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Identification of the causal agent of Armillaria root rot in South African forest plantations

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SUMMARY

Armillaria root rot has been known to occur on economically important *Pinus* and *Eucalyptus* spp. grown in plantations in South Africa, since the early 1900's. *Armillaria* spp. have been well studied in North America and Europe, but have received minimal attention in South Africa. Most reports of Armillaria root rot in South Africa have suggested that *A. mellea* (Vahl.:Fr.) P. Kumm. is the causal agent. The name *A. heimii* Pegler has also been used in more recent reports. The taxonomic disposition of *Armilliaria* in South Africa, therefore, remains unknown. The aim of this study was to identify and characterise *Armillaria* isolates from forest plantations in South Africa. Isolates were collected from infected trees in different forestry regions, using established isolation techniques. The intergenic spacer region (IGS), between the large subunit (LSU) and 5S gene of the ribosomal DNA (rDNA) operon, was amplified for all isolates using the primers P-1 and 5S-2B. The polymerase chain reaction (PCR) products were digested with the restriction endonuclease *Alu*I. Fragments resulting from the digests were separated on agarose gels to detect restriction fragment length polymorphisms (RFLPs). The IGS region for the selected isolates was also sequenced using a ABI PRISM™ 377 DNA sequencer. RFLP profiles and sequence data revealed that the isolates originating from the plantations in South Africa are distinct from *A. mellea* and *A. heimii*. We believe that they either represent an undescribed species of *Armillaria*, or possibly *A. fuscipes*.

INTRODUCTION

Armillaria root rot has been reported to occur in South Africa's forestry plantations since the early 1900's (Bottomley, 1936). The disease has been ascribed to the species *A. mellea* (Vahl.) Fries. In recent years, the name *A. heimii* Pegler, a species commonly occurring in

Africa, has been used (Wingfield and Knox-Davies, 1980). Uncertainty regarding the identity of the *Armillaria* species responsible for root rot in South African arose, after it was shown that the RFLP profiles of the IGS region between the South African isolates thought to represent *A. heimii,* differed (Coetzee *et al.*, 1997).

The taxonomy of the genus *Armillaria* has been thoroughly studied in the northern hemisphere (Anderson and Ulrich, 1979; Anderson, *et al.*, 1980; Roll-Hansen, 1985; Guillaumin, *et al.*, 1993). Biological species determination as well as morphological and molecular studies have shown that *Armillaria* comprises of at least 36 different species. Many of these species were previously aggregated in *A. mellea sensu lato* (Volk and Burdsall, 1995). However, much remains to be done regarding the taxonomic status of the African *Armillaria* species.

Armillaria root rot in the temperate regions of Africa has arbitrarily been attributed to *A. mellea* (Mohammed, 1994). This situation, however, has been partially resolved as the result of a number of investigations of the occurrence and identification of species of *Armillaria* in African countries. Studies on *Armillaria* in Cameroon, Congo, Gabon, Kenya, the Ivory Coast, Malawi, Tanzania, Zambia and Zimbabwe (Agustian *et al.*, 1994; Guillaumin *et al.*, 1994; Mohammed *et al.*, 1994; Mwangi *et al.*, 1989; 1994; Mwenje and Ride, 1993) have shown that *A. fuscipes* Petch, *A. heimii* Pegler, *A. mellea* and *A. mellea* var. *camerunensis* Henn. occur on commercially grown forest species, fruit trees and on many indigenous forest tree species.

The identification of African *Armillaria* species can be problematic due to the absence of rhizomorphs in soil in temperate regions of this continent (Swift, 1968), as well as the scarcity of the fruiting bodies during most of the year. However, it has been shown that molecular and biochemical techniques can be used as an alternative to morphology, in the identification of *Armillaria* spp. Differences in isozyme profilès (Morrison *et al.*, 1985), Restriction Fragment Length Polymorphisms (RFLP's) of different types of DNA (Smith and Anderson, 1989; Anderson *et al.*, 1989; Harrington and Wingfield, 1995) and IGS DNA sequence data (Anderson and Stasovski, 1992) have been successfully used to identify *Armillaria* spp. from the northern hemisphere.

No extensive molecular studies have been reported using *Armillaria* isolates from Africa. Isoenzyme analysis is the only molecular technique which has been used as an alternative to morphological and biological studies, in delineating African *Armillaria* spp. This method is, however, time consuming and large quantities of fungal material are needed to obtain reliable results (Bonde *et al.*, 1993). This is in contrast to the rapid and reliable technique developed by Harrington and Wingfield (1995) for the identification of northern hemisphere *Armillaria* spp. using PCR, RFLP's.

Harrington and Wingfield (1995) amplified the IGS region of representative *Armillaria* isolates from North America and Europe. The resulting PCR fragments were then digested with restriction endonucleases. One or more restriction fragment patterns could be obtained with the restriction endonuclease *Alu*I. Such RFLP profiles differed between species and could be used in species delineation. It was thus apparent that a single technique such as PCR RFLP could be useful in identifying isolates of *Armillaria* from Africa.

The objective of this study was two fold. Firstly, we aimed to identify and characterise the *Armillaria* isolates from forest plantations in South Africa. Secondly, we were interested in determining the relationship between species present in South Africa and *Armillaria* spp. from other parts of the Africa. To accomplish these objectives, the IGS region of the rRNA operon was amplified, subjected to RFLP analysis and sequenced. Also, morphological comparisons of the isolates at our disposal were undertaken.

MATERIALS AND METHODS

Fungal isolation and cultivation

Infected tissue was surface sterilised and pieces of the white mycelial fans found between the bark and the cambium were removed. The mycelia were placed on a selective medium (Harrington *et al.*, 1992) and incubated at 22 °C for two weeks in the dark. Tips from the rhizomorphs produced in culture were transferred to MYA (2% Biolab malt extract, 0,2% Biolab yeast extract and 1,5 % Biolab agar) plates and incubated at 22 °C for two weeks in the dark. Isolates were examined and grouped according to their culture morphology. All isolates used (Table 1) are maintained in the culture collection of the Tree Pathology Co-operative Programme, University of the Free State, Bloemfontein, South Africa.

DNA extraction

Isolates were cultivated in MY (2 % malt extract and 0,3 % yeast extract) liquid medium at 22 °C in the dark for two weeks. Mycelium was harvested by centrifugation (14000 rpm, 20 min) and freeze dried. DNA was extracted according to the fungal DNA extraction method of Raeder and Broda (1985). DNA quantification was done by UV spectroscopy (Maniatis *et al.*, 1982).

Table 1. List of isolates used in this study.

I.D.	Culture number	Other isolate numbers	Host	Origin	Collected by
Armillaria sp.	CMW 2717	A01-SA	*Pinus elliottii*	Sabie, South Africa	Wingfield, M.J.
Armillaria sp.	CMW 2740	B07-SA	*P. patula*	Entabeni, South Africa	Wingfield, M.J.
Armillaria sp.	CMW 3762	C05-SA	*P. patula*	Sabie, South Africa	Coetzee, M.P.A.
Armillaria sp.	CMW 3950	E01-SA	*Litchi chinensis*	Tzaneen, South Africa	Coetzee, M.P.A.
Armillaria sp.	CMW 3955	F01-Zim	*Acacia xanthophloea*	Harare, Zimbabwe	Wingfield, M.J. & Coetzee, M.P.A.
Armillaria sp.	CMW 3952	F04-Zim	unknown	Harare, Zimbabwe	Wingfield, M.J. & Coetzee, M.P.A.
A. heimii	CMW 3167	B934	*P. elliottii*	Sabie, South Africa	Ivory, M.
A. heimii	CMW 3164	B933	*Pelargonium asperum*	Saint-Denis, La Réunion	Fabregue, C.
A. heimii	CMW 3173	B932	*Tectona grandis*	Dola Hill, Zambia	Ivory, M.
A. heimii	CMW 3152	B935	-	Western Province, Cameroon	Watling, R.

Amplification of IGS (intergenic spacer region)

PCR (Saiki *et al.*, 1988) fragments of the intergenic spacer region (IGS) were generated with Primers P-1 and 5S-2B (Table 2). The PCR reaction mix consisted of 200 mM of each dNTP, 2.65 mM $MgCl_2$, Expand HF buffer with $MgCl_2$ supplied with the enzyme, 0.375 mM of each primer, 2.6 U Expand™ High Fidelity PCR System enzyme mix (Boehringer Mannheim, South Africa) and approximately 80 ng of the isolated DNA. The final reaction volume was 100 µl. The primer annealing temperature was 56 °C. The PCR products were separated on a 0.8% (wt/v) agarose (Promega, Madison, Wisconsin) gel stained with ethidium bromide and visualised under UV illumination.

Table 2. List of primers used in this study

Primer	Sequence
P-1	5' TTG CAG ACG ACT TGA ATG G 3'
5S-2B	5' CAC CGC ATC CCG TCT GAT CTG CG 3'
MCP-2	5' GGT ATG GAT CCA AGC GTA TTG 3'
MCP-2R	5' CAA TAC GCT TGG ATC CAT ACC 3'
MCP-2A	5'GAC TTGTAC TTG GAC TTG 3'
MCP-2AR	5'CAA GTC CAA GTA CAA GTC 3'
MCP-3	5' TGG ATT GCG GAC TTG GAC AGA 3'
MCP-3R	5' TCT GTC CAA GTC CGC AAT CCA 3'
5S-3MC	5' CAA TTC TGC CAA CAA GTC CC 3'
5S-3MCR	5' GGG ACT TGT TGG CAG AAT TG 3'
5S-5MC	5'ACT TGG GCA TTG AGG GCT TG 3'
5S-5MCR	5'CAA GCC CTC AAT GCC CAA GT 3'

Restriction enzyme digestion

Amplified IGS PCR fragments were digested with the restriction endonuclease *Alu*I (Harrington and Wingfield, 1995). The resulting fragments were electrophoresed on a 3% (wt/v) agarose (Promega, Madison, Wisconsin) gel stained with ethidium bromide and visualised with UV light.

Sequencing and DNA sequence data analysis

Both manual and autosequencing were used to obtain the DNA sequence of the IGS region of the *Armillaria* isolates from different geographical regions (Table 1). Both strands of the DNA were sequenced with the use of appropriate primers (Table 2). Primers other than P-1 and 5S-2B were derived as the sequence data became available. A Sequenase PCR Product Sequencing Kit with Sequenase Version 2.0 DNA Polymerase (Amersham Life Science, Cleavland, Ohio) was used for manual sequencing. Auto sequencing reactions were carried out with the use of an ABI PRIMS™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase, FS (Perkin Elmer, Warrington, U.K.) according to the protocols of the manufacturer. Sequence data were manually aligned and phylogenetic analysis was done using Phylogenetic Analysis Using Parsimony (PAUP) (Swofford, 1985).

RESULTS

Isolations, cultivation and morphological characteristics

The use of established techniques led to the successful isolation of *Armillaria* isolates from numerous infection sites in South Africa. Examination of the culture morphology of these isolates revealed whitish, flat mycelia in the centre of the inoculum. Cylindrical, brown

rhizomorphs were abundantly produced in culture (Fig. 1). In general the rhizomorphs branched monopodialy although a dichotomous branching type was sometimes observed (Fig. 1).

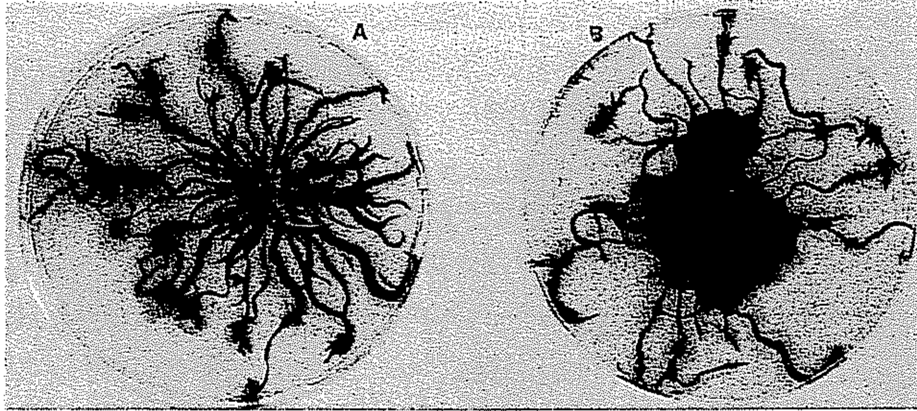

Figure 1. An *Armillaria* isolate from *P. elliotii* in a South African forestry plantation, cultivated on MYA for four weeks. (A) culture from above; (B) underside of the plate.

Figure 2. Basidiocarps of an *Armillaria* sp. found in Frankfort pine plantation, Sabie, South Africa.

Basidiomes were collected in April 1997 at Frankfort forestry plantation, Sabie (altitude 700 - 1000 m). The morphology of the basidiomes was as follows : *Pileus* up to 51 mm (average 35 mm) in diam., convex later broadly plane, centre broadly umbonate sometimes depressed encircling zone, yellow brown in centre, becomes pale brown to shades of light cream at margin, brown squamules, margin incurved, straite. *Lamellae* white, decurrent, close. *Stipe* 64 - 87 mm x 2 - 4.5 mm, central, cylindrical, solid, blackish brown, becomes light towards the ring, dark cream just above the ring, becomes light cream towards the apex, greyish

flocci from base to ring, brownish white inner tissue. *Ring* close to apex, membranous sacchate, circumsessile, whitish. No rhizomorphs were observed on roots or in the soil associated with dying trees.

DNA Amplification

The intergenic spacer region (IGS) between the 3' end of the large subunit (LSU) ribosomal RNA (rRNA) gene and the 5' end of the 5S gene for the *Armillaria* isolates used in this study was amplified using PCR. Primers CL12R (Veldman *et al.*, 1981) and O-1 (Duchesne and Anderson, 1990), as suggested by Harrington and Wingfield (1995), were originally used. These primers, however, led to poor amplification and often no amplification was obtained. The use of primers P-1 and 5S-2B generated single DNA amplification products for the respective isolates.

Two different fragment sizes were obtained for the different isolates. PCR fragments of approximately 1200 bp (base pairs) in size were obtained for isolates as well as for basidiospores of *Armillaria* from South Africa (Fig. 3). Isolates from Zambia (CMW 3173), Cameroon (CMW 3152) and from Zimbabwe (CMW 3955 & CMW 3952) had DNA fragment sizes of approximately 900 pb in size (Fig. 3).

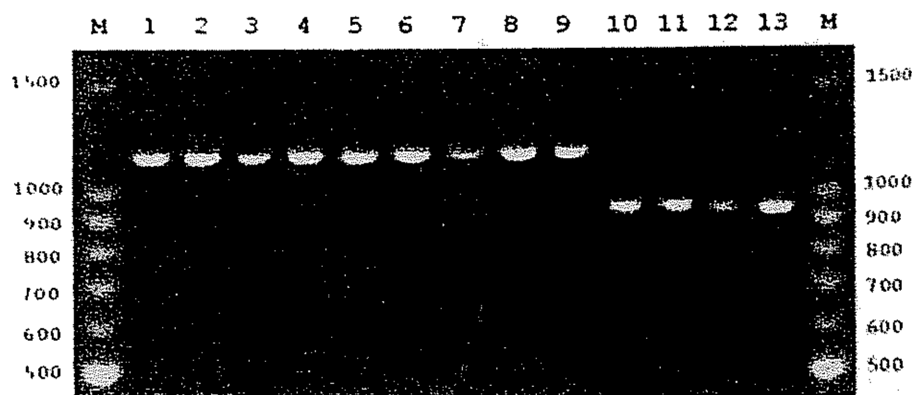

Figure 3. A 1.5 % ethidium bromide stained agarose gel. A 100 bp high molecular marker standard was loaded in lanes labelled M. IGS PCR fragments amplified with primers P-1 and 5S-2B are seen in lanes 1 to 13. Lanes 1 to 5 represents the PCR products of the South African isolates CMW 2717, CMW 2740, CMW 3762, CMW 3950, CMW 3167. Lanes 6 and 7 fragments from basidiospores from two different basidiocarps. Lane 8 from basidiocarp tissue. Lane 9 CMW 3164 from La Réunion. Lanes 10 and 11 CMW 3955 and CMW 3952 from Zimbabwe. Lane 12 CMW 3173 from Zambia. Lane 13 CMW 3152 from Cameroon.

Restriction Fragment Length Polymorphisms

Restriction digestion with the endonuclease *Alu*I resulted in DNA fragments of different sizes. These fragments were scored against a 100 bp molecular marker to determine the length of each of the fragments (Fig. 4). Fragments smaller than 100 bp were not determined because of the difficulty in visualising these bands under UV light.

All the South African isolates used in this study had similar RFLP fragments of 365, 245 and 135 bp in size respectively. Isolates obtained from Zimbabwe had RFLP profiles with fragment sizes of 530, 220 and 175 bp respectively. Determination of the RFLP profiles of the isolates identified as *A. heimii*, however, revealed differences in the DNA fragment sizes between the respective isolates. The RFLP profile of the *A. heimii* isolate originating from La Réunion (B933) was similar to that of the South African *Armillaria* isolates. *A. heimii* from Zambia (B932) and the Cameroon (B935) had the same RFLP profiles with DNA fragments of 520, 220 and 175 bp in size.

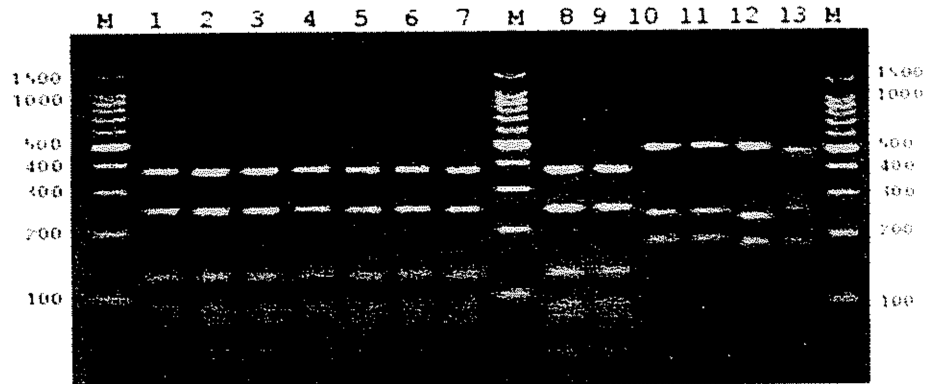

Figure 4. A 3 % agarose gel stained with ethidium bromide showing *AluI* restriction fragments for isolates of *Armillaria* spp. Lanes 1 - 8 show profiles of the South African collections : CMW 2717, CMW 2740, CMW 7362, CMW 3950, CMW 3167, basidiospores from a basidiocarp, basidiospores from a different basidiocarp and basidiocarp tissue respectively. Lane 9 CMW 3164 from La Réunion. Lanes 10 and 11 CMW 3955 and CMW 3952 from Zimbabwe. Lane 12 CMW 3173 from Zambia. Lane 13 CMW 3152 from Cameroon.

DNA sequence analysis

Isolates used in this study grouped in two major clades (Fig. 5) when subjected to PAUP analysis. *Armillaria* isolates from South Africa and La Réunion clustered together but were separated from *A. heimii* isolates originating in Zimbabwe, Zambia and Cameroon. Isolates of *A. heimii* from Zimbabwe and Zambia were more closely related to each other than to the isolate from Cameroon.

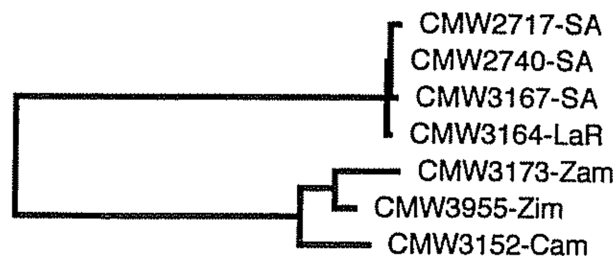

Figure 5. Most parsimonious phylogenetic tree generated by the branch and bound option of PAUP 3.1.1. for IGS DNA sequence data for representative isolates of *Armillaria* used in this study.

DISCUSSION

Armillaria root rot has been known as a problem in plantations since the earliest days of South African forestry (Bottomley, 1936). Despite this fact, almost no attention has been afforded to the identity of the causal agent of the disease. We believe that this is primarily due to the fact that sporocarps are extremely rare and, in some cases it has been stated that they do no exist (Wingfield and Knox-Davies, 1980). Thus, the disease attributed to *Armillaria* is based on typical symptoms of Armillaria root rot (Lundquist, 1987). The absence of rhizomorphs in the field is also well recognised and thought to be related to environmental conditions such as soil composition, temperature and moisture (Bottomley, 1937; Lückhoff, 1964; Swift, 1968; Rishbeth, 1978). In this study we were fortunate to be able to collect basidiocarps of the *Armillaria* sp. responsible for root rot and to consider its morphology in some detail. These observations have convinced us that the name *A. heimii*, that has been used for the fungus in recent literature (Ivory, 1987), is not appropriate.

Isolates of *Armillaria* from forest plantations in South Africa appear to represent a single taxon. We base this observation on the similarity of culture morphologies and also on molecular comparisons that we have made in this study. Given the fact that at least three taxa are thought to exist in nearby countries such as Zimbabwe (Mwenje and Ride, 1996) this might be strange. It is possible that other species occur within the boundaries of South Africa, although these are almost certainly not found in forest plantations. It is our intention to survey remnant native forest, particularly in areas such as the southern Cape for *Armillaria* spp. in the near future.

Macro-morphological features of the basidiocarps collected in the Frankfort forestry plantation, showed similarities with that of *A. heimii* as described by Pegler (1977). It was, however, notable that the stipes and caps were much larger than those described for *A. heimii* basidiomes. Basidiocarps of *A. heimii* are small with a pileus of up to 3 cm diam (Pegler, 1977), whereas the pileus of the basidiocarps collected in Frankfort were up to 5 cm diam. Stipe lengths differed also considerably with stipe lengths of 6.4 to 8.7 cm for the basidiocarps in South Africa and 2.5 to 4.5 cm for *A. heimii*. More striking, was the prominent blackish colour and the presence of the greyish flocci on the stipe of the basidiocarps collected in South Africa. This is in contrast to the darker ochraceous colour described for *A. heimii*. These morphological differences suggest that the basidiocarps collected in South Africa are not representative of *A. heimii*.

Both Anderson and Stasovski (1992) and Harrington and Wingfield (1995) amplified the IGS with the use of primer set CL12R and O-1. Primer CL12R binds on the 3'end of the LSU and O-1 on the 5'end of the 5S gene. Amplifying the IGS region of the South African isolates of *Armillaria* and cultures thought to represent *A. heimii* was impossible, using these primers.

The fact that the 5S gene is highly conserved (Hori *et al.*, 1977) and that primer O-1 is specific for Basidiomycetes (Anderson and Stasovski, 1992), made these results difficult to interpret. This phenomenon was, however, also observed in amplifying the IGS region of *Heterobasidion* spp. in which the 5S gene is inverted (T.C. Harrington, personal communication). Successful amplification of the IGS region of the isolates used in this study was only possible using primer 5S-2B (T.C. Harrington, personal communication). This primer binds to the 3'end of the 5S gene. Both PCR and later DNA sequencing results confirmed that the 5S gene in *A. heimii* and the South African isolates of *Armillaria* is in a opposite orientation, relative to the other genes in the rRNA operon.

Similar RFLP profiles from the amplified IGS region were observed for the South African isolates of *Armillaria,* indicating that these isolates belong to the same species. Because Zimbabwe is geographically close to parts of South Africa where our isolates were collected, it was suspected that the Zimbabwean *Armillaria* would be the same as that from South Africa. RFLP profiles of the Zimbabwean isolates were the same as each other but differed from those of the South African isolates. Isolates from Zimbabwe (Harare), therefore, belong to the same species but are different to those in South African forest plantations. This finding is consistent with that of Mwenje and Ride (1996) where only one group (Group III) was found to be present in Harare. It is clear that a more representative collection of Zimbabwean *Armillaria* isolates will be needed to determine the relationship between *Armillaria* spp. in forestry plantations in South Africa and those present in Zimbabwe.

Unexpectedly, the RFLP profiles of the isolates representing *A. heimii* were dissimilar from the *Armillaria* isolates originating from South Africa. Thus, isolates thought to represent *A. heimii* from Zambia and Cameroon had the same RFLP profiles, indicating that these isolates are of the same species. This was in contrast to the RFLP pattern of the La Réunion *A. heimii* isolate which differed from the other *A. heimii* isolates. This indicates that the *A. heimii* from La Réunion is a different species. The RFLP patterns of the Zimbabwean *Armillaria* were the same as those of *A. heimii* from Zambia and Cameroon. Similar RFLP profiles were observed for the South African *Armillaria* isolates and the *A. heimii* isolate from La Réunion but, these differed from the RFLP profiles of *A. heimii* from Cameroon and Zambia. Based on these results we believe that *Armillaria* isolates from South African forestry plantations and the isolate from La Réunion belong to a species other than *A. heimii.*

The composition of the tree generated from DNA sequence data for the LSU, 5S gene and the whole of the IGS confirmed RFLP results. The representative isolates grouped into two major clades, separated from each other with a long branch. The first clade included all of the South African isolates as well as the *A. heimii* isolate from La Réunion. *Armillaria* isolates from South African forestry plantations are, therefore, of the same species and probably also the

same as the isolate identified as *A. heimii* from La Réunion. The correct identification of the La Réunion isolate must now be resolved as it is clearly not the same as isolates of *A. heimii* from Zambia and Cameroon.

The second clade in the phylogenetic tree included *A. heimii* isolates from Zambia and Cameroon as well as the *Armillaria* isolate from Zimbabwe. As might have been expected, the *Armillaria* isolate from Zimbabwe showed closer phylogenetic relationship to *A. heimii* isolates from Zambia than to the isolate from Cameroon. Heim (1963) reported *A. heimii* to occur in Cameroon in his description of *Clitocybe* (Armillariella) *elegans*. We expect that the *A. heimii* isolates from Cameroon and Zambia used in this study probably represents this species. We are, however, presently attempting to amplify the IGS region from herbarium material of the holotype of *A. heimii* from Madagascar, which should resolve this issue conclusively.

In this study, we have shown that the *Armillaria* sp. common in pine plantations of South Africa probably does not represent *A. heimii*. The correct identity of the fungus must still be resolved. In our view, it either represents an undescribed species or *A. fuscipes* which is a species that has been thought to be a synonymy of *A. heimii* (Kile and Watling, 1988), although this is not commonly agreed upon (Watling *et al.*, 1991; Volk and Burdsall, 1995). We are currently studying authentic material of *A. fuscipes*, and hope to resolve this question in the near future.

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