Mycol. Res. **109** (0): 1–12 (■ 2005). © The British Mycological Society
doi:10.1017/S095375620500393X *Printed in the United Kingdom.*

Phylogenetic analyses of DNA sequences reveal species partitions amongst isolates of *Armillaria* from Africa

Martin P. A. COETZEE[1], Brenda D. WINGFIELD[1], Paulette BLOOMER[2] and Michael J. WINGFIELD[1]

[1] *Department of Genetics, Tree Protection Co-operative Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria 0002, South Africa.*
[2] *Department of Genetics, Molecular Ecology and Evolution Programme (MEEP), University of Pretoria, Pretoria 0002, South Africa.*
E-mail: brenda.wingfield@fabi.up.ac.za

Received 31 January 2005; accepted 17 July 2005.

The basidiomycete genus *Armillaria* cause root rot and death to woody plants in boreal, temperate and tropical regions of the world. Armillaria root rot has been described from various parts of Africa on many different hosts. However, very little is known regarding the evolutionary relationships among *Armillaria* species in Africa. The aim of this study was to determine the phylogenetic relationships between isolates originating from different regions in Africa using nDNA sequences from two non-coding gene regions. The ITS and the IGS-1 regions of the ribosomal DNA operon were sequenced and analysed using different phylogenetic tree searching methods. Phylogenetic trees grouped the African taxa in two strongly supported clades. One of these represented *A. fuscipes* and the other an undescribed but distinct species.

INTRODUCTION

Armillaria species (*Basidiomycota, Agaricales, Tricholomataceae*) cause root rot on various plant species. Generally they do not show strong host specificity and occur worldwide in natural forests and on planted woody crops (Hood, Redfern & Kile 1991, Kile, McDonald & Byler 1991, Termorshuizen 2001). The impact of the disease is exacerbated by the ability of *Armillaria* species to survive either as saprobes or necrotrophs (Gregory, Rishbeth & Shaw 1991), depending on the available substrate in a particular niche. Consequently, Armillaria root rot poses a significant problem for forestry and agricultural industries worldwide and the species warrant accurate identification.

The manner in which species are delineated in fungi depends heavily upon the species concept that is employed (Taylor *et al.* 2000). Where emphasis is placed on morphology (Regan 1926), specimens with similar basidiome form are considered the same species. When the biological species concept (Mayr 1942) is applied, species are viewed as a group of organisms that are sexually and reproductively isolated from those outside the group. A third perspective is provided by the various species definitions embodied within the phylogenetic species concept. One of these, the genealogical concordance species concept, defines a species as a group of organisms with a shared, exclusive genealogical history (Baum & Shaw 1995). Disparities that arise through the application of these different species concepts have led to a lack of consensus regarding the identification of African *Armillaria* species.

Numerous taxonomic studies have been undertaken on African *Armillaria* species (Mohammed, Guillaumin & Berthelay 1989, Mohammed & Guillaumin 1993, Mwenje & Ride 1993, 1996, 1997, Agustian *et al.* 1994, Mohammed *et al.* 1994, Abomo-Ndongo & Guillaumin 1997, Chillali *et al.* 1997, Coetzee *et al.* 2000a). These have included species identifications based on *in vitro* cultural characteristics, basidiome morphology, biochemical properties, and sexual or interspecific somatic incompatibility tests. Because basidiomes are short-lived and seldom encountered in *Armillaria* species, especially those in tropical Africa (Swift 1972), basidiome morphology has been of limited use in mapping species in the continent. Taxonomic studies based on interspecific somatic incompatibility tests, on the other hand, have been more successful.

In *Armillaria*, interspecific somatic incompatibility differs from intraspecific somatic incompatibility, as epitomised by Rayner (1991). In the latter case, a clear zone made up of sparse hyaline mycelium is produced between incompatible strains (Korhonen 1978, Mallett,

Hopkin & Blenis 1989). This interaction has been used in population studies to determine the clonal diversity of *Armillaria* species (e.g. Korhonen 1978, Kile 1983, Rizzo & Harrington 1993). In interspecific somatic incompatibility interactions, vegetative mycelium produces a black-pigmented demarcation line between incompatible isolates, which reflect different biological species (Korhonen 1978, Rishbeth 1982, Mallett & Hiratsuka 1986, Mallett *et al.* 1989). This interaction can, therefore, be used to distinguish between different biological species. Sexual compatibility studies, based on successful diploidization of a haploid tester strain from haploid or diploid strains, are not possible for homothallic taxa due to the absence of a haploid phase. Consequently, interspecific somatic compatibility tests have been used to distinguish homothallic *Armillaria* species from Africa (Guillaumin, Mohammed & Abomo-Ndongo 1994, Abomo-Ndongo & Guillaumin 1997).

Results of interspecific somatic incompatibility tests have suggested that *Armillaria* isolates from Africa can be separated into at least four groups, referred to as somatic incompatibility groups (SIGs) (Mohammed *et al.* 1989, 1994, Abomo-Ndongo & Guillaumin 1997). Of these, SIG I includes isolates from Kenya, Tanzania and São Tomé that represent the homothallic African form of *A. mellea*. Isolates considered to represent *A. heimii* from East, Central, West and South Africa have been designated as SIG II. Isolates residing in SIG III originated from Kenya, but were not assigned to a morphological species (Guillaumin *et al.* 1994, Mohammed *et al.* 1994, Abomo-Ndongo & Guillaumin 1997). One isolate from Kenya was incompatible with all other isolates and placed in SIG IV (Guillaumin *et al.* 1994, Mohammed *et al.* 1994).

Although isolates of *Armillaria* referred to as *A. heimii* have been considered as belonging to the same biological species (SIG II), they display considerable variation. This variation includes differences in their mating systems (Abomo-Ndongo, Mohammed & Guillaumin 1997), mycelial-mat morphology, and rhizomorph characteristics (Mwenje & Ride 1993, Mohammed *et al.* 1994), optimal growth temperatures (Mohammed & Guillaumin 1993, Mohammed *et al.* 1994), randomly amplified polymorphic DNA (RAPD) patterns (Mohammed 1994, Otieno, Pérez Sierra & Termorshuizen 2003), isozyme electrophoresis profiles (Agustian *et al.* 1994, Mwenje & Ride 1997), ITS and IGS RFLPs (Chillali *et al.* 1997, Coetzee *et al.* 2000a, Otieno *et al.* 2003) and inter-simple sequence repeat (ISSR) polymorphisms (Otieno *et al.* 2003). Collectively, these studies have shown that the isolates can be differentiated into at least three subgroups. In the light of these findings, *A. heimii* is referred to as *A. heimii s. lat.* as it possibly comprises several species (Mohammed & Guillaumin 1993).

In addition to *A. heimii* and *A. mellea*, studies on the taxonomy of African *Armillaria* species have included *A. fuscipes*. This species was first reported from Sri Lanka on *Acacia decurrens* (Petch 1909). After considering the micromorphology of *A. fuscipes*, Chandra & Watling (1981) suggested that this taxon and *A. heimii* were conspecific, and Pegler (1986) subsequently synonymised *A. heimii* with *A. fuscipes*, with the latter being the earlier named species. Pegler (1986) also suggested that *A. fuscipes* had been introduced into Sri Lanka on tea. After examining the type specimen of *A. heimii*, Kile & Watling (1988) and Watling (1992) supported the treatment of these taxa by Pegler (1986). These authors, however, suggested that conspecificity of the two species should be verified using cultural and interfertility studies. Although the similarity in basidiome morphology of *A. heimii* and *A. fuscipes* provides a strong case for their synonymy, this has not been generally accepted (Mohammed & Guillaumin, 1993, Otieno *et al.* 2003, Pérez-Sierra *et al.* 2004). Notwithstanding the apparent preference for the name *A. heimii* in the literature, nomenclaturally and from the perspective of the morphological species concept there is no reason to use this name instead of *A. fuscipes*. Consequently, we use the latter name in this study for isolates that have been treated as *A. heimii* in earlier publications.

As mentioned above, several studies have strongly suggested that *A. heimii s. lat.* from Africa, i.e. *A. fuscipes*, comprises several distinct species. Two recent studies (Coetzee *et al.* 2000a, Mwenje *et al.* 2003) confirmed this view. Phylogenetic analysis of IGS-1 sequence data showed that isolates of *A. fuscipes* from different African countries reside in two strongly resolved monophyletic groups. One of these groups includes isolates from South Africa, Zimbabwe (Group I of Mwenje & Ride 1996) and La Reunion and the other isolates from Zambia, Zimbabwe (Group II and III of Mwenje & Ride 1996) and Cameroon. These studies have suggested that the two groups represent at least two different species (Coetzee *et al.* 2000a, Mwenje *et al.* 2003). Some of the isolates included were, however, previously shown to represent the same interspecific somatic compatibility group and, therefore, the same biological species, even though they belong to different phylogenetic lineages. The possibility remains that the groups recognised by Coetzee *et al.* (2000a) and Mwenje *et al.* (2003) might reflect intraspecific genetic variation within *A. fuscipes*. Hence, the objective of this study was to re-evaluate the suggestion that *A. fuscipes* encompasses more than one species. This objective was accomplished by extending the number of isolates considered previously (Coetzee *et al.* 2000a, Mwenje *et al.* 2003) and conducting phylogenetic analysis of both ITS and IGS-1 sequence data.

MATERIALS AND METHODS

Fungal strains

Armillaria isolates used in this study originated from eight countries in sub-Saharan Africa and a wide range

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

Culture number[a]	Alternative number[b]	Host	Origin	GenBank accession nos	
				IGS[c]	ITS[c]
CMW2717	A04-SA, CBS*****	*Pinus elliottii*	South Africa	AF204821	AY882971
CMW2740	B07-SA, CBS*****	*P. patula*	South Africa	AF204822	AY882970
CMW3152[c]	CA1, B935, CBS*****	Unknown	Cameroon	AF204826	AY882980
CMW3164[c]	LR3, B933, CBS*****	*Pelargonium asperum*	La Reunion	AF204824	AY882975
CMW3173[c]	ZM1, B932	*Tectona grandis*	Zambia	AF204825	AY882981
CWM4455	40	*Camellia sinensis*	Zimbabwe	AF489486	AY882985
CMW4456[c]	Z1	*Brachystegia utilis*	Zimbabwe	AF489485	AY882984
CMW4871[c]	M1, CBS*****	*Widdringtonia whytei*	Malawi	AY882959	AY882976
CMW4873[c]	M3	Indigenous shrub	Malawi	AY882960	AY882977
CMW4874[c]	Z2, CBS*****	*Araucaria cunninghamii*	Zimbabwe	AF489481	AY882967
CMW4949[c]	T1, CBS*****	*Pinus elliottii*	Tanzania	AY882961	AY882978
CMW4950[c]	T2	*Pinus strobes*	Tanzania	AY882962	AY882979
CMW4953[c]	LR2, CBS*****	*Pelargonium asperum*	La Reunion	AY882963	AY882974
CMW5844	WG11, CBS*****	*P. patula*	Ethiopia	AY172032	AY882969
CMW5846	WG2E, CBS*****	*P. patula*	Ethiopia	AY172030	AY882968
CMW7184	K52, CBS*****	*Cypress* sp.	Kenya	AY882965	AY882973
CMW7187	K65	*Camellia sinensis*	Kenya	AY882964	AY882972
CMW10115	55, CBS*****	*Acacia albida*	Zimbabwe	AF489483	AY882983
CMW10116	56, CBS*****	*Newtonia buchananii*	Zimbabwe	AF489484	AY882982
CMW10165	P7	*Prunus persica*	Zimbabwe	AF489482	AY882966

[a] CMW numbers refer to the collection numbers in the fungal culture collection of the Tree Protection Co-operative Programme (TPCP), FABI (Pretoria).
[b] Alternative numbers refer to culture numbers used for isolates in previous publications.
[c] Isolates used in Mohammed *et al.* (1989, 1994), Abomo-Ndongo & Guillaumin (1997).

of hosts (Table 1). All isolates other than those from South Africa and Ethiopia were from the collections of Caroline Mohammed (CSIRO, Forestry and Forestry Products, Hobart) and Tom C. Harrington (Iowa State University, Ames). These cultures are preserved in the collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria. Representative isolates have also been deposited with the Centraalbureau voor Schimmelcultures (CBS), Utrecht.

Isolates that have been treated as *A. heimii* and placed in SIG II based on their basidiome morphology *in vitro*, phenotypic similarity and interspecific somatic compatibility (Mohammed *et al.* 1989, 1994, Abomo-Ndongo & Guillaumin 1997) are indicated in Table 1. The isolates from Ethiopia (CMW5844 and CMW5846) and South Africa (CMW2717 and CMW2740) were identified as *A. fuscipes* based on basidiome morphology and IGS-1 sequence data (Coetzee *et al.* 2000a, Gezahgne *et al.* 2004). The Zimbabwean isolates were previously characterised based on their morphological, biochemical and IGS-1 sequence data and found to represent three groups: Group I (CMW4874 and CMW10165); Group II (CMW4455 and CMW4456); Group III (CMW10115 and CMW10116) (Mwenje & Ride 1996, Mwenje *et al.* 2003). Isolates CMW4456 (from Group II) and CMW4874 (from Group I) were shown to be somatically compatible with isolates in SIG II (Mohammed *et al.* 1989, 1994, Abomo-Ndongo & Guillaumin 1997).

DNA extractions

Isolates were grown in liquid MY (1.5% malt extract and 0.2% yeast extract) medium for 4 wk in the dark at 24 °C. The mycelium was harvested using a strainer, lyophilised and ground to a fine powder in liquid nitrogen. Extraction buffer (1 ml, 100 mM Tris-Cl pH 8.4; 1.4 M NaCl; 25 mM EDTA pH 8; 2% CTAB, hexadecyltrimethylammonium bromide) was added to *ca* 0.5 g powdered mycelium and incubated at 60 ° for 2 h. The mycelium powder-buffer suspension was divided into two parts and centrifuged (17900 ***g***, 20 min) to precipitate cell debris. Isoamyl alcohol: chloroform (1:24 v/v) extractions were performed on the aqueous phase until a clean interphase was obtained. Nucleic acids were precipitated using 96% ice-cold ethanol. The precipitate was collected by centrifugation (17900 ***g***, 30 min), washed with cold 70% ethanol, dried and eluted in sterile distilled water. Contaminating RNA was removed by adding RNAse A (0.01 mg μl^{-1}) (Roche, ■). AQ1

Amplification of the ITS and IGS-1 regions of the rDNA

The ITS1 and ITS2 regions between the 3′ end of the SSU and the 5′ end of the LSU rRNA gene as well as the IGS-1 between the 3′ end of the LSU and the 5′ end of the 5S gene were amplified using PCR. The ITS regions were amplified using primer pair ITS1 and ITS4 (White *et al.* 1990). Primer pair P-1 (Hsiau 1996) and

5S-2B (Coetzee *et al.* 2000a) were used to amplify the IGS-1 region for the *Armillaria* isolates. Reaction conditions and the PCR reaction mix were the same as those previously described by Coetzee *et al.* (2000b). ITS and IGS-1 amplicons were visualised under UV illumination after electrophoresis on an agarose (Promega, Madison, WI) gel (0.8%) stained with ethidium bromide.

DNA sequencing

ITS and IGS-1 DNA sequences were obtained using an ABI PRISM automated sequencer. A QIAquick PCR purification kit (Qiagen, Hilden, Germany) was used to purify PCR products from unincorporated nucleotides, excess primer and salts as well as primer dimers, prior to sequencing. Sequence reactions were carried out with the ABI Prism® BigDyeTM Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase, FS (Perkin Elmer, Warrington, UK) following the protocol supplied by the manufacturer. DNA sequences for the ITS region were generated using primers ITS1 and ITS4 as well as internal primers CS2B and CS3B (Coetzee *et al.* 2001). The IGS-1 region was sequenced using primers P-1, 5S-2B and internal primers MCP2, MCP2R, MCP3, MCP3R, 5S-3MC and 5S-4MCR (Coetzee *et al.* 2000a).

Cloning of IGS-1 amplicons

IGS-1 PCR products from isolates that gave poor sequences were cloned into vector pCR®4-TOPO® after purification, as outlined above. Cloning reactions were done using a TOPO TA Cloning® Kit for Sequencing (Invitrogen Life Technologies, Carlsbad, CA) with One Shot® TOP10 Chemically Competent *Escherichia coli* cells following the manufacturer's directions. The IGS-1 region was amplified directly from transformed *E. coli* cells to verify positive inserts. PCR mixtures included dNTPs (250 µM each), *Taq* Polymerase (2.5 U) (Roche Diagnostics, Mannheim, Germany), PCR buffer with $MgCl_2$ (supplied by the manufacturer), primers P-1 and 5S-2B (0.1 µM each), brought to a final volume of 50 µl with water. PCR conditions were as follows: 1 cycle at 95 ° for 1 min (denaturation), 35 cycles of 60 ° for 30 s (primer annealing), 70 ° for 30 s (elongation), and 95 ° for 30 s (denaturation). A final elongation step was allowed at 70 ° for 7 min. PCR products were visualized under UV illumination on a 1% agarose gel stained with ethidium bromide. The IGS-1 insert from one clone that was successfully amplified from positively transformed cells was sequenced for each isolate as described above.

Sequence and phylogenetic analyses

Phylogenetic analyses were done on three data matrices: (1) *Armillaria* ITS including all African isolates and two representative sequences for each *Armillaria* sp. available on GenBank (accession numbers in Fig. 1) with *A. mellea* as outgroup; (2) African *Armillaria* ITS including ITS sequences determined in this study with *A. hinnulea* (AF394918 and AF329907) as outgroup taxon; and (3) African *Armillaria* IGS-1 including sequences obtained in this study and sequences available on GenBank from previous studies (Table 1). An outgroup was not available for rooting phylogenetic trees generated from IGS-1 sequence data as a result of an inverted 5S gene that is present only in the African taxa (see Results). Alignment was done using Clustal X (Thompson *et al.* 1997) and manually corrected using BioEdit Sequence Alignment Editor version 5.0.9 (Hall 1999). Indels larger than two base pairs in the African ITS and IGS-1 data matrices were coded using a multistate-character system (Coetzee *et al.* 2001).

Parsimony analyses were conducted on the African ITS and IGS-1 data matrices using PAUP* version 4.10 (Swofford 1998). Missing, parsimony uninformative and ambiguously aligned regions were excluded from the data sets before analyses. Gaps were treated as a fifth character, 'newstate'. Parsimony trees were generated by heuristic searches with random addition of sequences (100 replicates), TBR (tree bisection reconnection) branch swapping and MULPARS active. MaxTrees was set to auto-increase after 100 trees were generated and branches collapsed if negative branch lengths were obtained. Bootstrap analysis (1000 replicates) using the same settings as above but with *A. hinnulea* as reference taxon, and sequential addition of sequences was employed to obtain confidence of branch nodes (Felsenstein 1985) for trees generated from the African ITS data matrix.

Phylogenetic trees based on distance methods were generated for all data matrices using PAUP*. Missing data and ambiguously aligned or gapped regions were excluded from the data sets prior to analysis. Trees were obtained using a neighbour-joining (NJ) tree-building algorithm (Saitou & Nei 1987) that incorporated a Kimura 2-parameter nucleotide substitution model (Kimura 1980). Confidence values for branching nodes were determined for the African ITS and *Armillaria* ITS datasets using bootstrap analysis (1000 replicates) with the same settings as above.

Relative nucleic substitution rate heterogeneity among lineages based on ITS sequence data was determined for the African taxa. A relative rate test (Robinson *et al.* 1998) was performed using RRTree version 1.1 (Robinson-Rechavi & Huchon 2000). In the test the Kimura 2-parameter substitution model was applied and *A. hinnulea* was used as reference taxon.

Intra-specific nucleotide diversity and evolutionary distance comparisons

The intra-specific nucleotide diversity and evolutionary distances between the two African taxa were compared

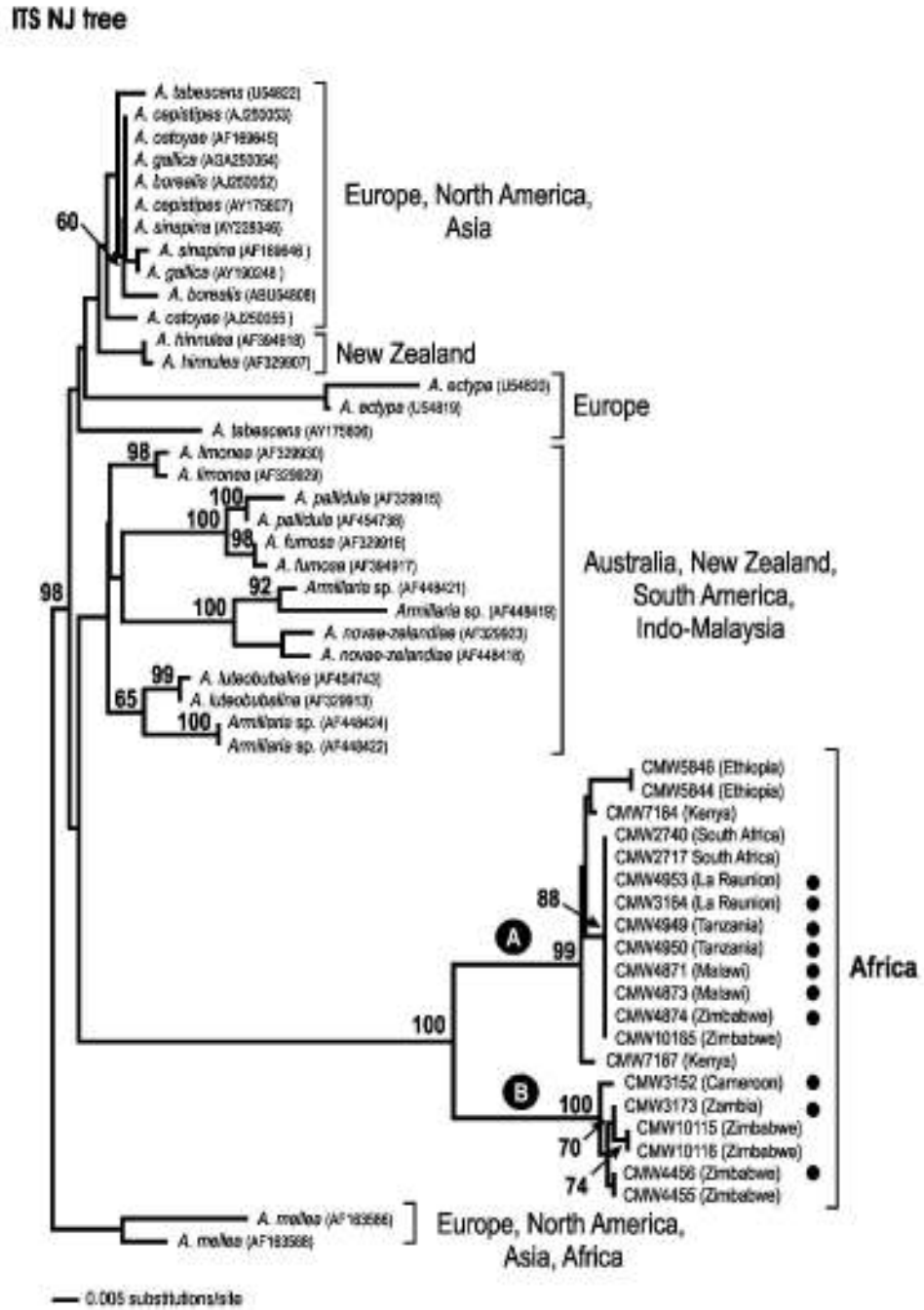

Fig. 1. Neighbour-joining tree generated from the *Armillaria* ITS data matrix. Numbers in brackets are GenBank accession numbers. Bootstrap values are given above the tree branches. Dots (●) denote isolates previously shown to belong to SIG II (Mohammed *et al.* 1989, 1994, Abomo-Ndongo & Guillaumin 1997). Bar = nucleotide substitutions per site.

against those for geographically separated groups in *Armillaria luteobubalina*, *A. mellea*, and *A. novae-zelandiae* (Table 2). Sequences for each species were aligned in a separate dataset using methods described above. Characters from the extreme 5′ and 3′ ends of the ITS sequences were deleted from some taxa to obtain individual datasets that had identical start and end positions. Missing and gapped regions were excluded prior to analyses. Mean nucleotide diversity (π) (Nei 1987) was calculated over all taxa for each data set and mean evolutionary distances between and within groups within a specific dataset using a Kimura 2-parameter nucleotide substitution model in MEGA version 2.1 (Kumar *et al.* 2001).

RESULTS

Amplification of ITS and IGS-1

The ITS region was successfully amplified for all African isolates. The ITS amplicon size for all isolates was approximately 650 bp. The IGS-1 region was successfully amplified for isolates CMW4871, CMW4873, CMW4949, CMW4950, CMW4953, CMW7187 and

Table 2. GenBank accession numbers, origin and grouping of *Armillaria* species included in intra-specific nucleotide diversity and evolutionary distance comparisons.

Species	Group	Origin	GenBank accession nos	
			ITS	IGS-1
A. luteobubalina	Australia (west)	WA, Australia	AF329913	–
A. luteobubalina	Australia (west)	Cape Arid, WA, Australia	AF454741	–
A. luteobubalina	Australia (west)	Popanyinning, WA, Australia	AF454742	–
A. luteobubalina	Australia (east)	VIC, Australia	AF329909	–
A. luteobubalina	Australia (east)	VIC, Australia	AF329910	–
A. luteobubalina	Australia (east)	South Australia	AF329912	–
A. luteobubalina	Australia (east)	Traralgon, VIC, Australia	AF454743	–
A. mellea	Europe	Cambridgeshire, UK	AF163578	AF162602
A. mellea	Europe	Hungary	AF163581	AF163605
A. mellea	Europe	Iran	AF163583	AF163606
A. mellea	Europe	France	AF163585	AF163600
A. mellea	USA (west)	Orinda, CA	AF163595	AF163608
A. mellea	USA (west)	Berkeley, CA	AF163596	AF163607
A. mellea	USA (west)	CA	AF163597	AF163609
A. mellea	USA (east)	Durham, NH	AF163587	AF163616
A. mellea	USA (east)	Durham, NH	AF163588	AF163617
A. mellea	USA (east)	Boston, MA	AF163589	AF163614
A. mellea	USA (east)	Provincetown, MA	AF163590	AF163615
A. mellea	Asia	South Korea	AF163591	AF163611
A. mellea	Asia	South Korea	AF163592	AF163612
A. mellea	Asia	South Korea	AF163593	AF163613
A. mellea	Asia	Japan	AF163594	AF163610
A. novae-zelandiae	Australia	Australia	AF329923	–
A. novae-zelandiae	Australia	Mt Wellington, Tasmania	AF454739	–
A. novae-zelandiae	New Zealand	South Island, New Zealand	AF329925	–
A. novae-zelandiae	New Zealand	North Island, New Zealand	AF239926	–
A. novae-zelandiae	South America	Grand Isla de Chiloe, Chile	AF448417	–
A. novae-zelandiae	South America	Grand Isla de Chiloe, Chile	AF448418	–

CMW7184. The IGS-1 amplicon sizes for these isolates were approximately 1200 bp.

Phylogenetic analyses

Armillaria *ITS data matrix and neighbour-joining tree*

The *Armillaria* ITS data matrix included 52 taxa with 1183 character sites after alignment with inclusion of gaps. Eighty-eight missing and ambiguously aligned characters as well as 656 gaps were excluded prior to the analysis. The final analysis included 439 characters.

The NJ-tree (Fig. 1) generated from the *Armillaria* ITS dataset grouped the African isolates into a strongly supported (100% bootstrap) cluster. The isolates incorporated in this African cluster were further separated into two strongly supported groups. Lengths for branches separating these two major groups were longer or nearly equal to those separating other *Armillaria* species in the NJ-tree.

African ITS data matrix

This dataset included 880 characters after alignment by inserting gaps. The absolute sequence lengths for the African taxa was 625–630 bp, and for *Armillaria hinnulea* 851–853 bp. Ten indel regions equalling 241 characters were replaced with multistate characters. Prior to cladistic and distance analysis 94 missing and ambiguously aligned characters were excluded. After exclusion of parsimony-uninformative and constant characters, 129 characters were included in the parsimony analysis. Distance analysis was based on 515 characters after exclusion of gapped regions and multi-state characters.

African IGS-1 data matrix

This dataset included 1259 characters after alignment with inclusion of gaps. The absolute sequence lengths of the IGS-1 sequences for the isolates included in this analysis ranged between 851 and 1133 bp. We replaced

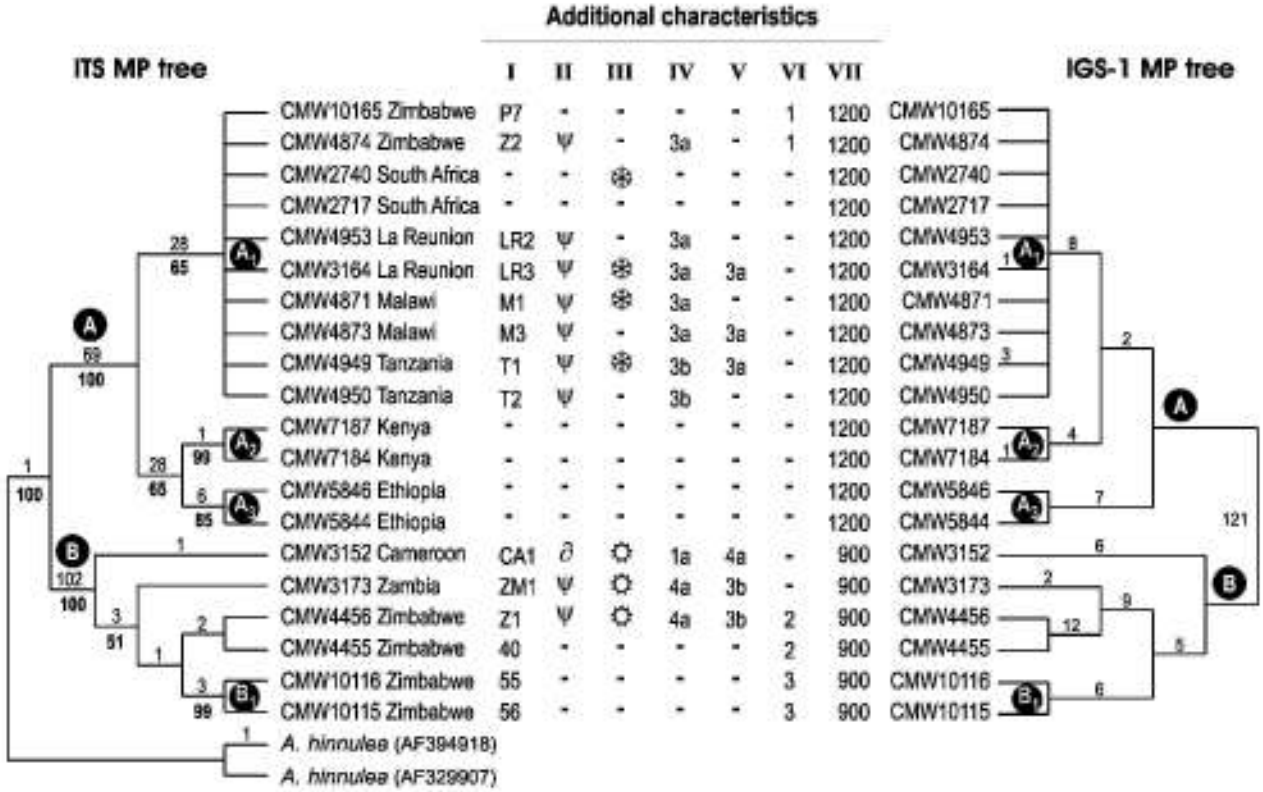

Fig. 2. Cladograms generated from the African ITS and IGS-1 data matrixes. ITS MP tree: One of seven most parsimonious (MP) trees obtained from the ITS dataset, tree length = 161 steps, CI = 0.959, and RI = 0.983. IGS-1 MP tree: one of two MP trees obtained from the IGS-1 data matrix, tree length = 187 steps, CI = 0.936 and RI = 0.983. Bootstrap values are given below and branch lengths above the tree branches. Additional characteristics: I, isolate numbers from previous publications; II, sexual system, Ψ = homothallic and ∂ = heterothallic (Mohammed *et al.* 1989, Abomo-Ndongo *et al.* 1997); III temperature sensitivity, ❄ = mesothilic (optimum growth temperature 24–26 °C) and ☼ = thermophilic (optimum growth temperature 28–30 °) (Guillaumin 1992); IV, grouping of African isolates based on protein, esterase and RAPD analysis as well as Southern hybridization (Mohammed 1992); V, grouping of African isolates according to their physiological, morphological and sexual systems (Guillaumin 1992); VI, grouping of Zimbabwean isolates based on their morphological and biochemical characteristics (Mwenje & Ride 1996); and VII, IGS-1 amplicon sizes in bp. (Coetzee *et al.* 2000a, Mwenje *et al.* 2003, and this study).

46 indel regions of 549 characters by multi-state characters, yielding a total of 756 characters available for analysis. We excluded 81 missing and ambiguously aligned regions before cladistic and distance analysis. Parsimony analysis was based on 156 parsimony-informative characters after exclusion of 20 parsimony-uninformative and 580 constant characters. Distance analysis included 675 characters after exclusion of gaps and multi-state characters.

African cladograms and neighbour-joining trees

Heuristic searches on the African ITS data matrix yielded seven equally most parsimonious (MP) trees with similar topology. Two MP trees with similar topologies were generated from the African IGS-1 data matrix after a heuristic search. The overall topologies of the MP trees (Fig. 2) obtained from these two datasets were congruent. The general topology of the NJ trees (Fig. 3) generated from the African ITS and IGS-1 data matrixes were similar and reflected those of the MP trees.

Parsimony and NJ trees generated from ITS sequence data separated the African isolates into two highly supported clades (labelled A and B), both with 100% bootstrap support (bs). Isolates in Clade A clustered in three sub-groups (A_1, A_2 and A_3). Clade A_1 included isolates from Zimbabwe, South Africa, La Reunion, Malawi and Tanzania (MP: 65% bs, NJ: 91% bs). Isolates in group A_2 originated from Kenya (MP: 99% bs, NJ: 89% bs). Clade A_3 included isolates from Ethiopia (MP: 85% bs, NJ: 99% bs). Clade B included isolates from Cameroon, Zambia and Zimbabwe. Two isolates from Zimbabwe grouped in a strongly supported B_1 sub-clade (MP: 99% bs, NJ: 94% bs).

Relative rate heterogeneity test

Treating individual isolates as representing independent lineages yielded *P* values ranging between 0.115583 and 1. The relative rate test thus indicated that substitution rate heterogeneity in the ITS regions among isolates included in this study is not statistically

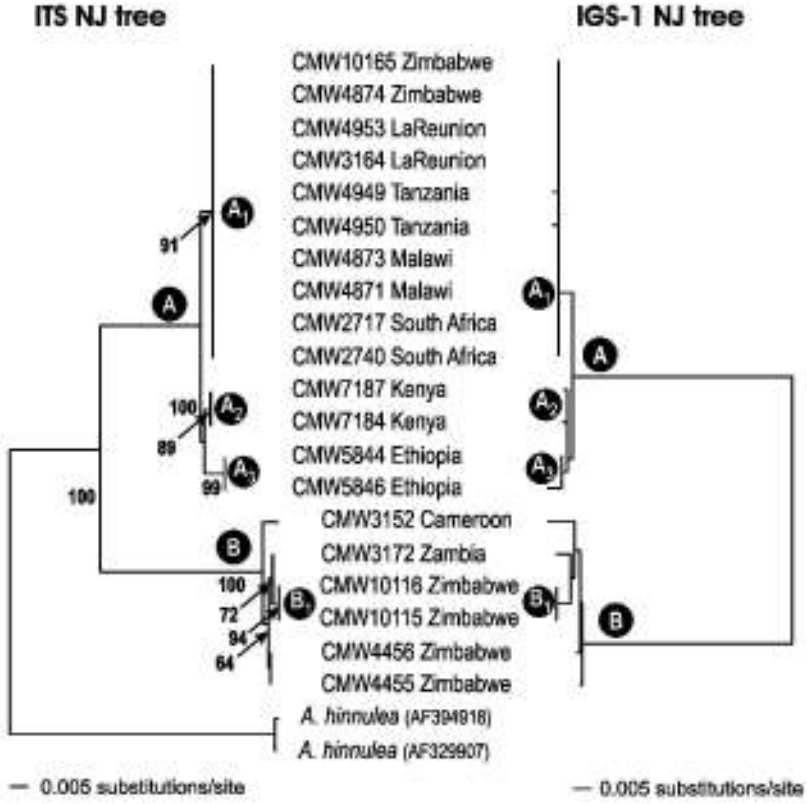

Fig. 3. Neighbour-joining trees generated from the African ITS and IGS-1 data matrixes. Bootstrap values are given below the tree branches. Bar = nucleotide substitutions per site.

significant. The ITS region within the African taxa therefore evolved at a constant rate.

Intraspecific nucleotide diversity and evolutionary distance comparisons

Nucleotide diversity (π) in the ITS sequence data sets was the highest for the African taxa (0.0368 ± 0.0056) followed by *Armillaria mellea* (0.0321 ± 0.0042), *A. novae-zelandiae* (0.0193 ± 0.0035), and *A. luteobubalina* (0.0089 ± 0.0021) (Fig. 4a). The overall nucleotide diversity in the IGS-1 datasets was the highest for the African taxa (0.0638 ± 0.0067), followed by *A. mellea* (0.0537 ± 0.0064) (Fig. 4b). Evolutionary distances between the two African groups were the highest in both ITS and IGS-1 sequence analysis (Fig. 4a–b). The evolutionary distance between the two African groups A and B, based on ITS data, was 1.8–2.4 times greater than between the groups in *A. mellea*, 4.2–8.8 times greater than between the groups in *A. novae-zelandiae*, and 11.3 times greater than the groups in *A. luteobubalina*. The distance between the two African groups based on IGS-1 sequence data was 1.5–2.9 times greater than between groups in *A. mellea*.

DISCUSSION

An overall objective of this study was to test two opposing views regarding the taxonomy of *Armillaria fuscipes*, which we have previously as a synonym of *A. heimii*, from Africa. Using interspecific somatic incompatibility tests, isolates have been shown to represent a single somatic compatibility group, and thus the same biological species (Mohammed *et al.* 1989, 1994, Abomo-Ndongo & Guillaumin 1997). In contrast, phylogenetic studies have suggested that *A. fuscipes* includes more than one species (Coetzee *et al.* 2000a). The present study provides additional evidence supporting the view that isolates treated as *A. fuscipes* represent at least two species.

Isolates representing *A. fuscipes*, also referred to as *A. heimii* or somatic incompatibility group II (Mohammed *et al.* 1989, 1994, Abomo-Ndongo & Guillaumin 1997), all have an inverted 5S gene. The IGS-1 region of seven isolates was amplified in this study using primer 5S2B that binds to the complimentary 5′ position of the inverted 5S gene (Coetzee *et al.* 2000a). Successful amplification with this primer thus indicates that the 5S gene is inverted for these isolates. Inversion of the 5S gene has previously been reported for other isolates included in this study (Coetzee *et al.* 2000a, Mwenje *et al.* 2003). This study, together with those on other *Armillaria* spp. (Anderson & Stasovski 1992, Terashima *et al.* 1998, Coetzee *et al.* 2001, 2003), indicates that this phenomenon is restricted to *A. fuscipes s. lat.* Prior to our own studies, inversion of the 5S gene had previously been reported only for *Coprinus comatus* (Cassidy & Pukkila 1987) and is, therefore, highly unusual in basidiomycetes.

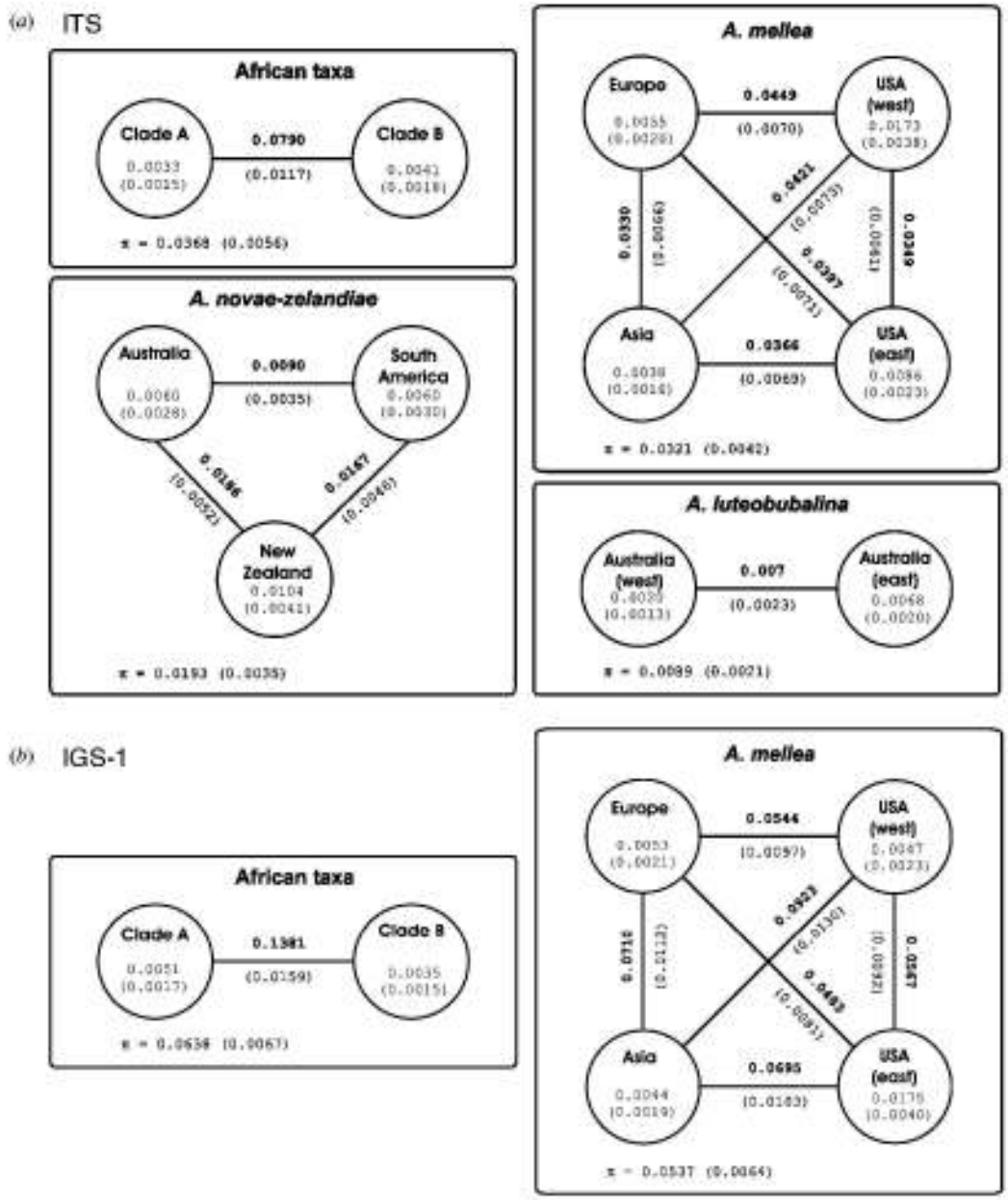

Fig. 4. Nucleotide diversity (π) within global populations and mean evolutionary distances between and within groups based on ITS (*a*) and IGS-1 (*b*) sequence data. Values within circles are the intragroup distance and those above the lines connecting the groups, the intergroup distances. Standard deviations are in brackets.

The NJ-tree generated from the ITS data matrix grouped the African taxa in a strongly supported cluster separated from other *Armillaria* species included in the study. The length of the branch connecting the African isolates with the remainder of the taxa was exceptionally long in comparison with other branches in the NJ-tree. Nucleic substitution rate homogeneity among lineages was not tested for taxa in the *Armillaria* ITS data set; consequently it is uncertain if all lineages presented in the NJ-tree evolved at the same evolutionary rate. However, interpretation of the NJ-tree suggests strongly that the African group has undergone a very long period of independent evolution.

Cladograms and NJ-trees generated from the ITS datasets separated the African isolates into two highly supported sister groups. Some biogeographic structure was observed with isolates from Kenya and Ethiopia grouping in two different subgroups within one of the sister groups. A third subgroup within the other sister group represented two isolates from Zimbabwe that are distinct from the other Zimbabwean isolates in terms of their cultural, molecular and biochemical characteristics (Mwenje & Ride 1996, Mwenje *et al.* 2003). Cladograms and NJ-trees generated from the IGS-1 data set yielded topologies similar to those from the ITS data sets. Because of the absence of an outgroup, it was, however, not possible to gain statistical support for these results.

Isolates from Cameroon, La Reunion, Malawi, Tanzania, Zambia, and Zimbabwe, that have been

treated as *A. heimii* (Mohammed *et al.* 1989, 1994, Abomo-Ndongo & Guillaumin 1997), and considered here to be *A. fuscipes*, could be delineated in two phylogenetic groups. The question emerging from our results is, therefore, whether these two African lineages represent infraspecific taxa or distinct species displaying considerable biological similarity. In an attempt to address this question, we regarded the African isolates as a single taxon and investigated the genetic variation within it. We considered the evolutionary distances within and between these two phylogenetic groups, which are sympatric, and compared them with those determined in this study for allopatric groups in *A. luteobubalina*, *A. mellea* and *A. novae-zelandiae*. Analyses of the ITS and IGS-1 sequence data revealed variation within the sympatric African taxa that was nearly equivalent to or higher than that within the heterothallic allopatric global populations for the other *Armillaria* species included.

Earlier studies have shown that African isolates included in this study are homothallic (Mohammed *et al.* 1989, Abomo-Ndongo *et al.* 1997), with exception of the isolate from Cameroon that is heterothallic (Mohammed *et al.* 1989, Abomo-Ndongo *et al.* 1997). The sexual system is not known for isolates from South Africa, Kenya, Ethiopia, and some Zimbabwean ones. Homothallic (self-fertilising) species are expected to maintain low intraspecific genetic variation and this reduces the amount of variation with every new generation. In contrast, heterothallic species display higher levels of genetic variation as a result of gene flow and subsequent recombination between individuals. Thus, the overall intraspecific sequence diversity would be lower for homothallic than for heterothallic species. The fact that isolates in the African group, most of which are known to be homothallic, display higher nucleotide diversity than the heterothallic populations to which they were compared, suggests that this group does not represent a single species.

The distances between the two major African lineages emerging from the ITS and IGS-1 sequence data were 1.8–8.8 times greater than between the allopatric groups in *A. luteobubalina*, *A. mellea and A. novae-zelandiae* included in this study. Furthermore, the distances within the two African groups were generally lower than those within the sympatric groups of *A. mellea*, *A. luteobubalina* and *A. novae-zelandiae*. Variation in the tandem arrays, especially within the ITS and IGS region, are usually observed between species, whilst they are relatively conserved among individuals of the same species (Hillis & Dixon 1991). Concerted evolution occurs through the processes of unequal crossing over and (or) gene conversion (Dover 1982, Arnheim 1983). Because of concerted evolution, mutations occurring within the rDNA spacer and gene regions are homogenised throughout the tandem array. They consequently become fixed in populations characterised by unrestricted gene flow, thereby maintaining low intraspecific variation (Hillis & Dixon 1991). If the isolates from the two African groups represent the same species, with unrestricted gene flow between the groups, it would be reasonable to expect that mutations within the ITS and IGS-1 regions would be homogenised. The results would be low intergroup variation. Hence, the evolutionary distances between the two African groups should be shorter than the distance between allopatric populations belonging to the other *Armillaria* spp. However, the results indicate that the ITS and IGS-1 sequences are conserved within, but highly variable between, the two African groups. Mutations that occurred in the ITS and IGS-1 regions of isolates from one group did not become fixed in isolates from the other group. Our data, therefore, suggests a lack of gene flow and subsequent genetic recombination between isolates from the respective groups in their natural environments, despite their being somatically compatible *in vitro*.

The observed phylogenetic partition and apparent lack of genetic recombination in ITS and IGS-1 loci for the African isolates might be attributed to their homothallic nature. If this is the case, the two major phylogenetic lineages could represent clonal lineages within a species. Neighbour-joining trees generated from the *Armillaria* ITS data matrix showed, however, that branches connecting the two main groups are longer than or equivalent in length to branches separating species from Australasia, South America, and the Northern Hemisphere. Relative rate heterogeneity tests indicated that the two lineages evolved independently, and at the same rate, from their common ancestor. These results, together with the geographic distribution of the African taxa and the sister relationships of the two main groups, indicate that sympatric speciation has occurred and that the two lineages represent two closely related species.

Recognition of the two main phylogenetic lineages emerging from this study as discrete species led to a decision to search previous publications for diagnostic characters linked to isolates used in this study. Characters identified during this search included: sexual systems (Mohammed *et al.* 1989, Abomo-Ndongo *et al.* 1997); temperature sensitivity (Guillaumin 1992), groupings according to protein, esterase and RAPD analysis as well as Southern hybridization (Mohammed 1992); groupings based on physiology and morphology (Guillaumin 1992); grouping of isolates from Zimbabwe using morphological and biochemical characteristics (Mwenje & Ride 1996); and IGS-1 amplicon sizes (Coetzee *et al.* 2000a, Mwenje *et al.* 2003, and this study) (Fig. 2). Amplicon size differences and temperature sensitivity were found to be the most diagnostic characters for recognizing the two species. Isolates within the two major phylogenetic lineages can be distinguished by amplicon sizes of approximately 1200 bp. and 900 bp, respectively. Although temperature sensitivity has been reported for only a few isolates, there is a strong indication that isolates belonging to the one phylogenetic lineage (Clade A) are

mesothilic, whereas those in the other lineage (Clade B) are thermophillic.

Phylogenetic trees generated from ITS and IGS-1 sequence data in this study clearly separated the isolates into two major lineages. If the biological species concept is adopted, isolates within these lineages would represent the same species. Comparisons in terms of basidiome morphology would probably lead to the same conclusion. Applying the morphological species concept, but taking into account biological characteristics other than basidiome morphology, however, suggests that they represent different species. This possibility is confirmed when the phylogenetic species concept is employed. One of these two lineages (Clade A) represents the widely distributed *A. fuscipes*, which has also been referred to as *A. heimii* in some previous studies (Mohammed & Guillaumin 1993, Otieno *et al.* 2003, Pérez-Sierra *et al.* 2004). Isolates in the second phylogenetic lineage (Clade B) represent an as yet unnamed species.

ACKNOWLEDGEMENTS

We thank the members of the Tree Protection Co-operative Programme (TPCP), the Mellon Foundation, the National Research Foundation (NRF) and the THRIP initiative of the Department of Trade and Industry (DTI) South Africa for funding. We are grateful to Caroline Mohammed and Tom C. Harrington who generously provided cultures. We also thank Jan Perold for valuable suggestions and assistance in preparing the manuscript.

REFERENCES

Abomo-Ndongo, S. & Guillaumin, J.-J. (1997) Somatic incompatibility among African *Armillaria* isolates. *European Journal of Forest Pathology* **27**: 201–206.

Abomo-Ndongo, S., Mohammed, C. & Guillaumin, J.-J. (1997) Sexual behaviour of *Armillaria heimii* and *A. mellea* isolates from Africa. *European Journal of Forest Pathology* **27**: 207–224.

Agustian, A., Mohammed, C., Guillaumin, J.-J. & Botton, B. (1994) Discrimination of some African *Armillaria* species by isozyme electrophoretic analysis. *New Phytologist* **128**: 135–143.

Anderson, J. B. & Stasovski, E. (1992) Molecular phylogeny of Northern Hemisphere species of *Armillaria*. *Mycologia* **84**: 505–516.

Arnheim, N. (1983) Concerted evolution of multigene families. In *Evolution of Genes and Proteins* (M. Nei & R. K. Koehn, eds): 38–61. Sinauer Associates, Boston, MA.

Baum, D. A. & Shaw, K. L. (1995) Genealogical perspectives on the species problem. In *Experimental and Molecular Approaches to Plant Biosystematics* (P. C. Hoch & A. G. Stephenson, eds): 289–303. Missouri Botanical Gardens, St Louis, MO.

Cassidy, J. R. & Pukkila, P. J. (1987) Inversion of 5S ribosomal RNA genes within the genus *Coprinus*. *Current Genetics* **12**: 33–36.

Chandra, A. & Watling, R. (1981) Studies in Indian *Armillaria* (Fries per Fries) Staude (*Basidiomycotina*). *Kavaka* **10**: 63–84.

Chillali, M., Idder-Ighili, H., Agustian, A., Guillaumin, J.-J., Mohammed, C. & Botton, B. (1997) Species delimitation in the African *Armillaria* complex by analysis of the ribosomal DNA spacers. *Journal of General and Applied Microbiology* **43**: 23–29.

Coetzee, M. P. A., Wingfield, B. D., Coutinho, T. A. & Wingfield, M. J. (2000a) Identification of the causal agent of Armillaria root rot of *Pinus* species in South Africa. *Mycologia* **92**: 777–785.

Coetzee, M. P. A., Wingfield, B. D., Harrington, T. C., Dalevi, D., Coutinho, T. A. & Wingfield, M. J. (2000b) Geographical diversity of *Armillaria mellea* s. s. based on phylogenetic analysis. *Mycologia* **92**: 105–113.

Coetzee, M. P. A., Wingfield, B. D., Bloomer, P., Ridley, G. S., Kile, G. A. & Wingfield, M. J. (2001) Phylogenetic relationships of Australian and New Zealand *Armillaria* species. *Mycologia* **93**: 887–896.

Coetzee, M. P. A., Wingfield, B. D., Bloomer, P., Ridley, G. S. & Wingfield, M. J. (2003) Molecular identification and phylogeny of *Armillaria* isolates from South America and Indo-Malaysia. *Mycologia* **95**: 285–293.

Dover, G. (1982) Molecular drive: a cohesive mode of species evolution. *Nature* **299**: 111–116.

Felsenstein, J. (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**: 783–791.

Gezahgne, A., Coetzee, M. P. A., Wingfield, B. D., Wingfield, M. J. & Roux, J. (2004) Identification of the Armillaria root rot pathogen in Ethiopian plantations. *Forest Pathology* **34**: 133–145.

Gregory, S. C., Rishbeth, J. & Shaw, C. G. (1991) Pathogenicity and virulence. In *Armillaria Root Disease* (C. G. Shaw & G. A. Kile, eds): 76–87. [USDA Agricultural Handbook No. 691.] USDA, Washington DC.

Guillaumin, J.-J. (1992) Investigation of *Armillaria* species from western, eastern and central Africa. In *Investigation of* Armillaria *Species in Western, Eastern and Central Africa. Proceedings of the Workshop 3–7th February 1992 held at the University of Zimbabwe, Harare, Zimbabwe* (C. Mohammed, ed.): 20–57.

Guillaumin, J.-J., Mohammed, C. & Abomo-Ndongo, S. (1994) Vegetative incompatibility and sexual systems of *Armillaria* isolates from tropical Africa. In *Proceedings of the Eighth International Conference on Root and Butt Rots* (M. Johansson & J. Stenlid, eds): 349–354. Swedish University of Agricultural Sciences, Uppsala.

Hall, T. A. (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**: 95–98.

Hillis, D. M. & Dixon, M. T. (1991) Ribosomal DNA: molecular evolution and phylogenetic inference. *Quarterly Review of Biology* **66**: 411–453.

Hood, I. A., Redfern, D. B. & Kile, G. A. (1991) *Armillaria* in planted hosts. In *Armillaria Root Disease* (C. G. Shaw & G. A. Kile, eds): 122–149. [USDA Agricultural Handbook No. 691.] USDA, Washington DC.

Hsiau, P. T.-W. (1996) *The taxonomy and phylogeny of the mycangial fungi from Dendroctonus brevicomis and D. frontalis (Coleoptera: Scolytidae)*. PhD thesis, Iowa State University, Ames.

Kile, G. A. (1983) Identification of genotypes and the clonal development of *Armillaria luteobubalina* Watling and Kile in eycalypt forests. *Australian Journal of Botany* **31**: 657–671.

Kile, G. A. & Watling, R. (1988) Identification and occurrence of Australian *Armillaria* species, including *A. pallidula* sp. nov. and comparative studies between them and non-Australian tropical and Indian *Armillaria*. *Transactions of the British Mycological Society* **91**: 305–315.

Kile, G. A., McDonald, G. I. & Byler, J. W. (1991) Ecology and disease in natural forests. In *Armillaria Root Disease* (C. G. Shaw & G. A. Kile, eds): 102–121. [USDA Agricultural Handbook No. 691.] USDA, Washington DC.

Kimura, M. (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* **16**: 111–120.

Korhonen, K. (1978) Interfertility and clonal size in the *Armillariella mellea* complex. *Karstenia* **18**: 31–42.

Kumar, S., Tamura, K., Jakobsen, I. B. & Nei, M. (2001) MEGA2: molecular evolutionary genetics analysis software. *Bioinformatics* **17**: 1244–1245.

Mallett, K. I. & Hiratsuka, Y. (1986) Nature of the 'black line' produced between different biological species of the *Armillaria mellea* complex. *Canadian Journal of Botany* **64**: 2588–2590.

Mallett, K. I., Hopkin, A. A. & Blenis, P. V. (1989) Vegetative incompatibility in diploid isolates of *Armillaria* North American biological species I and V. *Canadian Journal of Botany* **67**: 3083–3089.

Mayr, E. (1942). *Systematics and the Origin of Species*. Columbia University Press, New York.

Mohammed, C. (1992) Biochemical and molecular taxonomy of African *Armillaria*. In *Investigation of Armillaria Species in Western, Eastern and Central Africa. Proceedings of the Workshop 3–7th February 1992 held at the University of Zimbabwe, Harare, Zimbabwe* (C. Mohammed, ed.): 68–79.

Mohammed, C. (1994) The detection and species identification of African *Armillaria*. In *Modern Assays for Plant Pathogenic Fungi: identification, detection and quantification* (A. Schots, F. M. Dewey & R. P. Oliver, eds): 141–147. CAB International, Wallingford.

Mohammed, C. & Guillaumin, J.-J. (1993) *Armillaria* in tropical Africa. In *Aspects of Tropical Mycology* (S. Isaac, J. C. Frankland, R. Watling & A. J. S. Whalley, eds): 207–217. Cambridge University Press, Cambridge, UK.

Mohammed, C., Guillaumin, J.-J. & Berthelay, S. (1989) Preliminary investigations about the taxonomy and genetics of African *Armillaria* species. In *Proceedings of the Seventh International Conference on Root and Butt Rots* (D. J. Morrison, ed.): 447–457. Forestry Canada, Victoria, BC.

Mohammed, C., Guillaumin, J.-J., Botton, B. & Intini, M. (1994) Species of *Armillaria* in tropical Africa. In *Proceedings of the Eighth International Conference on Root and Butt Rots* (M. Johansson & J. Stenlid, eds): 402–410. Swedish University of Agricultural Sciences, Uppsala.

Mwenje, E. & Ride, J. P. (1993) Characterisation of Zimbabwean *Armillaria* by morphological features and isozyme analysis of cell wall degrading enzymes. In *Proceedings of the Eighth International Conference on Root and Butt Rots* (M. Johansson & J. Stenlid, eds): 393–401. Swedish University of Agricultural Sciences, Uppsala.

Mwenje, E. & Ride, J. P. (1996) Morphological and biochemical characterization of *Armillaria* isolates from Zimbabwe. *Plant Pathology* **45**: 1036–1051.

Mwenje, E. & Ride, J. P. (1997) The use of pectic enzymes in the characterization of *Armillaria* isolates from Africa. *Plant Pathology* **46**: 341–354.

Mwenje, E., Wingfield, B. D., Coetzee, M. P. A. & Wingfield, M. J. (2003) Molecular characterisation of *Armillaria* species from Zimbabwe. *Mycological Research* **107**: 291–296.

Nei, M. (1987). *Molecular Evolutionary Genetics*. Columbia University Press, New York.

Otieno, W., Pérez Sierra, A. & Termorshuizen, A. (2003) Characterization of *Armillaria* isolates from tea (*Camellia sinensis*) in Kenya. *Mycologia* **95**: 160–175.

Pérez-Sierra, A., Guillaumin, J.-J., Spooner, B. M. & Bridge, P. D. (2004) Characterization of *Armillaria heimii* from Africa. *Plant Pathology* **53**: 220–230.

Pegler, D. N. (1986) *Agaric Flora of Sri Lanka* [*Kew Bulletin Additional Series* No. 12.] HMSO, London.

Petch, T. (1909) New Ceylon fungi. *Annals of the Royal Botanic Garden, Peradeniya* **4**: 299–307.

Rayner, A. D. M. (1991) The challenge of the individualistic mycelium. *Mycologia* **83**: 48–71.

Regan, C. T. (1926) Organic evolution. *Report on the British Association for Advancement of Science* **1925**: 75–86.

Rishbeth, J. (1982) Species of *Armillaria* in southern England. *Plant Pathology* **31**: 9–17.

Rizzo, D. M. & Harrington T. C. (1993) Delineation and biology of clones of *Armillaria ostoyae*, *A. gemina* and *A. calvescens*. *Mycologia* **85**: 164–174.

Robinson, M., Gouy, M., Gautier, C. & Mouchiroud, D. (1998) Sensitivity of the relative-rate test to taxonomic sampling. *Molecular Biology and Evolution* **15**: 1091–1098.

Robinson-Rechavi, M. & Huchon, D. (2000) RRTree: relative-rate tests between groups of sequences on a phylogenetic tree. *Bioinformatics* **16**: 296–297.

Saitou, N. & Nei, M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4**: 406–425.

Swift, M. J. (1972) The ecology of *Armillaria mellea* in the indigenous and exotic woodlands of Rhodesia. *Forestry Oxford* **45**: 67–86.

Swofford, D. L. (1998) *PAUP*: phylogenetic analysis using parsimony (*and other methods)*. Version 4. Sinauer Associates, Sunderland, MA.

Terashima, K., Cha, J. Y., Yajima, T., Igarashi, T. & Miura, K. (1998) Phylogenetic analysis of Japanese *Armillaria* based on the intergenic spacer (IGS) sequences of their ribosomal DNA. *European Journal of Forest Pathology* **28**: 11–19.

Termorshuizen, A. J. (2001) Ecology and epidemiology of *Armillaria*. In *Armillaria Root Rot: biology and control of honey fungus* (R. T. V. Fox, ed.): 45–63. Intercept, Andover, UK.

Taylor, J. W., Jacobson, D. J., Kroken, S., Kasuga, T., Geiser, D. M., Hibbett, D. S. & Fisher, M. C. (2000) Phylogenetic species recognition and species concepts in fungi. *Fungal Genetics and Biology* **31**: 21–32.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **24**: 4876–4882.

Watling, R. (1992) *Armillaria* Staude in the Cameroon Republic. *Persoonia* **14**: 483–491.

White, T. J., Bruns, T., Lee, S. & Taylor, J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols: a guide to methods and applications* (M. A. Innis, D. H. Gelfand, J. J. Sninsky & T. J. White, eds): 315–322. Academic Press, San Diego.

Corresponding Editor: J. W. G. Cairney