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Phylogenetic relationships among *Armillaria* species inferred from partial elongation factor 1-alpha DNA sequence data

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Abstract. *Armillaria* species are important root rot pathogens with a wide host range and a worldwide distribution. The taxonomy of these fungi has been problematic for many years but the understanding of the relationships between them has been substantially improved through the application of DNA sequence comparisons. In this study, relationships between different *Armillaria* species were determined using elongation factor 1-alpha DNA sequence data for the first time. A total of 42 isolates, representing the majority of *Armillaria* species, with diverse geographic distributions and hosts, were included in this study. PCR amplification yielded products of 600 bp for all the isolates. Phylogenetic trees resulting from parsimony analysis showed that this gene region is useful for studying relationships between species. Generally, results were similar to those emerging from previous comparisons using ITS and IGS-1 sequence data. Phylogenetic trees generated from the dataset grouped the African taxa in a strongly supported clade, basal to the rest of the *Armillaria* species included in the study. The *Armillaria* species originating from the Northern Hemisphere formed a monophyletic group. Within this group, isolates of *A. mellea* constituted four subclades, representing their geographical origin. The phylogenetic relationships among species from the Southern Hemisphere were not entirely resolved. However, *A. pallidula*, *A. fumosa* and *A. hinnulea* grouped in a strongly supported clade and isolates of *A. limonea* formed a sister clade with those of *A. luteobubalina*. This is the first time a single-copy protein coding gene has been used to study phylogenetic relationships in *Armillaria*, and overall the data support previously held views regarding the relationships between species.

Additional keywords: Armillaria root rot, EF 1-α, evolution, basidiomycetes, Tricholomataceae, taxonomy.

Introduction

Species of *Armillaria* are important plant pathogens that can cause serious root disease problems in plantations, natural forests and in fruit tree orchards. These pathogens cause the plant disease known as Armillaria root rot. The taxonomy of *Armillaria* has been surrounded by considerable confusion and debate. The taxonomic status of *Armillaria* is, however, now reasonably well recognised and there are at least 38 species included in the genus, based on morphological characteristics or reproductive isolation (Volk and Burdsall 1995).

A number of techniques have been employed to identify *Armillaria* species. Traditionally, macro- and micromorphological characters of the basidiocarps were used for this purpose. Although this method is reasonably easy to apply, the rare occurrence of basidiocarps and their relatively short-lived nature, limits the use of morphology for identification (Swift 1972; Kile and Watling 1981). In addition to these problems some species, for example *A. gemina* and *A. ostoyae*, have identical basidiocarp morphologies (Bérubé and Dessureault 1989). These limitations led to the introduction of mating compatibility tests to facilitate identification (Korhonen 1978; Ullrich and Anderson 1978; Anderson and Ullrich 1979). This method is, however, time consuming and results are commonly ambiguous. Yet, despite their disadvantages morphology and mating tests have played an important role in *Armillaria* taxonomy and are still commonly used.

In an attempt to overcome the problems associated with morphology and mating compatibility tests, identification employing biochemical and genotypic characteristics have emerged. Biochemical characters obtained from isozyme and protein profiles (Morrison *et al*. 1985; Lin *et al*. 1989; Whalström *et al*. 1991; Mwenje and Ride 1996) as well as

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monoclonal and polyclonal antibodies (Burdsall *et al*. 1990) have been used. Genotypic characters from mtDNA, nDNA and amplified IGS-1 as well as ITS region RFLP analyses (Anderson *et al*. 1989; Harrington and Wingfield 1995; Coetzee *et al*. 2000*b*), DNA–DNA hybridisation (Miller *et al*. 1994) and AFLP (Pérez-Sierra *et al*. 2004) have yielded useful results.

Comparisons of DNA sequence data are increasingly being used in order to gain knowledge concerning the phylogenetic relationships among *Armillaria* species. Sequence data for this purpose have largely emerged from the IGS-1 (Anderson and Stasovski 1992; Coetzee *et al*. 2000*a*, 2000*b*, 2001; Pérez-Sierra *et al*. 2004) and ITS regions (Coetzee *et al*. 2000*a*, 2001; Chillali *et al*. 1998; Pérez-Sierra *et al*. 2004). Piercey-Normore *et al*. (1998) used combined sequence data of four anonymous DNA regions to determine the phylogeny of North American biological species (NABS) of *Armillaria*. The combined anonymous dataset gave a more resolved phylogenetic tree than those based on ITS and IGS-1 sequence data. No studies have been reported employing DNA sequence data for a protein-coding gene for phylogenetic analyses including a wide variety of *Armillaria* species.

The objectives of this study were to generate DNA sequence data for the translational elongation factor 1-α (EF 1-α) gene for *Armillaria* spp. from different parts of the world. This gene is involved in protein synthesis in eukaryotes, transporting amino-acyl tRNAs to the ribosomes (Slobin 1980). It has been successfully used in taxonomic and phylogenetic studies on ascomycetes and basidiomycetes, both at the intra- and interspecific levels (Baayen *et al*. 2000; Kauserud and Schumacher 2001; Jiménez-Gasco *et al*. 2002). Data from the current study would provide an additional gene region on which to test taxonomic groupings and phylogenetic relationships previously identified using other gene regions.

Materials and methods

Cultivation of isolates

Isolates included in this study were obtained from the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa (Table 1). Isolates were grown on MYA (2 g/L Biolab malt extract, 0.2 g/L Biolab yeast extract and 1.5 g/L Biolab agar) in Petri dishes for two weeks at 23°C in the dark.

DNA extraction

Isolates were transferred to liquid MY (2 g/L Biolab malt extract and 0.2 g/L Biolab yeast extract) in 500-mL Erlenmeyer flasks and allowed to grow for three weeks at 23°C in the dark. Mycelium was harvested through filtration, freeze-dried and ground into a fine powder in liquid nitrogen. Approximately 0.6 g of powdered mycelium was added to 1 mL of extraction buffer (200 mM Tris-HCl, pH 8; 25 mM EDTA; 250 mM NaCl; 0.5% SDS) and incubated at 57°C for one hour. The aqueous phase was separated from cell debris by centrifugation (15 300 *g*, 30 min). Phenol : chloroform (1 : 1) extractions were performed until a clean interphase was obtained. Excess phenol was removed through a final chloroform extraction. DNA was precipitated overnight at −20°C using cold ethanol (2 : 1 v/v) and collected by centrifugation (15 300 *g*, 15 min). The precipitated DNA was washed with 70% ethanol and recollected by centrifugation. The DNA was dried at 55°C and resuspended in sterile distilled water. DNA concentrations were determined using a Beckman Du Series 7500 UV spectrophotometer following the procedure outlined in Maniatis *et al*. (1982).

Amplification of the partial EF 1-α gene region

Approximately 100 ng of DNA extracted from the *Armillaria* isolates was used as template for amplification of a region of the EF 1-α gene. Amplicons were generated using primers EF595F (5′ CGT GAC TTC ATC AAG AAC ATG 3′), which binds at the 5′ end of the exon, and EF1160R (5′ CCG ATC TTG TAG ACG TCC TG 3′), which is complimentary to the 3′ end of the exon (Kauserud and Schumacher 2001) (Fig. 1). The PCR reaction mixture included 1 mM of each dNTP; 2.5 mM $MgCl_2$; PCR buffer supplied with the polymerase enzyme; 0.1 μM of each primer; 100 ng DNA and 2.5 U of *Taq* polymerase (Boehringer Mannheim, South Africa). The final reaction volume was 50 μL. The PCR reaction conditions were: an initial denaturation at 94°C for 2 min; 30 cycles of denaturation at 94°C for 30 s; annealing at 56°C for 30 s and an extension step at 72°C for 30 s. The final elongation step was allowed to proceed for 7 min at 72°C. PCR products were electrophoresed on a 1% (w/v) ethidium bromide-stained agarose gel and the bands were visualised under UV illumination.

DNA sequencing

PCR products were purified prior to sequencing using a QIAquick PCR purification kit (QIAGEN, Hilden, Germany). Primers EF595F and EF1160R were used in separate reactions to sequence both DNA strands. Sequencing reactions were conducted using a ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase, FS (Perkin Elmer, Warrington, UK) according to the manufacturer's instructions. DNA sequences for the partial EF 1-α gene were determined using an ABI PRISM 3100 automatic DNA sequencer (Applied Biosystems, Foster City, CA). Sequences were deposited in GenBank (Accession numbers: DQ435623–DQ435620).

DNA sequence analysis

Sequencing results were analysed using Sequence Navigator version 1.01 (ABI PRISM^{TM}). The DNA sequences were aligned using the program ClustalX, version 1.8 (Thompson *et al*. 1997) and manually adjusted. Phylogenetic analysis was performed using PAUP* version 4.0b10 (Swofford 1998). DNA sequences from the basidiomycete *Schizophyllum commune* (GenBank accession number X94913) were used to provide an outgroup. Missing and ambiguous characters were excluded from the analysis. Phylogenetic signal (Hillis and Huelsenbeck 1992) was determined for 1000 random trees. Phylogenetic trees were generated based on parsimony using heuristic searches with random stepwise addition of sequences (10 replicates) and TBR (tree bisection reconnection) branch swapping with MulTree active. Characters were reweighted according to the mean consistency index (CI) after each tree search until the number of trees stabilised, to reduce homoplasy. Confidence at the branch points was obtained through bootstrap analysis (1000 replicates) (Felsenstein 1985). Settings were the same as above except that simple addition of sequences was used.

Results

DNA amplification

A DNA amplicon was successfully amplified for all the isolates used in this study. All amplifications yielded a single

Table 1. List of *Armillaria* isolates used in the study

Species	Isolate number	Alternative culture collection number	Collector/Supplier	Origin
A. borealis	CMW3172	B370	K. Korhonen	Finland
	CMW3182	B373	K. Korhonen	Germany
A. cepistipes	CMW6909	33/82144	D. Morrison	USA
A. fumosa	CMW4955	123/1	C. Mohammed	Australia
	CMW4960	Q/COLL.9.4	C. Mohammed	Australia
A. fuscipes	CMW3164	B933	J. M. Sung	La-Reunion
	CMW4953	LR2	C. Fabregue	La-Reunion
A. gallica	CMW3171	B110	K. J. Smereka	USA
	CMW6901	21A	M. T. Banik	USA
A. gemina	CMW3181	B485	J. Anderson	USA
	CMW6888	5/JJW223	J. J. Worrall	USA
A. hinnulea	CMW4980	119/DAR	C. Mohammed	Australia
	CMW4981	LOT3/2	C. Mohammed	Australia
A. limonea	CMW4680	C3.28/0.1	I. A. Hood	New Zealand
	CMW4991	3522/2	G. S. Ridley	New Zealand
A. luteobubalina	CMW4977	SA(6)	C. Mohammed	Australia
	CMW8876	Chile-1	M. J. Wingfield	Chile
A. mellea	CMW11265	426	—	Italy
	CMW3956	B497	J. Anderson	East USA
	CMW3961	B730	T. Terashita	Japan
	CMW3964	B927	T. Bruns	West USA
	CMW4605	B282	T. C. Harrington	East USA
	CMW4610	B916	J. M. Sung	South Korea
	CMW4611	B917	J. M. Sung	South Korea
	CMW4613	B1205	M. Saber	Iran
	CMW4620	B1218	—	West USA
A. nabsnona	CMW6905	28/HB-20/SS5	D. Morrison	USA
A. novae-zelandiae	CMW4722	G3.0.34.4	I. A. Hood	New Zealand
	CMW4967	NSW3(4)	C. Mohammed	Australia
	CMW5448	7365/2	R. H. Petersen	Australia
A. ostoyae	CMW3162	B481	J. Anderson	USA
A. pallidula	CMW4971	3984	C. Mohammed	Australia
A. tabescens	CMW3158	B898	T. Volk	USA
	CMW3165	B531	J.-J. Guillaumin	France
Zimbabwe Group II	CMW4455	40	E. Mwenje	Zimbabwe
	CMW4456	Z1	M. Ivory	Zimbabwe
Zimbabwe Group III	CMW10115	56	E. Mwenje	Zimbabwe
	CMW9954	P21	E. Mwenje	Zimbabwe
Unknown	CMW4143	—	M. J. Wingfield	Indonesia
	CMW4994	4698/10	G. S. Ridley	New Zealand
	CMW5446	7348/10	R. H. Petersen	Argentina
	CMW5597	A35–4	I. A. Hood	New Zealand

fragment. The amplification products for the isolates were ~600 bp in length.

Sequence data and analysis

The total number of characters included in the data matrix was 562 after alignment by inserting gaps. Twenty-five missing or ambiguously aligned characters and 329 constant characters were excluded from the analysis. The number of parsimony informative characters was 173. A $g1$ value of -1.40443 was obtained, indicating that there was phylogenetic signal. Heuristic searches yielded eight parsimonious trees with a length of 462 steps. The CI and retention index (RI) were 0.680 and 0.852, respectively. Five parsimonious trees were generated (length = 314 steps, CI = 0.787 and RI = 0.897) after reweighting. Generally the trees had similar topologies but differed in branch length. One of the five most parsimonious trees (Fig. 2) was randomly chosen for presentation.

Bootstrap values supported the separation of species into distinct clades. In the analyses, two major and well-supported groups were detected. One of these represented isolates from Africa (100% bootstrap support) and the other

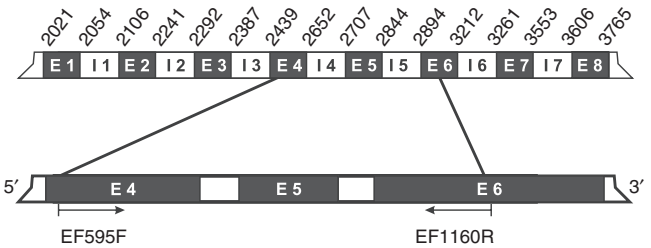

Fig. 1. Diagram showing the structure of the elongation factor 1-α gene from the basidiomycete *Schizophyllum commune*. Exons and introns are presented with black and white boxes, respectively. The binding positions for primers EF595F and EF1160R are indicated on the enlarged diagram. The numbers indicate the positions of the bases in the EF 1-α gene open reading frame of *S. commune*.

incorporated all isolates from other parts of the world. These are referred to as the African and non-African clades. The Northern Hemisphere species grouped within a well-supported subclade (83% bootstrap support) within the non-African clade.

Isolates within the African clade formed two subclades. Isolates CMW3164 and CMW4953 representing *A. fuscipes* grouped within one subclade with 74% bootstrap support. The remainder of the isolates CMW4455, CMW4456, CMW10115 and CMW9954 representing Zimbabwean groups II and III (Mwenje and Ride 1996) formed the second subclade with 99% bootstrap support. Isolates CMW9954 and CMW10115 formed a group within the second subclade with 63% bootstrap support.

Isolates representing *A. borealis*, *A. gallica*, *A. nabsnona*, *A. cepistipes* and *A. tabescens* grouped together in one clade with a 63% support value. This clade comprised three less well-supported clades, namely the *A. borealis* clade, *A. tabescens* clade and a clade comprising *A. gallica*, *A. nabsnona*, and *A. cepistipes*. *Armillaria gemina* and *A. ostoyae* grouped in a sister clade with 100% bootstrap support. These two major clades had 75% bootstrap support.

Isolates representing *A. mellea* formed a monophyletic group within the Northern Hemisphere clade. Isolates CMW3956 and CMW4605 from eastern North America formed a separate subclade with a 96% bootstrap support value. Isolates CMW3964 and CMW4620 from western North America grouped together with 100% bootstrap support. *Armillaria mellea* isolates CMW4613 and CMW11265 from Europe resolved into a clade with 100% bootstrap support. Isolates CMW3961, CMW4610 and CMW4611 from Asia formed a separate clade with 99% bootstrap support.

Southern Hemisphere isolates CMW4955 and CMW4960, representing *A. fumosa*, and isolate CMW4971, representing *A. pallidula*, grouped together with 100% bootstrap support. The clade with *A. hinnulea* isolates CMW4980 and CMW4981 had 100% bootstrap support and formed a sister clade to *A. fumosa* and *A. pallidula* (78% bootstrap support). Isolate CMW8876 of unknown identity grouped with *A. luteobubalina* isolates CMW4977 and CMW5446 (98% bootstrap support). *Armillaria limonea* isolates CMW4680 and CMW4991 resolved into a clade with 100% support and formed a sister group to *A. luteobubalina* (98% bootstrap support). Isolates CMW4967, CMW4722 and CMW5448 representing *A. novae-zelandiae* and CMW4143 representing an unknown species formed a clade with a bootstrap value of 100%. Two isolates CMW4994 and CMW5597 of unknown identity formed a distinct subclade with 100% bootstrap support.

Discussion

DNA sequence data for the EF 1-α gene were successfully generated and analysed for a range of *Armillaria* spp. in this study. To the best of our knowledge, this is the first time that this gene region has been used to consider phylogenetic relationships in *Armillaria*. All isolates yielded PCR products of similar size, indicating that the amplified gene region does not include large indels, and is therefore a suitable choice of gene region for phylogenetic studies. The aligned sequences showed considerable homology among *Armillaria* spp. but various species-specific nucleotide substitution and indels were observed. Little sequence variation was observed within species with noticeable variation between different species. This is consistent with various previous studies employing IGS-1 and ITS rDNA operon DNA sequence data in taxonomic studies of *Armillaria* spp. (Anderson and Stasovski 1992; Chillali *et al*. 1998; Coetzee *et al*. 2001, 2003).

Phylogenetic comparisons based on EF 1-α sequence data showed that *Armillaria* spp. thought to be native to Africa, reside in a clade strongly separated from all other species. This so-called African clade has previously been identified based on isozyme analysis (Mwenje and Ride 1997) and IGS-1 sequence data (Mwenje *et al*. 2003). Thus, the sequence data for a new protein-coding gene region reflect the same patterns that have emerged from previous molecular-based comparisons.

Isolates from Africa that have previously been shown to represent different taxonomic groups (Mwenje *et al*. 2003) resided in a strong monophophyletic assemblage and are regarded as the African *Armillaria* group. These isolates were previously thought to represent a single species treated as *A. heimii sensu lato* and were shown to have high levels of intraspecific variation (Mohammed *et al*. 1989). Recent studies based on IGS-1 and ITS sequence data, however, suggest that the African isolates represent at least two different species: *A. fuscipes* (syn. *A. heimii*) and an unnamed species (Coetzee *et al*. 2000*b*; Mwenje *et al*. 2003; Coetzee *et al*. 2005). Pérez-Sierra *et al*. (2004) reported similar results but viewed the two groups as different populations of *A. fuscipes*, which they referred to as *A. heimii* and an unknown species. Results of the present study also show that the African isolates reside in subclades, representing the three taxonomic groups suggested by Mwenje *et al*. (2003).

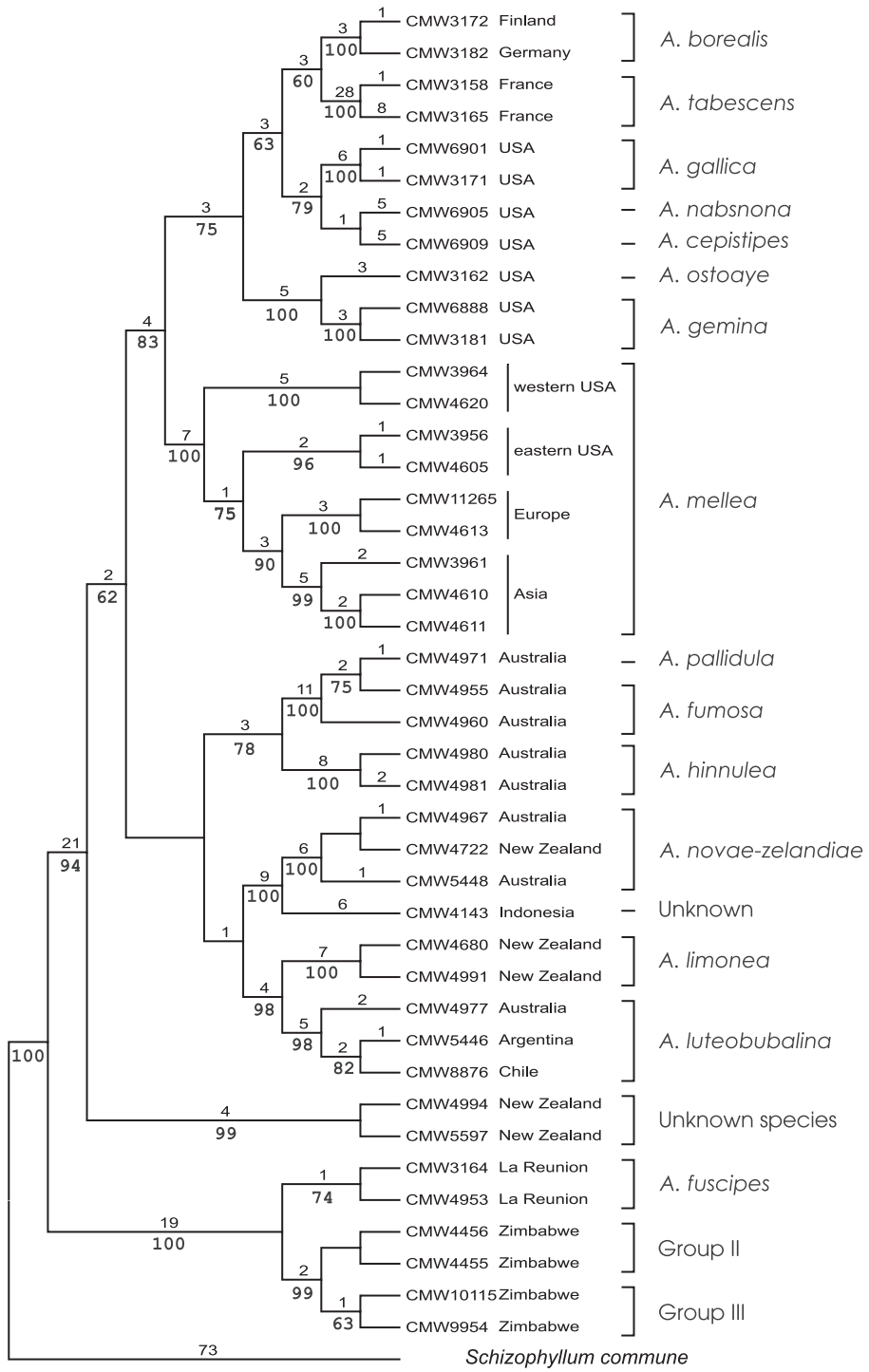

Fig. 2. One of the most parsimonious trees generated after a heuristic search using the elongation factor 1-α DNA sequence data with ambiguous and missing data excluded. Branch length values are shown above the tree branches and percentage bootstrap (1000 replicates) values greater than 50% are shown below the tree branches. Number of parsimony informative characters = 173, tree length = 314 steps, CI = 0.787 and RI = 0.897.

Isolates within the *A. mellea* clade formed strongly supported monophyletic groups consistent with the geographical origin of the isolates. These were isolates from Asia, Europe, eastern North American and western North America. Differences between geographically separated isolates of *A. mellea* have been observed in a number

of previous studies. Anderson *et al*. (1989) showed that *A. mellea* isolates from Europe and eastern North America differ in their *Eco*RI, *Bam*HI and *Sal*I digestion patterns of the rRNA operon. Intraspecies variation pertaining to IGS-1 RFLP patterns was similarly reported for this species by Harrington and Wingfield (1995). Likewise, differences have been observed in the mating systems of isolates from Europe, North America and Japan (Anderson *et al*. 1980; Ota *et al*. 1998). The subdivision of isolates of *A. mellea* according to their origin is congruent with the study of Coetzee *et al*. (2000*a*), showing that isolates of *A. mellea* from various Northern Hemisphere origins represent Asian, European, eastern North American and western North American lineages. Of these, isolates from Europe, North America and Asia have been shown to be sexually compatible and thus reported to be the same biological species (Anderson *et al*. 1980, 1989; Ota *et al*. 1998). The separation of the isolates into geographic groups may reflect intraspecific variation due to allopatric separation. Alternatively, these lineages may represent sibling species in the process of allopatric speciation (Coetzee *et al*. 2000*a*) with incompletely developed intrinsic genetic isolation mechanisms. Inclusion of sequence data from a gene region not previously considered, adds strong additional support for the view that *A. mellea* from different geographic areas are genetically distinct and probably represent sibling species.

Results of this study showed that isolates in the *A. ostoyae* clade included those representing *A. ostoyae* and *A. gallica*. *Armillaria ostoyae* and *A. gemina*, have previously shown to be phylogenetically closely related (Anderson and Stasovski 1992; Miller *et al*. 1994). These two species also have identical basidiocarp morphology (Bérubé and Dessureault 1989). They can however, be differentiated from other species based on vegetative features (Bérubé and Dessureault 1989) and on mating tests (Anderson and Ullrich 1979).

Isolates representing *A. borealis*, *A. gallica*, *A. nabsnona*, *A. cepistipes* and *A. tabescens* grouped in the *A. gallica* clade. These data are congruent with studies showing that *A. gallica* and *A. cepistipes* are ecologically (Korhonen 1995) and morphologically (Termorshuizen and Arnolds 1987; Marxmüller 1992; Korhonen 1995) similar and that they can only be differentiated using mating tests (Termorshuizen and Arnolds 1987). They are also consistent with the fact that *A. gallica* and *A. cepistipes* have previously been shown to be phylogenetically closely related based on DNA data (Anderson and Stasovski 1992; Miller *et al*. 1994; Chillali *et al*. 1998). Furthermore, *A. nabsnona* has been found to be related to *A. gallica* based on DNA reassociation data (Miller *et al*. 1994) and our new sequence data confirm this view.

The grouping of *A. borealis* in the *A. gallica* clade in this study is an interesting result. Previous studies based on ITS and IGS-1 sequence data analysis of the rDNA operon showed that this species is phylogenetically most closely related to *A. ostoyae* and *A. gemina* (Anderson and Stasovski 1992; Chillali *et al*. 1998). Also, Korhonen (1995) placed *A. borealis* within the '*A. ostoyae* clade' based on morphological similarities with *A. ostoyae* and *A. gemina*. The lack of correlation between the ribosomal phylogeny and that of the EF 1-α gene suggests that the evolutionary histories of these two gene regions are not the same. This highlights the danger of using single gene phylogenies to infer phylogenetic relationships.

The grouping of *A. tabescens* within the *A. gallica* clade was unexpected. Previous work based on DNA reassociation showed that *A. mellea* and *A. tabescens* are most closely related (Miller *et al*. 1994). Chillali *et al*. (1998) further showed, using ITS sequence data, that *A. mellea* and *A. tabescens* are basal to the rest of Northern Hemisphere species. Miller *et al*. (1994) contended that *A. tabescens* is the more basal species and, therefore, more ancient than *A. mellea*. These studies, however, did not include species from the Southern Hemisphere. The grouping of *A. tabescens* with *A. gallica*, *A. cepistipes* and *A. nabsnona* may be explained by the different rates at which ribosomal and protein-coding genes evolve. The different evolutionary rates lead to these species grouping together in one way when ribosomal genes are used and in another way when protein-coding genes are employed, as was observed in this study.

Armillaria fumosa, *A. pallidula*, *A. novae-zelandiae*, *A. luteobubalina*, *A. limonea*, *A. hinnulea* and the undescribed species from New Zealand have been reported only from the Southern Hemisphere (Kile and Watling 1983, 1988). Cladograms generated from EF1-α sequences confirm that these species are closely related and that they group basal to those from the Northern Hemisphere. This is consistent with the results of previous studies based on IGS-1 and LSU sequence data, suggesting that the Southern Hemisphere species are ancestral to those from the Northern Hemisphere (Coetzee *et al*. 2001; Dunne *et al*. 2002). The new sequence dataset, therefore, provides further evidence for the hypothesis that *Armillaria* or the ancestor of this genus originated in Gondwana (Coetzee *et al*. 2001; Dunne *et al*. 2002).

Armillaria fumosa and *A. pallidula* grouped together in a one clade in this study. This supports the findings of Coetzee *et al*. (2001) who reported that these species are closely related and cannot be distinguished using ITS sequence information. Kile and Watling (1988), using interfertility tests and morphology, showed that these are distinct species although they share some morphological similarities. The results of the present study together with those of Coetzee *et al*. (2001) indicate that the species have recently diverged from a common ancestor and have not accumulated sufficient differences at the DNA level to differentiate between them.

The grouping of *A. hinnulea* outside the Northern Hemisphere clade in this study was of particular interest. This species has been reported from Tasmania, southeast Australia and New Zealand and it is to be expected that it

should be phylogenetically closely related to those species occurring in the Southern Hemisphere. Phylogenetic studies based on ITS sequence data, however, showed that it is most closely related to species from the Northern Hemisphere (Coetzee *et al*. 2001; Dunne *et al*. 2002). Dunne *et al*. (2002) suggested that it may have evolved from a common ancestor with *A. cepistipes* [= *A. bulbosa*]. This result is supported by the findings of Kile and Watling (1983), who showed that the basidiocarp morphology of *A. hinnulea* is in various aspects similar to those of the European species *A. cepistipes*. The fact that the EF 1-α gene sequences gave results different from those from studies based on the ITS region might reflect the fact that EF 1-α has evolved more slowly than the ITS region. More rapid evolution of the ITS region could have resulted in the inclusion of synapomorphic characters, leading to *A. hinnulea* grouping with Northern Hemisphere species. In contrast, the EF 1-α gene nucleotides for this species may have retained the ancestral character states of species in the Southern Hemisphere, resulting in the grouping of this species basal to those from the Northern Hemisphere.

Isolates representing *A. luteobubalina* from Chile and Australia had similar DNA sequences and grouped together in one clade. An isolate, tentatively identified as *A. luteobubalina* based on IGS-1 and ITS sequences (Coetzee *et al*. 2003), grouped with the isolates representing this species. The identification of this isolate as *A. luteobubalina* has been controversial, as this species had never before been reported from South America. Results of the current study provide additional evidence that the isolates from Chile represent *A. luteobubalina* and that the species is present in South America. Coetzee *et al*. (2003) also showed that despite their large geographic separation, isolates in this clade retained a high level of ITS and IGS-1 sequence similarity. These authors therefore postulated that this is an ancient species with its origin in the Gondwana supercontinent.

Phylogenetic trees obtained in this study showed that *A. luteobubalina* is closely related to *A. limonea*. Isolates representing the latter species grouped in a monophyletic clade. This relationship supports the findings of Kile and Watling (1988) that the two species share some morphological characters such as a yellow pigment in their pileus.

An unidentified isolate from Indonesia grouped with *A. novae-zelandiae* in a strongly supported clade. This isolate represents a set of isolates that were obtained from infected *Eucalyptus grandis* trees but for which no basidiocarps were found. A description based on morphology or identification using mating tests was thus not possible (Coetzee *et al*. 2003). The set of isolates were considered by Coetzee *et al*. (2003) who attempted to identify them. In their study the authors showed that the isolates either represent *A. novae-zelandiae* or a previously undescribed species that is closely related to that species. The phylogenetic trees generated in this study thus support the finding of Coetzee *et al*. (2003).

Armillaria novae-zelandiae is common in New Zealand and Australia (Kile and Watling 1983). Isolates from both areas appear to represent a single species (Coetzee *et al*. 2001). These isolates are morphologically similar (Kile and Watling 1983) and are sexually compatible (Kile and Watling 1983). Our results using sequence data from a new gene region support the view that these isolates represent the same taxon.

Two undescribed isolates from New Zealand formed a distinct clade and showed no relationship to any known species, but fell in the Southern Hemisphere group. These isolates were shown by Coetzee *et al*. (2001) to probably represent an undescribed species. Results of the present study provide additional support for the view that these isolates represent a discrete taxon that awaits description.

This study presents the first EF 1-α DNA sequence data for *Armillaria* species. It is also the first protein-coding gene and first single-copy gene to be investigated for phylogenetic analysis of this genus. Sequence data from the majority of isolates belonging to the different species showed unique species-specific substitutions, allowing the isolates to be differentiated into clades representing the species. Results of this study demonstrate that the EF 1-α region is useful for phylogenetic analysis and classification of *Armillaria* species. They have also added considerable confidence to relationships identified based on DNA sequences for other gene regions.

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