences and determined their phylogenetics relationships with other *Armillaria* species. However, these authors had only a limited number of isolates available for this study. Overall taxonomic studies are hampered by the lack of cultures that are available for most of the species known from South America. In addition, it is probable that many species have gone undiscovered as a result of the lack of distinguishing morphological characteristics between closely related species.

In the Patagonia region of Argentina, *Armillaria* has been reported from dead wood and stumps of mainly *Nothofagus* Blume (Singer 1953, 1969; Garrido 1988). *Nothofagus* is a dominant genus in Patagonian forests and one of the key taxa among Gondwanan floristic elements (Dimitri et al. 1997; Craw et al. 1999). Other host plants reported from this area include several exotic *Pinus* species used for commercial timber production (Ramirez et al. 1992).

The genus *Nothofagus* is distributed in the southern hemisphere from South America to Oceania (Van Steenis 1971; Hill 2001). The distribution of these trees over the southern hemisphere has been attributed to continental drift during the break up of Gondwana during the Mesozoic era. Species of *Nothofagus* from Australia and New Zealand have been reported to be hosts of *A. hinnulea* Kile & Watling, *A. luteobubalina*, *A. limonea* and *A. novae-zelandiae* (Kile and Watling 1981, 1983, 1988).

The *Armillaria* diversity from Patagonian forests is largely unknown. With the exception of the study by Coetzee et al. (2003), little research has been conducted on the *Armillaria* species from this region. The phylogenetic relationships between *Armillaria* isolates from South America and the rest of the Southern Hemisphere, as well as from different *Nothofagus* species is also not known. The objectives of this study were, therefore, to determine the identity based on DNA sequence of a collection of isolates from Patagonia and to assess their phylogenetic relationships with other *Armillaria* species using ITS, LSU and EF1-α sequence data.

Materials and methods

Fungal isolates

Thirty-two isolates were collected from native and cultivated trees at four national reserves during April to June 2007 (Table 1). The reserves are located in Chubut, Neuquén and Río Negro provinces in the Andes Mountains in the continental Patagonia region of Argentina (Fig. 1). Additional isolates representing *A. novae-zelandiae*, *A. luteobubalina*, *A. hinnulea*, *A. pallidula* Kile & Watling and *A. fumosa* Kile & Watling, an isolate putatively identified as *A. montagnei* from basidiocarps as well as isolates from *Armillaria* spp. from Africa, were obtained from the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI) and the personal collection of R.H. Petersen (Table 2).

Fungal cultures were obtained from the basidiocarps by transferring small pieces from the pileus or stipes of basidiocarps to selective medium dichloran-benomyl-streptomycin (DBS) that included streptomycin (0.01%),

Table 1 *Armillaria* isolates collected from Patagonia, Argentina, during this study

CIEFAP no.	Alternative no.	Lineage designation	Host	Location	Collector	ITS GenBank access no.	LSU GenBank access no.
Arg.5		IV	*Nothofagus dombeyi*	Torrecillas glacier, "Los Alerces" National Park, Chubut province	M.B. Pildain, M. Rajchenberg	FJ660952	FJ711616
Arg.7	CMW29505	IV	*N. dombeyi*	Menendez lake coast, "Los Alerces" National Park Chubut province	M.B. Pildain, M. Rajchenberg	FJ660951	FJ711617
Arg.11		IV	*N. dombeyi*	Arrayanes river coast, "Los Alerces" National Park, Chubut province	M.B. Pildain	FJ711600	-
Arg.12	CMW29507	IV	*N. dombeyi*	Arrayanes river coast, "Los Alerces" National Park, Chubut province	M.B. Pildain, M. Rajchenberg	FJ660948	FJ711618
Arg.17		IV	*N. dombeyi*	Arrayanes river coast, "Los Alerces" National Park, Chubut province	M.B. Pildain	FJ711598	-
Arg.21		III	*N. dombeyi*	Torrecillas glacier, "Los Alerces" National Park, Chubut province	M.B. Pildain	FJ711597	-
Arg.25	CMW29509	III	*N. dombeyi*	Sagrario harbor, "Los Alerces" National Park, Chubut province	M.B. Pildain, M. Rajchenberg	FJ660946	FJ711621
Arg.26		IV	*N. dombeyi*	Sagrario harbor, "Los Alerces" National Park, Chubut province	M.B. Pildain	FJ711605	-
Arg.28		IV	*N. dombeyi*	Sagrario harbor, "Los Alerces" National Park, Chubut province	M.B. Pildain	FJ711604	-
Arg.29	CMW29511	III	*N. dombeyi*	Toro harbor, "Los Alerces" National Park, Chubut province	M.B. Pildain, M. Rajchenberg	FJ660947	FJ711632
Arg.30		IV	*N. dombeyi*	Toro harbor, "Los Alerces" National Park, Chubut province	M.B. Pildain	FJ711601	-
Arg.32		IV	*N. dombeyi*	Los Cántaros Path, "Puerto Blest", "N. Huapí" National Park, Neuquen province	M.B. Pildain	FJ711606	-
Arg.40		IV	*N. dombeyi*	Frias lagoon, "Puerto Blest", "N. Huapí" National Park, Río Negro province	M.B. Pildain	FJ711599	-
Arg.44		IV	*N. dombeyi*	Frias lagoon, "Puerto Blest", "N. Huapí" National Park, Río Negro province	M.B. Pildain	FJ711603	-
Arg.45	CMW29519	IV	*Fitzroya cupressoides*	Frias lagoon, "Puerto Blest", "N. Huapí" National Park, Río Negro province	M.B. Pildain, M. Rajchenberg	FJ660949	FJ711620
Arg.46		I	*Pinus radiata*	Victoria isle, "N. Huapí" National Park, Río Negro province	M.B. Pildain	FJ711614	-
Arg.48	CMW29514	IV	*N. dombeyi*	Correntoso lake coast, "N. Huapí" National Park, Neuquen province	M.B. Pildain, M. Rajchenberg	FJ660950	FJ711619
Arg.49	CMW29521	I	*N. dombeyi*	Correntoso lake coast, "N. Huapí" National Park, Neuquen province	M.B. Pildain, M. Rajchenberg	FJ660935	FJ711629
Arg.52		I	*N. alpina*	Chachin waterfall coast, "Lanin" National Park, Neuquen province	M.B. Pildain	FJ711613	-
Arg.53	CMW29523	I	*N. alpina*	Quechuquina farm, "Lanin" National Park, Neuquen province	M.B. Pildain, M. Rajchenberg	FJ660936	FJ711631
Arg.55		I	*N. obliqua*	Quillén lake coast, "Lanin" National Park, Neuquen province	M.B. Pildain	FJ711612	-
Arg.56		I	*N. obliqua*	Quillén lake coast, "Lanin" National Park, Neuquen province	M.B. Pildain	FJ711615	-
Arg.57	CMW29524	I	*Pseudotsuga menziesii*	Quechuquina farm, "Lanin" National Park, Neuquen province	M.B. Pildain, M. Rajchenberg	FJ660937	FJ711627
Arg.142		IV	*N. dombeyi*	Lacar lake coast, "Lanin" National Park, Neuquen province	M. Rajchenberg	FJ711602	-
Arg.143		II	*N. antarctica*	"La 106" farm, Futaleufú dep. Chubut province	M. Rajchenberg	FJ711611	-
Arg.269	CMW29528	III	*N. pumilio*	Bagilt lake coast, Provincial Reserve, Chubut province	M. Rajchenberg	FJ660945	FJ711622

Table 1 (continued)

CIEFAP no.	Alternative no.	Lineage designation	Host	Location	Collector	ITS GenBank access no.	LSU GenBank access no.
Arg.270		II	*N. pumilio*	Bagilt lake coast, Provincial Reserve, Chubut province	M. Rajchenberg	FJ711609	-
Arg.271		III	*N. dombeyi*	Los Cántaros Path, "Puerto Blest", "N. Huapi" National Park, Neuquen province	M. Rajchenberg	FJ711596	-
Arg.272		II	*N. dombeyi*	Los Cántaros Path, "Puerto Blest", "N. Huapi" National Park, Neuquen province	M. Rajchenberg	FJ711610	-
Arg.281		II	*N. oblicua*	Lacar lake coast, "Lanin" National Park, Neuquen province	M. Rajchenberg	FJ711608	-
Arg.309	CMW29532	II	*N. dombeyi*	Nahuel Huapi lake coast, "N. Huapi" National Park, Río Negro province	M. Rajchenberg	FJ660939	FJ711625
Arg.311		II	*N. dombeyi*	Nahuel Huapi lake coast, "N. Huapi" National Park, Río Negro province	M. Rajchenberg	FJ711607	-

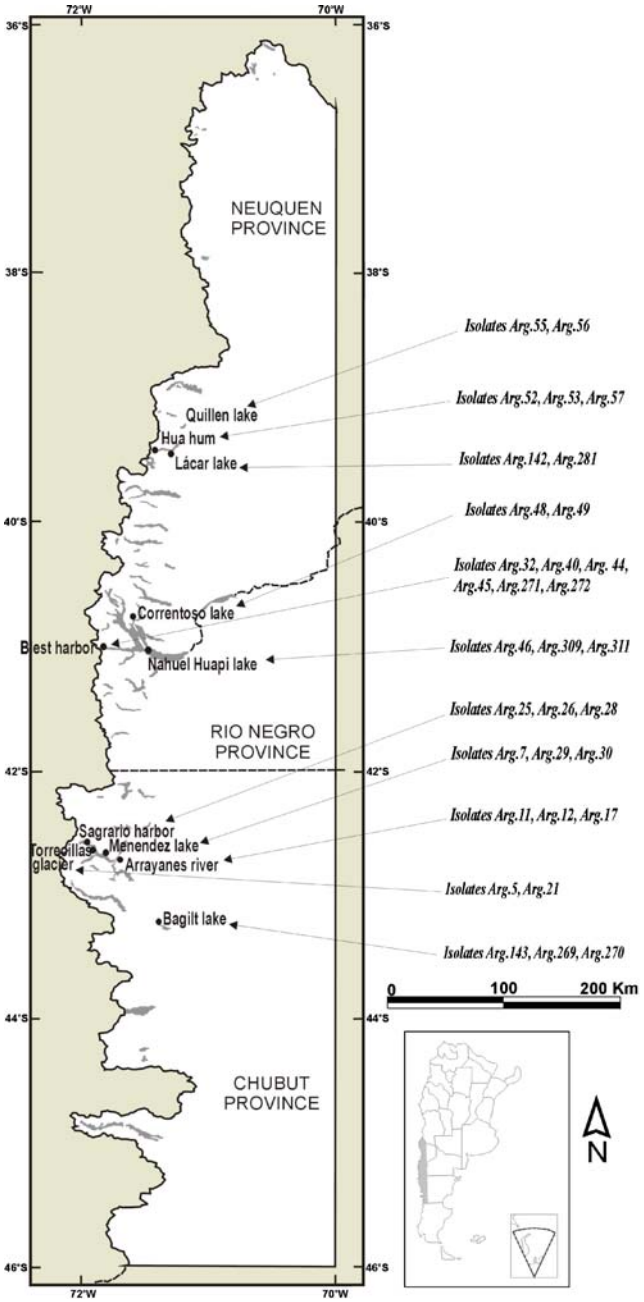

Fig. 1 Map showing the sites from where the isolates of *Armillaria* were collected

benomyl (0.004%), dichloran (0.002%), malt extract (1.5%) and agar (1.5%) (Harrington et al. 1992). The isolates were grown at 25°C in the dark for 3 weeks. Tips from the rhizomorphs produced by the cultures were transferred to MEA (2% malt extract and 1.5% agar; Merck) and incubated at 25°C in the dark for 3 weeks. Rhizomorph tips were transferred to fresh MEA until the cultures were pure. Stock cultures of all the isolates are maintained in the culture collection of "Centro de Investigación y Extensión Forestal Andino Patagónico (CIEFAP)" and the culture collection of FABI.

Table 2 Additional *Armillaria* isolates or sequences obtained from GenBank included in this study

Armillaria species	Culture number[a]	Alternative number	Host	Location	Collector	ITS GenBank access no.	LSU GenBank access no.
A. fumosa	CMW4955	123.1	*Eucalyptus* sp.	Tasmania, Australia	GA Kile	AF329918	DQ338552
A. fumosa	CMW4959	Qld.Coll.8(1)	*Pinus radiata*	Queensland, Australia	GA Kile	AF329920	-
A. fuscipes	CMW2740	CBS118115	*P. patula*	South Africa	MPA Coetzee	AY882970	-
A. fuscipes	CMW4953	LR3	*Pelargonium asperum*	La Reunion	C Fabregue	AY882974	AY882963
A. hinnulea	CMW4980	CBS164.94	*Eucalyptus obliqua*	Tasmania, Australia	C Mohammed	-	DQ338555
A. hinnulea	CMW4988	5, JJW223	*Nothofagus* sp.	S. Island, N. Zealand	GS Ridley	AF329906	-
A. hinnulea	CMW4990	3512/13	*Nothofagus* sp	S. Island, N. Zealand	GS Ridley	AF329905	-
A. hinnulea	RP7088[b]	-	Unknown	S. Island, N. Zealand	GS Ridley	FJ711636	-
A. hinnulea	RP3906[b]	-	Unknown	Tasmania, Australia	RH Petersen	FJ711637	-
A. hinnulea	RP2596[b]	-	Unknown	S. Island, N. Zealand	RH Petersen	FJ711635	-
A. limonea	CMW4678	A3.4.26.3	*Beilschmiedia tawa*	N. Island, N. Zealand	IA Hood	AF329929	-
A. limonea	CMW4680	C3.28.0.1	*B. tawa*	N. Island, N. Zealand	IA Hood	AF329930	DQ338560
A. limonea	RP2817[b]	-	Unknown	S. Island, N. Zealand	RH Petersen	FJ711640	-
A. luteobubalina	CMW4977	SA(6)	Unknown	Victoria, Australia	Unknown	AF329912	DQ338559
A. luteobubalina	CMW4978	MtCole1(18)	Unknown	Victoria, Australia	Unknown	AF329909	-
A. luteobubalina	CMW5446	7348/10	*Nothofagus* sp.	Neuquen, Argentina	RH Petersen	AF445068	DQ338562
A. luteobubalina	CMW8876	Chile-1	*P. radiata*	Temuco, Chile	MJ Wingfield	AF448423	-
A. luteobubalina	CMW8879	Chile-4	*P. radiata*	Temuco, Chile	MJ Wingfield	AF448424	AF448424
A. montagnei	CMW29530[b]	RP8377	*Nothofagus* sp.	Neuquen, Argentina	RH Petersen	FJ660940	FJ711623
A. novae-zelandiae	CMW5448	7365/2	*Nothofagus* sp.	Isla de Chiloe, Chile	RH Petersen	AF448417	-
A. novae-zelandiae	RP2615[b]	-	Unknown	S. Island, N. Zealand	RH Petersen	FJ660942	-
A. novae-zelandiae	CMW3951	O-1	*Acacia mangium*	Malaysia	MJ Wingfield	AF448419	DQ338553
A. novae-zelandiae	CMW4143	-	*Eucalyptus grandis*	Sumatra, Indonesia	MJ Wingfield	AF448421	
A. novae-zelandiae	CMW4722	G3.0.34.4	*B. tawa*	N. Island, N. Zealand	IA Hood	AF329926	DQ338551
A. novae-zelandiae	CMW4963	ATCC66127	*Antherosperma moschatum*	Tasmania, Australia	GA Kile	AF329922	-
A. novae-zelandiae	CMW4964	Qld.Coll.(10)3	*P. radiata*	Queensland, Australia	GA Kile	AF329924	-
A. novae-zelandiae	CMW4986	3505/15	*Nothofagus* sp.	S. Island, N. Zealand	GS Ridley	AF329925	-
A. novae-zelandiae	CMW29536[b]	RP8306	*N. pumilio*	Chubut, Argentina	RH Petersen	FJ660943	FJ711628
A. novae-zelandiae	CMW29538[b]	RP8367	*Nothofagus* sp.	Neuquen, Argentina	RH Petersen	FJ660944	FJ711630
A. novae-zelandiae	CMW5450	7365/4	*Nothofagus* sp.	Isla de Chiloe, Chile	RH Petersen	AF448418	-
A. novae-zelandiae	RP7562	-	Unknown	N. Island, N. Zealand	RH Petersen	FJ711641	-

Table 2 (continued)

Armillaria species	Culture number[a]	Alternative number	Host	Location	Collector	ITS GenBank access no.	LSU GenBank access no.
A.novae-zelandiae	RP2560[b]	-	Unknown	S. Island, N. Zealand	RH Petersen	FJ660941	FJ711626
A. pallidula	CMW4968	ATCC66124	*P. caribaea*	Australia	P Gordon	AF329915	DQ338550
A. pallidula	CMW4972	Qld5761	Unknow	Queensland, Australia	Unknow	AF329914	-
A. paulensis	SP308014	-	Unknown	São Paulo, Brazil	M. lima	EF639348	-
Armillaria sp.	CMW3152	-	Unknown	Cameroon	E Mwenje	AY882980	-
Armillaria sp.	CMW15584	6M1	*Camelia sinensis*	Kenya	M Ivory	AF513024	FJ711634
Armillaria sp.	CMW15585	13T2	*C. sinensis*	Kenya	E Mwenje	AF513022	FJ711633-
Armillaria sp.	CMW29529[b]	RP8371	*Nothofagus* sp.	Río Negro, Argentina	RH Petersen	FJ660938	FJ711624
Armillaria sp.	RP2577[b]	-	Unknown	S. Island, New Zealand	RH Petersen	FJ711638	-
Armillaria sp.	RP2600[b]	-	Unknown	S. Island, New Zealand	RH Petersen	FJ711639	-
Armillaria sp.	CMW4993	4698/9	*Nothofagus* sp.	N. Island, N. Zealand	GS Ridley	AF329933	DQ338561
Armillaria sp.	CMW4994	4968/10	*Nothofagus* sp.	N. Island, N. Zealand	GS Ridley	AF329932	-
Armillaria sp.	CMW5597	A35.4	*N. fusca*	N. Island, N. Zealand	IA Hood	AF329931	-
Armillaria sp.	CMW4456	-	*Brachystegia utilis*	Zimbabwe	E Mwenje	AY882484	-
Armillaria sp.	CMW10115	-	*Acacia albida*	Zimbabwe	E Mwenje	AY882983	-
Armillaria sp.	CMW10116	-	*Newtonia buchananii*	Zimbabwe	E Mwenje	AY882982	-

[a] CMW refers to the collection numbers in the fungal culture collection of the Tree Protection Co-operative Programme (TPCP), FABI (Pretoria); RP refers to the culture collection of RH Petersen

[b] Isolates not included in previous studies and identified based on basidiocarp morphology by RH Petersen or DNA sequence comparisons during this study

Molecular methods

DNA extraction Isolates were grown in liquid MY (2% malt extract and 0.3% yeast extract) medium in the dark for approximately 3 weeks at 22°C. Mycelium was harvested through filtration using sterilised filter paper and freeze dried. DNA was extracted from the mycelium following the method described by Coetzee et al. (2000). DNA concentration and quality were determined using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA.).

DNA amplification Three gene regions were amplified in this study including the internal transcribed spacer regions (ITS1, 5.8S and ITS4), ribosomal large subunit (LSU) gene and the transcription elongation factor 1 alpha (EF1-α) gene. The ITS region of the isolates was amplified using primers ITS1 and ITS4 (White et al. 1990). A region of the LSU was amplified with primers LR0R and LR7 (Moncalvo et al. 2000). A fragment of the EF1-α gene was amplified using the primers EF595F and EF1160R (Kauserud and Schumacher 2001). PCR reaction mixtures for amplification of the EF1- α gene and ITS regions were the same. The mixture included dNTPs (0.25 mM of each), 2.5 mM $MgCl_2$; PCR buffer supplied with the polymerase enzyme; 0.1 μM of each primer; 100 - 500 ng DNA; and 2.5U of Fast Start *Taq* polymerase (Roche Diagnostics, Mannheim, Germany). The final reaction volume was 50 μL. The PCR reaction conditions for ITS and IGS regions and EF1-α gene region were the same as those described by Coetzee et al. (2003) and Maphosa et al. (2006), respectively. PCR products were separated on a 1% (w/v) ethidium bromide-stained agarose gel and the bands were visualised under UV illumination.

DNA sequencing Amplified DNA fragments were purified using a MSB® Spin PCRapace purification kit (Invitek) following the instructions of the manufacturer. DNA sequencing reactions were done using a BigDye Terminator v3.1 cycle sequencing kit (ABI) following the protocol provided by the manufacturer. Sequences were determined on an ABI 3100 DNA automated sequencer. Both strands of the PCR fragments were sequenced using the respective primer pairs used in the intial amplification. DNA sequences for the internal

portion of the LSU gene region were obtained using additional primers LR16 and LR3R (Moncalvo et al. 2000).

Cloning and PCR amplifcation of cloned fragments EF1-α amplicons did not produce clear electropherograms. These fragments were, therefore, cloned to obtain single alleles of this gene region. PCR products were purified prior to cloning using a MSB® Spin PCRapace purification kit (Invitek) following the manufacturers instructions. PCR products were then cloned using a pGEM®-T easy vector system following the procedures outlined by the manufacturer (Promega, Madison, USA).

Cloned fragents were amplified directly from the transformed *Escherichia coli* cells using primers T7 and SP6. Standard PCR mixtures and conditions were used for the amplification reactions. PCR products were visualised under UV illumination after electrophoresis on agarose gels (1%, 1/v) stained with ethimdium bromide. The EF-1α inserts from clones that were succesfully amplified, were sequenced as described above.

Sequence analyses DNA sequences were analysed using the Invitrogen Vector NTI advance version 10 computer package. Sequences were deposited in GenBank under the accession numbers listed in Table 1. Searches for sequence identity in the GenBank DNA database were conducted using the Gapped BlastN search algorithm (NCBI) (Altschul et al. 1997). Additional sequences of *Armillaria* species, including the recently described *A. paulensis* Capelari (EF639348) (Lima et al. 2008), were retrieved from GenBank for phylogenetic analyses. All sequence alignments were performed using MAFFT version 5.0 (Katoh et al. 2005).

Phylogenetic analyses

Sequences for each gene region were analysed separately in the phylogenetics study. For the ITS, two datasets were generated; one with a large collection of isolates belonging to a number of *Armillaria* species together with all the isolates from Patagonia, while the other included a smaller selection of isolates from Patagonia and other *Armillaria* species. The large ITS dataset was used to ascertain the affiliation of all the isolates from Patagonia.

Neighbour-joining (NJ) trees were generated only from the large ITS sequence dataset using MEGA version 4.0 (Tamura et al. 2007). Gaps and missing data were deleted prior to the analysis. A maximum composite likelihood model was used to calculate the distances between taxa. The NJ tree was rooted to midpoint. Bootstrap analysis included 1,000 replicates using the same settings.

Phylogenetic trees were generated from the reduced ITS and the LSU dataset using maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI). Nucleotide substitution models for each of the datasets were determined with ModelTest version 3.06 (Posada and Crandall 1998) under an AIC selection criterion. *Cylindrobasidium laeve* (GenBank accession n° DQ234541) and *Oudemansiella radicata* (GenBank accession n° DQ071719) were used as outgroup species in the LSU dataset.

MP analyses were done using PAUP* 4.0b10 (Swofford 2000). Gaps were treated as missing characters and cladograms were generated using heuristic searches based on 1,000 random sequence additions, TBR branch swapping and MULPARS effective. Characters were weighted according to their mean consistency index to reduce the effect of homoplasy. Bootstrap analyses were made using unweighted and weighted characters in separate analyses. Settings for the bootstrap analyses were the same as above, but with a simple stepwise addition of sequences.

ML analyses were done using GARLI (genetic algorithm for rapid likelihood inference) version 0.951 for Mac OS X (Zwickl 2006). A general time reversible substitution model with gamma distribution and invariable sites (GTR+I+G) was incorporated in the search with model parameters optimised during the ML analysis. Starting tree topologies were obtained through a random tree building algorithm, and the genetic algorithm parameters were kept at their default settings. Support for nodes was accomplished using bootstrap (1,000 replicates) analysis with the same settings as above.

Bayesian inference of the phylogeny was assessed using MrBayes version 2.01 (Huelsenbeck et al. 2001) to gain support for the tree topology obtained from the MP and ML analyses. The Markov Chain was set to 10^7 generations with sampling intervals of every 100 generations. The best models for the ITS and LSU datasets were TVM+I+G and TIM+I+G, respectively. These models are related to the GTR+I+G model and prior settings for the latter model was, therefore, employed in the analysis. Four runs were done with different starting tree topologies. The log-likelihood scores of sampled trees were ploted against generation time using TRACER version 1.3 (http://evolve.zoo.ox.ac.uk/software.html) to determine the number of trees that should be excluded before the stationary point is reached. The analysis was repeated once for each gene to determine tree convergence. Nodes with a posterior probability of ≥ 0.95 were considered to be significantly supported by the data.

Results

PCR amplification

The ITS and LSU regions yielded single amplicons for all isolates. Sequence comparisons showed that the cloned

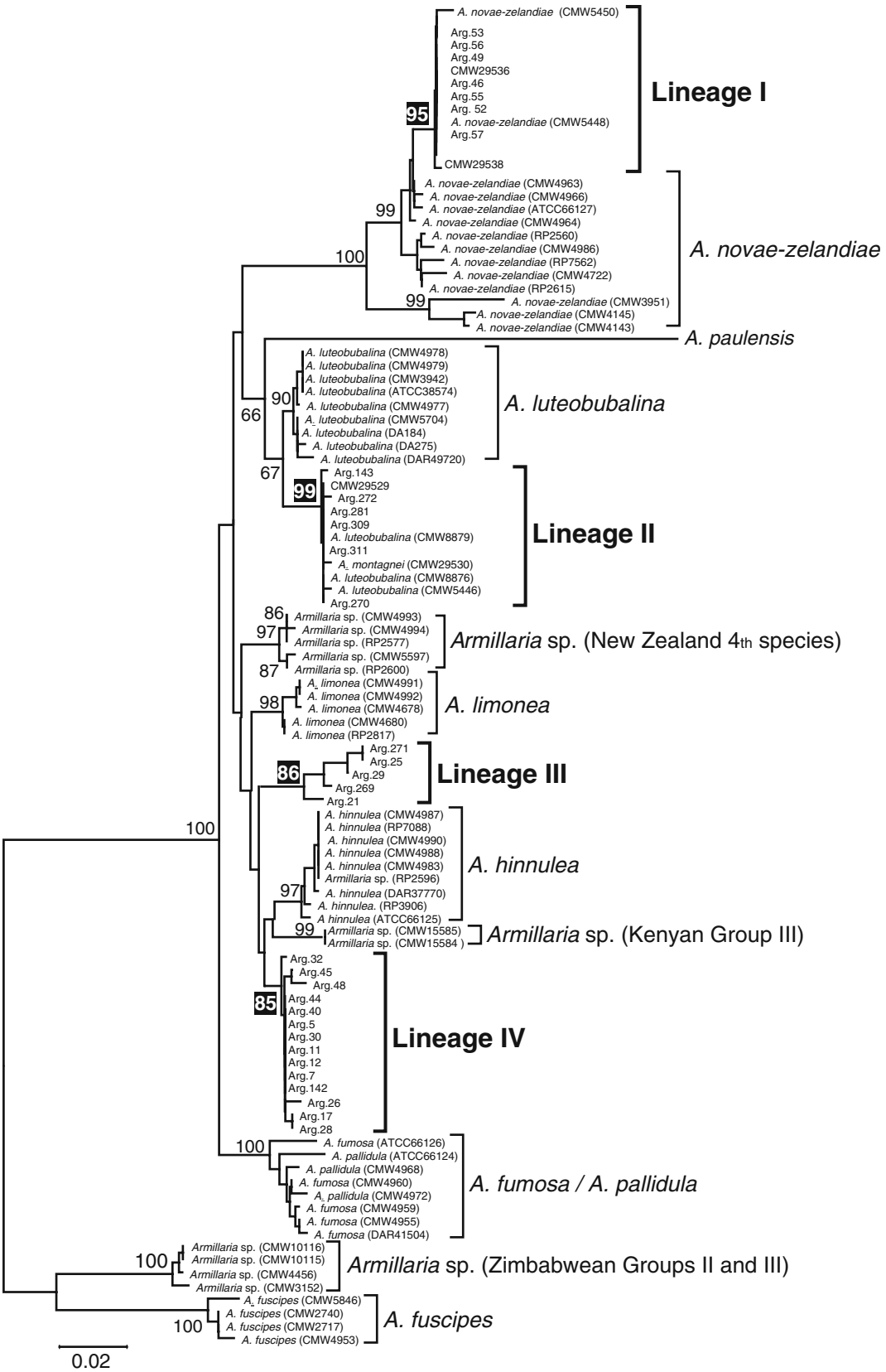

Fig. 2 Neighbour-joining tree generated from ITS DNA sequences for *Armillaria* species from Australia, New Zealand, Africa and South America. Bootstrap values supporting the grouping of isolates to their respective species or lineages are indicated next to the nodes. Bootstrap support for Argentinean lineages I to IV are indicated in the *filled boxes*. *Scale bar*: substitutions per site

Table 3 Bootstrap and Bayesian confidence support values (in %) for Argentinian *Armillaria* lineages I to IV

Lineage	ITS (large)		ITS			LSU	
	NJ[a]	MP[a]	ML[a]	BI[a]	MP	ML	BI
I	94	96	96	100	87	83	86 (ns)[b]
II	99	100	100	100	98	98	100
III	88	100	100	100	86	94	100
IV	85	95	96	100	80	85	88 (ns)

[a] *NJ* Neighbour joining, *MP* maximum parsimony, *ML* maximum likelihood, *BI* bayesian inference

[b] *ns* No support

EF1-α gene amplification fragments have apparently different origins and that this gene is probably present in more than one copy in the individual isolates. Sequences from this gene were, therefore, considered unsuitable for use in a phylogenetic analysis and they were not included for subsequent analyses.

Phylogenetic analyses

The large ITS dataset included 100 taxa and 1,191 characters after alignment. The NJ tree generated from this dataset grouped the isolates into four strongly supported major lineages that are henceforth referred to as lineage I to IV (Fig. 2). The grouping of isolates in the respective lineages was highly supported for most of the methods and datasets (Table 3, Figs. 2–6). The sequence of *A. paulensis* (EF639348) grouped sister to the *A. luteobubalina* isolates from Australia and South America (lineage II) and was separated from them by a long branch.

The aligned reduced ITS sequence dataset included 1,169 characters, with 113 of the characters being parsimony informative. Heuristic tree searches under maximum parsimony analysis resulted in 63 equally most parsimonious trees but were reduced to 12 trees after rescaling according to the mean consistency index. All trees had the same topology but differed in branch length. The tree lengths (TL) were 153 steps, consistency index (CI) and retention index (RI) were 0.772 and 0.951, respectively. One of these trees is presented in Fig. 3.

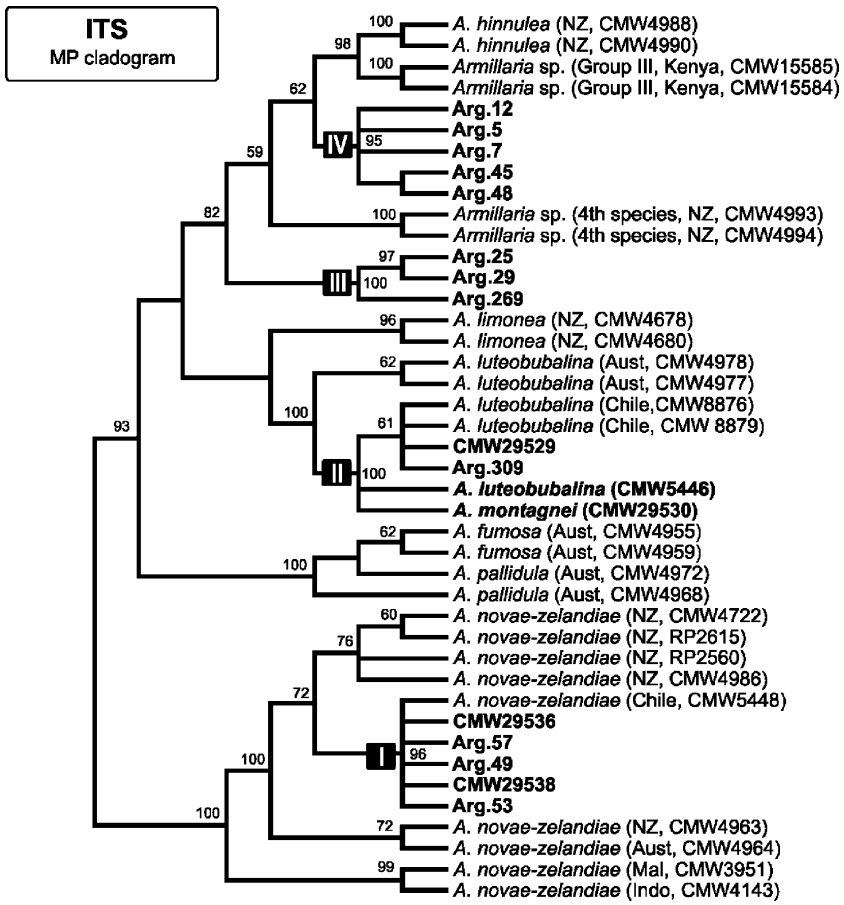

Fig. 3 Most parsimonious tree generated from ITS sequence data. *Numbers* at the nodes indicate bootstrap support values (< 50% are not indicated). *Roman numerals* indicate the lineage designation. Isolates in *bold* originated from Argentina. *Arg* Argentina, *Aust* Australia, *Indo* Indonesia, *Mal* Malaysia, *NZ* New Zealand

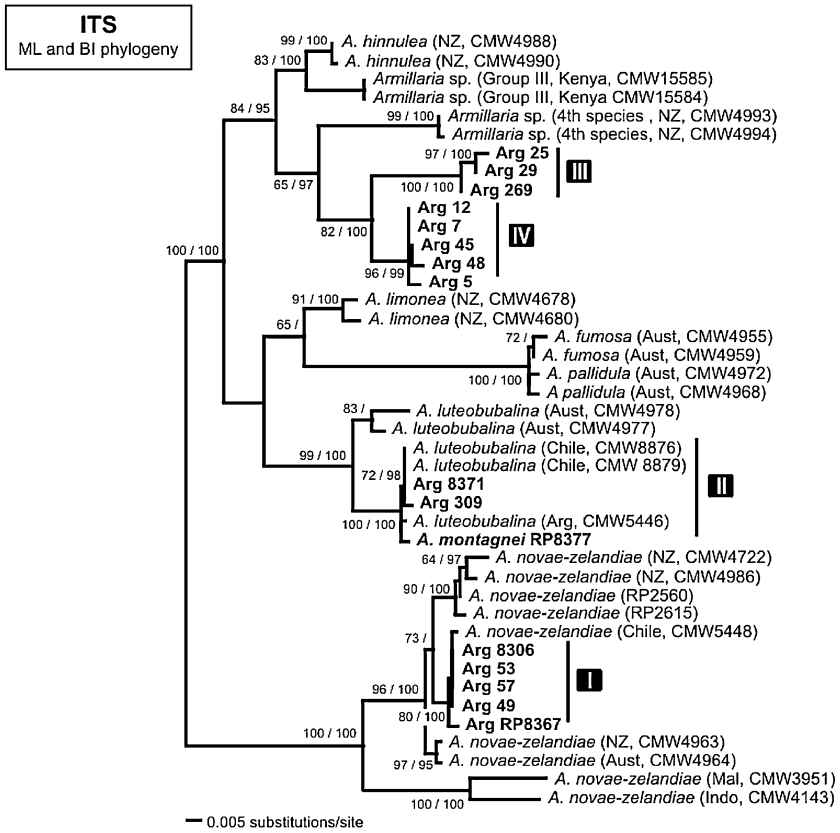

Fig. 4 Phylogram generated from ITS sequence data using maximum likelihood (ML) and Bayesian analyses. *Numbers* above the branches indicate bootstrap values for the nodes followed by the Bayesian posterior probability (in %). Bootstrap values lower than 50% and posterior probability values lower than 95% are not indicated. *Roman numerals* indicate the lineage designation. Scale bar shows the number of substitutions per site. The origin and culture number are indicated in brackets for the isolates of *Armillaria* originating outside Argentina. *Arg* Argentina, *Aust* Australia, *Indo* Indonesia, *Mal* Malaysia, *NZ* New Zealand

The LSU sequence data set included 1,438 characters after alignment of which 93 characters were parsimony informative. The heuristic search using unweighted characters yielded 7 most parsimonious trees. Two equally parsimonious trees with TL=110 steps and similar topology were obtained after reweighing characters according to their mean consistency indexes. The CI and RI for the trees were 0.765 and 0.912, respectively. One of these trees is presented in Fig. 5.

Lineage I formed a cluster that included isolates from Patagonia and representatives of *A. novae-zelandiae* from Australia and New Zealand (Figs. 2–6). This lineage formed a strongly supported monophyletic group based on ITS sequences with isolates from Chile (CMW5448 and CMW5450) previously identified as *A. novae-zelandiae* by Coetzee et al. (2003) (Figs. 2–4). The majority of trees generated from the datasets grouped the isolates from Argentina sister to those of *A. novae-zelandiae* from Australia and New Zealand. Exceptions were found in the BI tree from the ITS dataset (Fig. 4) as well as the MP and BI trees from the LSU dataset (Figs. 5 and 6), for which this relationship was unresolved.

Lineage II included isolates from Patagonia, together with isolates from Chile and Argentina that were identified as *A. luteobubalina* by Coetzee et al. (2003) (Figs. 2–6). An isolate (CMW29530) from a basidiocarp that was identified by R. Petersen as *A. montagnei* also resided in this lineage. This lineage formed a sister group with isolates of *A. luteobubalina* from Australia in the NJ, MP, ML and BI trees generated from the ITS datasets (Figs. 2–4). This relationship, however, was unresolved in the MP, ML and BI trees generated from the LSU dataset (Figs. 5 and 6).

The relationship of lineages III and IV with other *Armillaria* species could not be fully resolved because they were placed at different phylogenetic positions based on the different methods and genomic regions considered. The NJ tree showed that lineage IV forms a sister group with a cluster that included isolates representing *A. hinnulea* and an *Armillaria* species from Kenya (Group III, Mwenje et al. 2006), but was not supported by bootstrap (Fig. 2). The MP tree generated from the reduced ITS sequences placed the two lineages within a monophyletic clade that included isolates of *A. hinnulea*, *Armillaria* Kenyan Group III and

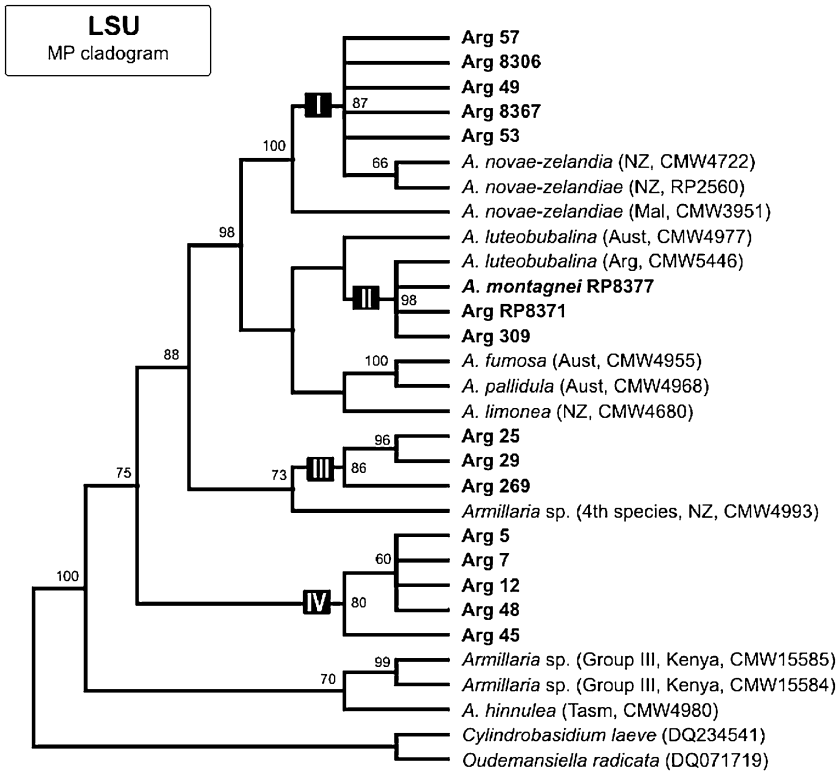

Fig. 5 Most parsimonious tree generated from LSU sequence data. *Numbers* at the nodes indicate bootstrap values (< 50% are not indicated). *Roman numerals* indicate the lineage designation. The origin and culture number are indicated in brackets for the isolates of *Armillaria* originating outside Argentina. *Arg* Argentina, *Aust* Australia, *Mal* Malaysia, *NZ* New Zealand

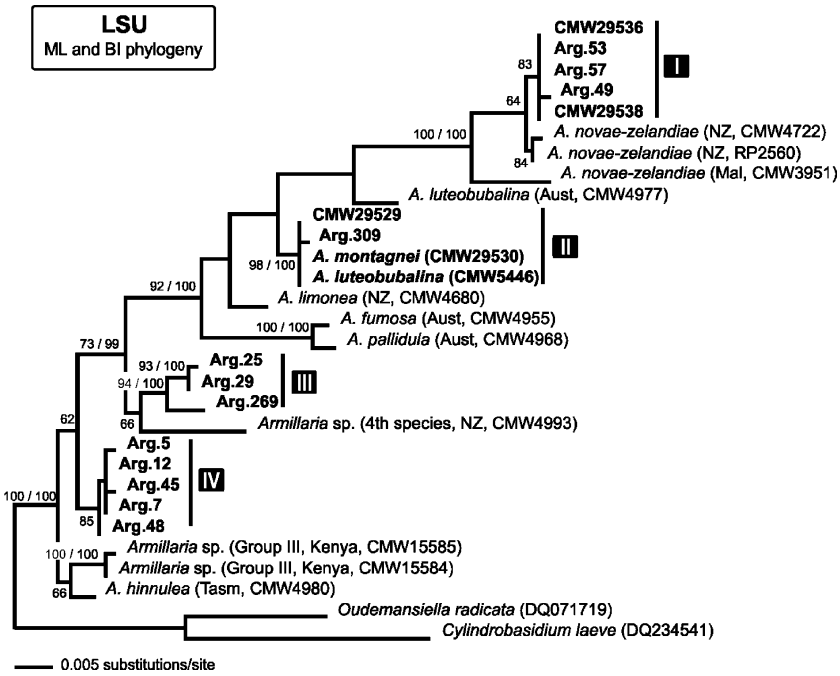

Fig. 6 Combined phylogram generated from LSU sequence data using maximum likelihood and Bayesian analyses. *Numbers* above the branches indicate bootstrap values for the nodes followed by the Bayesian posterior probability (in %). Bootstrap values lower than 50% and posterior probability values lower than 95% are not indicated. *Roman numerals* indicate the lineage designation. Isolates in *bold* originated from Argentina. *Scale bar* shows the number of substitutions per site. *Arg* Argentina, *Aust* Australia, *Indo* Indonesia, *Mal* Malaysia, *NZ* New Zealand

Armillaria 4th species from New Zealand (Fig. 3). Within this clade, the isolates representing lineage IV formed a sister group with *A. hinnulea* and *Armillaria* Kenyan Group III, but with low bootstrap support. Although not well supported, the MP tree generated from the reduced ITS sequence dataset placed the isolates belonging to lineage III sister to the group that included lineage IV, *A. hinnulea,* Kenyan Group III and the *Armillaria* 4th species from New Zealand (Fig. 3). Trees generated from the ITS sequence data based on ML and BI placed the isolates belonging to the two respective lineages sister to each other within a strongly supported group (Fig. 4). Both lineages formed a sister group with the *Armillaria* species from New Zealand with strong BI support (Fig. 4). The MP, ML and BI trees generated from the LSU sequence data placed the isolates in lineage III, sister to the unknown species from New Zealand (CMW4993), but there was no BI support for this relationship and the bootstrap values were low in the MP (73%) and ML (66%) trees (Figs. 5 and 6).

Discussion

The focus of this study was to assess the identity of *Armillaria* isolates collected from infected trees in Patagonia based on DNA sequence data and to determine their phylogenetic relationships with other *Armillaria* species. An earlier study, using DNA-based techniques, identified *A. luteobubalina* and *A. novae-zelandiae* from South America (Coetzee et al. 2003). In the present study, we have extended the work of Coetzee et al. (2003) by increasing the number of isolates and hosts. Results of the phylogenetic analyses confirm the findings of Coetzee et al. (2003), but also suggest the presence of two additional taxa in Patagonia.

Isolates collected in this study originated from infected trees, mainly *N. dombeyi* Blume, but samples were also obtained from *N. pumilio* (Poepp. et Endl.) Kraser, *N. alpina* (Poepp. et Endl.) Oest., *P. radiata* D. Don, *Pseudotsuga menziesii* (Mir.) Franco and *Fitzroya cupressoides* (Molina) Johnst. The data did not indicate any species-specific interaction between specific *Armillaria* species from Patagonia and their tree hosts. This is not surprising as it is known that *Armillaria* species are generally not host specific, and host shifting, e.g. between Pinaceae and Fagaceae, occurs frequently (Kile et al. 1991).

Phylogenetic trees generated in this study provide evidence that Lineage I probably represents *A. novae-zelandiae*. This fungus was reported by Singer (1969) and later by Coetzee et al. (2003) from South America and it has been recorded from Australasia in New Zealand, eastern Australia, Papua New Guinea (Kile et al. 1994) as well as from Indo-Malaysia (Coetzee et al. 2003). *Armillaria novae-zelandiae* was also previously reported on *Nothofagus* in South America and Australasia (Singer 1969; Kile and Watling 1988; Guillaumin et al. 1991). In the present study, isolates from Patagonia belonging to Lineage I were collected from different tree species including *N. dombeyi*. A number of species of *Nothofagus* have a southern transcontinental distribution and fossil material belonging to this genus has been found in Antarctica (Hill 2001). The association of *A. novae-zelandiae* with the tree genus suggests that this fungus is endemic to Southern Hemisphere.

Isolates belonging to *A. novae-zelandiae* showed substructure within the species clade reflecting the results from Coetzee et al. (2003) and geological history. All isolates from South America grouped in one cluster within a larger clade that included isolates from New Zealand and Australia. The South American – Australasian group formed a sister group with isolates from Indo-Malaysia. This is consistent with the fact that Malaysia separated from Gondwana followed by New Zealand and finally Australia and South America (Smith and Briden 1977). Also, a high level of sequence variation was observed among the groups. The results suggest strong biogeographic isolation between isolates, and populations in the process of allopatric speciation.

Singer (1969) suggested that *A. novae-zelandiae* is morphologically very similar to *A. montagnei* var. *montagnei* and *A. sparrei* var. *elaeodes*, species that are only known from southern South America. Therefore, results of the present study may indicate that *A. novae-zelandiae* represents a species complex. Further research based on detailed morphological and cultural studies as well as including mating tests will be needed to better characterise this species and possible sibling species.

Lineage II was closely related to *A. luteobubalina* in all trees generated in this study. The isolates from South America and those representing *A. luteobubalina*, however, formed two strongly supported monophyletic sister groups. Two isolates from South America, that were previously suggested by Coetzee et al. (2003) to belong to this species, also grouped with the isolates from Patagonia. An isolate (8377) collected from a basidiocarp that resembled *A. montagnei* (R.H. Petersen, data not shown) was also placed within the South American group. *Armillaria montagnei* was recorded by Singer (1956) on the trunks of *N. dombeyi* in Neuquén province where it was reported to occur frequently and to be consumed by people from the area. It is thus possible that Lineage II from Patagonia represents this fungus. However, isolate CMW5446 from Argentina was obtained from a basidiocarp thought to resemble *A. limonea* but it had the highest ITS, IGS-1 and EF1-α sequence similarity with *A. luteobubalina* (Coetzee et al. 2003; Maphosa et al. 2006). The grouping of two isolates from basidiocarps seemingly representing different species illustrates the difficulty that may be encountered when identification of *Armillaria* species is based soley on

morphology. The identity of this group will be resolved once the morphology of basidiocarps linked to this lineage can be thoroughly studied.

Recently, a new species, *A. paulensis*, was described from Sao Paulo in Brazil (Lima et al. 2008), based on a single specimen. The fungus showed a close affiliation, based on ITS sequences, with isolates identified as *A. luteobubalina* from South America and Australia (Lima et al. 2008). The NJ tree generated in the present study supported this relationship, placing *A. paulensis* sister to *A. luteobubalina*. None of the isolates collected in the present study could, however, be linked to this species based on DNA sequence similarity.

The phylogenetic relationships of isolates belonging to Lineage III and IV with other *Armillaria* species could not be resolved. However, results of this study, indicated that they represent distinct species. Phylogenetic trees generated from the LSU sequence data placed isolates from Lineage III in a monophyletic group that included an isolate representing the unnamed *Armillaria* 4th species from New Zealand (Coetzee et al. 2001). Lineage IV was placed at a more basal position, intermediate between Lineage III and a group that included *A. hinnulea* and *Armillaria* Kenyan Group III (Mwenje et al. 2006). Phylogenetic trees based on ITS sequences grouped isolates from these two lineages in a monophyletic group that included *A. hinnulea* and the unnamed *Armillaria* 4th species from New Zealand and Kenyan species Group III. Results of this study, therefore, suggest that Lineages III and IV have a phylogenetic relationship with *A. hinnulea* and the unnamed species from Kenya and New Zealand, but that they represent unique taxonomic entities.

Armillaria hinnulea and the species from Kenya and New Zealand were previously shown to be phylogenetically closely related to *Armillaria* species from the Northern Hemisphere (Coetzee et al. 2001; Mwenje et al. 2006). However, phylogenetic analysis of *Armillaria* species using partial EF1-α gene sequences placed *A. hinnulea* sister to the Australian species *A. fumosa* and *A. pallidula* (Maphosa et al. 2006). The conflicting position of *A. hinnulea* in the ITS and EF1-α tree topologies was ascribed by Maphosa et al. (2006) as the result of differences in the evolution of the two genomic regions in this species. *Armillaria hinnulea* is restricted to the Southern Hemisphere and has only been reported from New Zealand and Australia. The *Armillaria* species from Kenya was isolated from tea bushes and shown to be closely related to *A. hinnulea* (Mwenje et al. 2006). The grouping of Lineages III and IV with this group of species has increased the number of taxa from the Southern Hemisphere that are phylogenetically related (based on ITS DNA sequences) to *Armillaria* species from the northern hemisphere. The basis of this relationship is not clear and this question warrants further investigation.

Although *Armillaria* species from South America have been relatively well documented, the origins and evolutionary relationships of most species have yet to be inferred within a comprehensive molecular phylogenetic context. Our results show that the *Nothofagus* dominated forests of Patagonia have a great *Armillaria* diversity, including at the least four taxa, circumscribed as lineages I to IV. They could be linked to one of the several names available from the region, or alternatively, they might represent new taxa. Based on this conclusion, it will be important to correlate the phylogenetic groups emerging from this study with morphological characteristics of the basidiocarps, cultural characters, mating compatibility and pathogenicity tests in order to develop solid taxonomic concepts for this important group of fungi.

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