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Identification of the causal agent of Armillaria root rot of *Pinus* species in South Africa

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Abstract: Armillaria root rot was reported on economically important *Pinus* and *Eucalyptus* species grown in plantations in South Africa since the early 1900s. *Armillaria* species 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 suggest that *A. mellea* is the causal agent. The name *A. heimii* has also been used in more recent reports, although these have not been based on mycological studies. The taxonomic disposition of *Armillaria* in South Africa, therefore, remains unknown. The aim of this study was to identify and characterize *Armillaria* isolates from forest plantations in South Africa based on morphology, PCR-RFLP profiles and sequence data. Analysis of these characters revealed that the isolates originating from the plantations in South Africa are distinct from *A. mellea* and *A. heimii*. We believe that they represent *A. fuscipes*.

Key Words: *Armillaria fuscipes*, PCR-RFLP, IGS-1, phylogeny, rRNA

INTRODUCTION

Armillaria root rot has been known in South African forestry plantations since the early 1900s (Pole Evans 1933, Kotzé 1935, Bottomley 1937). This disease was originally ascribed to the species *A. mellea* (Vahl : Fr.) P. Kumm. (Lundquist 1987). However, the name *A. heimii* Pegler, a species commonly occurring in Africa, later replaced *A. mellea* (Ivory 1987). Uncertainty regarding the identity of the *Armillaria* species responsible for root rot in South Africa arose in a preliminary study (Coetzee et al 1997). In that study, it was shown that the RFLP (restriction fragment length polymorphism) profiles of the first intergenic spacer region (IGS-1) of the rRNA operon, differed between the South African isolates and apparently authentic isolates of *A. heimii*.

The taxonomy, biology and phylogeny of *Armillaria* has been thoroughly studied in the Northern Hemisphere (Anderson and Ulrich 1979, Anderson et al 1980, Roll-Hansen 1985, Guillaumin et al 1993b). Biological species determination as well as morphological and molecular studies have shown that *Armillaria* includes at least 36 different species (Volk and Burdsall 1995). Many of the species have previously been aggregated in *A. mellea* s. l.

In the temperate regions of Africa, Armillaria root rot 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 *Armillaria* species in African countries. Studies on *Armillaria* in Cameroon, Congo, Gabon, Kenya, the Ivory Coast, Malawi, Tanzania, Zambia and Zimbabwe (Augustain et al 1994, Guillaumin et al 1993a, Mohammed et al 1993, Mwangi et al 1989, 1993, Mwenje and Ride 1993, 1996) have suggested that *A. heimii* Pegler, *A. mellea* s. s., *A. mellea* (Vahl : Fr.) P.Kumm. subsp. *africana* Mohammed et al (invalidly published) and *A. mellea* (Vahl : Fr.) P.Kumm. var. *camerunensis* Henn. [= *A. camersunensis* (Henn.)] are present. These occur on commercially grown forest species, fruit trees and on many indigenous forest tree species.

Identification of African *Armillaria* species has been difficult due to the absence of rhizomorphs (Swift 1968), as well as the scarcity of the fruiting bodies during most of the year. Molecular and biochemical techniques have been shown to be useful as an alternative to morphology in the identification of *Armillaria* species. Differences in isozyme profiles (Morrison et al 1985), RFLPs of different types of DNA (Smith and Anderson 1989, Anderson et al

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1989, Harrington and Wingfield 1995) and IGS-1 DNA sequence data (Anderson and Stasovski 1992) have been successfully used to identify *Armillaria* species from the northern hemisphere. No DNA-based studies have been conducted using *Armillaria* isolates from Africa, other than the preliminary report of Coetzee et al (1998).

Isoenzyme analysis is the only molecular technique that has been used as an alternative to morphological and biological studies in delineating African *Armillaria* species. 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* species using PCR-RFLPs.

In the study of Harrington and Wingfield (1995), the IGS-1 region of representative *Armillaria* isolates from North America and Europe was amplified. Resulting IGS-1 amplicons were then digested with restriction endonucleases. Unique RFLP profiles were obtained for each species using the restriction endonuclease *AluI*. Such RFLP profiles were used in the delineation of taxa. It was thus apparent that a single technique such as PCR-RFLP might be useful to identify isolates of *Armillaria* from Africa.

The objective of this study was twofold. Our first aim was to identify and characterize the *Armillaria* isolates from forest plantations in South Africa. Secondly, we were interested in determining the relationship between species present in South Africa and those species from other parts of Africa. To accomplish these objectives, the IGS-1 region of a collection of isolates available to us was amplified, subjected to RFLP analysis and sequenced. Also, descriptions of the basidiocarps and culture morphology of the isolates were made and compared with those of type specimens of *A. fuscipes* and *A. heimii*.

MATERIALS AND METHODS

Fungal isolation and cultivation.—Isolates from South Africa were collected from infection centers in pine plantations in Mpumalanga, the Northern Province as well as Kwa-Zulu Natal (TABLE I, FIG. 1). *Armillaria* isolates from other countries in Africa were supplied by TC Harrington (TABLE I). Infected tissue was surface sterilized and small pieces (2 mm^2) from the white mycelial fans between the bark and the cambium were removed from infected tissue. The mycelial pieces were placed on a selective medium (Harrington et al 1992) and incubated at 22 C for 2 wk in the dark. Tips of the rhizomorphs produced in the primary cultures 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 2 wk in the dark. All cultures used

TABLE I. Isolates used in this study

Identity[a]	Culture number[b]	Other isolate numbers[c]	Host	Origin	Collector	GenBank
Armillaria sp.	CMW 2717	A01-SA	*Pinus elliottii*	Sabie, South Africa	Wingfield, M.J.	AF204821
Armillaria sp.	CMW 2740	B07-SA	*P. patula*	Entabeni, South Africa	Wingfield, M.J.	AF204822
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.	AF204827
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.	AF204823
A. heimii	CMW 3164	B933	*Pelargonium asperum*	Saint-Denis, La Reunion	Fabregue, C.	AF204824
A. heimii	CMW 3173	B932	*Tectona grandis*	Dola Hill, Zambia	Ivory, M.	AF204825
A. heimii	CMW 3152	B935	Unknown	Western Province, Cameroon	Watling, R.	AF204826

[a] Where species names are given, these are the names supplied by the culture collections noted.
[b] CMW refers to the culture collection of the Tree Pathology Co-operative Programme (TPCP).
[c] B refers to the culture collection of T.C. Harrington, Iowa State University, Ames, Iowa.

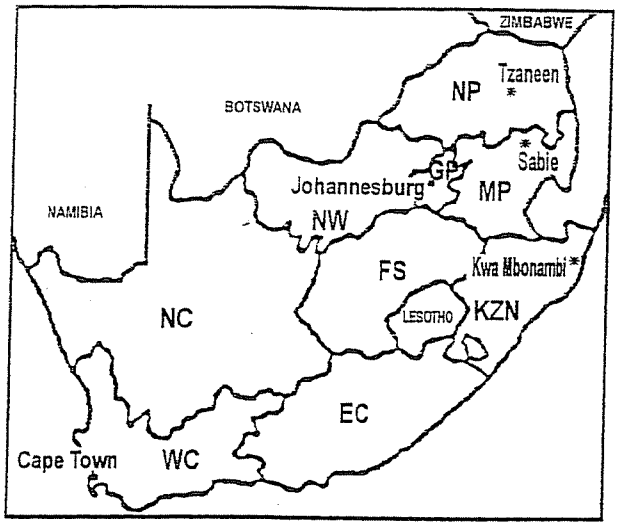


FIG. 1. Map of South Africa showing the relative location of the *Armillaria* isolates collected from different pine plantations. EC = Eastern Cape, FS = Free State, GP = Gauteng, KZN = Kwa-Zulu Natal, MP = Mpumalanga, NC = Northern Cape, NP = Northern Province, NW = Northern Cape, WC = Western Cape.

(TABLE I) are maintained in the culture collection of the Tree Pathology Co-operative Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI, University of Pretoria, Pretoria, South Africa).

Basidiocarp morphology.—The macromorphology of the basidiocarps obtained in Frankfort forestry plantation, South Africa, was examined and compared with the descriptions of *A. fuscipes* (Chandra and Watling 1981) and *A. heimii* (Pegler 1977). Hand sections of the dried material were made with a surgical scalpel for microscopic examination. The sections were mounted in 2% KOH and 1% phloxine or in Melzer's solution and examined using a light microscope (Zeiss Axioskop, Carl Zeiss, Germany). The sizes of the basidiospores, basidia and cheilocystidia were determined using a calibrated eyepiece micrometer. Color descriptions and codes are based on those of Rayner (1970).

Dried specimens were prepared for SEM (scanning electron microscopy) by fixing 5 mm^2 pieces in glutaraldehyde (3%) and OsO_4 (0.5%). This was followed by dehydration of the specimens in a graded acetone series and critical point drying using a critical point drier (BIO-RAD, Watford, England). The dried material was coated with gold palladium and examined in a scanning electron microscope (JEOL WINSEM, model JSM 6400) at 5 kV.

The type specimens of *A. heimii* and *A. fuscipes* were obtained from the Muséum National D'Histoire Naturelle (P) and the Royal Botanical Gardens (K), respectively. Both specimens were examined, but because of the importance of the material and the limited amount of material available for *A. heimii,* no microscopical examination was undertaken. Detailed descriptions of the macro- and microscopic morphology of both specimens were published by Heim (1963) (as *Clitocybe elegans,* synonym with *A. heimii*), Pegler (1977) and Kile and Watling (1988) for *A. heimii* and by Petch (1909) and Chandra and Watling (1981) for *A. fuscipes.* Comparisons of the macro- and micromorphological features of these two species with those of the basidiocarps obtained in South Africa were, therefore, based on descriptions in the literature.

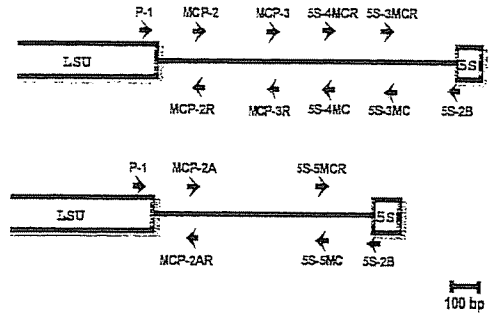


FIG. 2. Position of primers used to determine the DNA sequence of the IGS-1 on the rRNA operon.

DNA amplification and PCR-RFLPs.—Isolates were cultured in liquid MY (2% malt extract and 0.3% yeast extract) medium in the dark for approx 2 wk at 22 C. Mycelium was harvested by centrifugation (15 300 *g,* 20 min) and freeze dried. DNA from the mycelia was extracted according to the method published by Coetzee et al (2000). DNA quantification was done by UV spectroscopy using a Beckman Du Series 7500 Spectrophotometer.

PCR was used to amplify the first intergenic spacer region (IGS-1) 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. Primers CL12R (Veldman et al 1981) and O-1 (Duchesne and Anderson 1990), as suggested by Harrington and Wingfield (1995), were initially used. It was, however, not possible to obtain any amplification using these primers. Primers P-1 (Hsiau 1996) and 5S–2B (5′ CAC CGC ATC CCG TCT GAT CTG CG 3′) (FIG. 2) were, therefore, used since they resulted in good amplification for *Heterobasidion* species, which has been shown to have an inverted 5S gene (TC Harrington pers comm). The PCR reaction mix and reaction conditions were the same as those published by Coetzee et al (2000). The PCR products were visualized under UV illumination after electrophoresis on an agarose (Promega, Madison, Wisconsin) gel (0.8% wt/v) stained with ethidium bromide.

IGS-1 amplicons were digested by adding 10 units of the restriction endonuclease *AluI* and 1 µL (10 mg/mL) BSA (acetylated Bovine Serum Albumin) (Promega, Madison, Wisconsin) to 20 µL of the PCR reaction mix without purification. Digestion took place at 37 C for 6 h. The restriction fragments were visualized on a 3% (wt/v) agarose (Promega, Madison, Wisconsin) gel stained with ethidium bromide using UV illumination.

DNA sequencing.—Both strands of the IGS-1 region were sequenced using primers P-1, 5S–2B, MCP-2, MCP-2R, MCP-3, MCP-3R, 5S–3MC, 5S–3MCR, 5S–4MC, 5S–4MCR, MCP-2A, MCP-2AR, 5S–5MC and 5S–5MCR (FIG. 2) (Coetzee et al 1998). Primers other than P-1 and 5S–2B were derived continuously as the sequence data became available. An ABI

PRISM® 377 DNA sequencer was used for autosequencing. Sequencing reactions were carried out using an ABI PRISM® Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase, FS (Perkin Elmer, Warrington, UK) according to the protocol of the manufacturer.

Sequence data were manually aligned by inserting gaps and deposited in TreeBASE (data matrix SN328-949, phylogram SN328). All indels (insertions or deletions) were excluded from the analysis. Analysis of the sequence data was done using PAUP* version 4.0b2 (Swofford 1998). Search methods used to analyse the distance matrix and to generate a phylogram representing the relationship between the isolates was based on the neighbor-joining tree building method. The objective function was set to minimum evolution and the distance measures between the isolates were based on the HKY85 (Hasegawa et al 1985) distance measure model. Maximum likelihood search indicated that the substitution rate for variable sites is infinite and the rate for variable sites was, therefore, set to equal. The phylogram generated was rooted to midpoint given that no appropriate outgroup taxon is available for comparison.

RESULTS

Isolations and morphological characteristics.—Established techniques led to the successful isolation of *Armillaria* from numerous infection sites in South Africa. These isolates had whitish, flat mycelium in the center and cylindrical, brown rhizomorphs were abundantly produced in culture (FIG. 3). In general, the rhizomorphs branched monopodially although a dichotomous branching type was sometimes observed (FIG. 3).

Basidiomes were collected in Apr 1997 at Frankfort forestry plantation, Sabie (FIG. 1) (700–1000 m elev) (FIGS. 4–8). Using the terminology of Largent (1977) and Largent et al (1977) we describe the basidiomes as follow: *Pileus* (FIG. 4) up to 51 mm (average 35 mm) diam, convex later broadly plane, center broadly umbonate sometimes depressed encircling zone, citrine (21k) in center, becomes orange yellow (17b) to shades of light cream at margin, brussels brown (17'i) squamules, margin incurved, striate. *Lamellae* (FIG. 4) white, decurrent, close. *Stipe* (FIG. 4) 64–87 mm × 2–4.5 mm, central, cylindrical, solid, olive gray (23''''), becomes lighter towards the ring, buff (19''d) just above the ring, becomes citrine (21k) towards the apex, pale olive gray (23''''f) flocci from base to ring, brownish white inner tissue. *Ring* (FIG. 4) close to apex, membranous saccate, circumscissile, whitish. *Basidiospores* (FIGS. 5, 7, 8) 7–12 (9) × 4–7 (6) µm, elliptic-ovoid to elliptic-oblong, slightly thickened, smooth to slightly roughened, apiculated, hyaline, non-amyloid, cyanophilous and *Spore print* white in mass. *Basidia* (FIGS. 6, 7) 4 spored, 30–50 (40) × 10–12 (10) µm, clavate, thin walled, hyaline, no clamp connections on the base of the basidia. *Cheilocystidia* 38 × 8 µm, clavate, thin walled, hayline. *Hymenophoral trama* slightly bilateral, thin walled, hyaline. *Subhymenial trama* interwoven. No rhizomorphs were observed on roots or in the soil associated with dying trees.

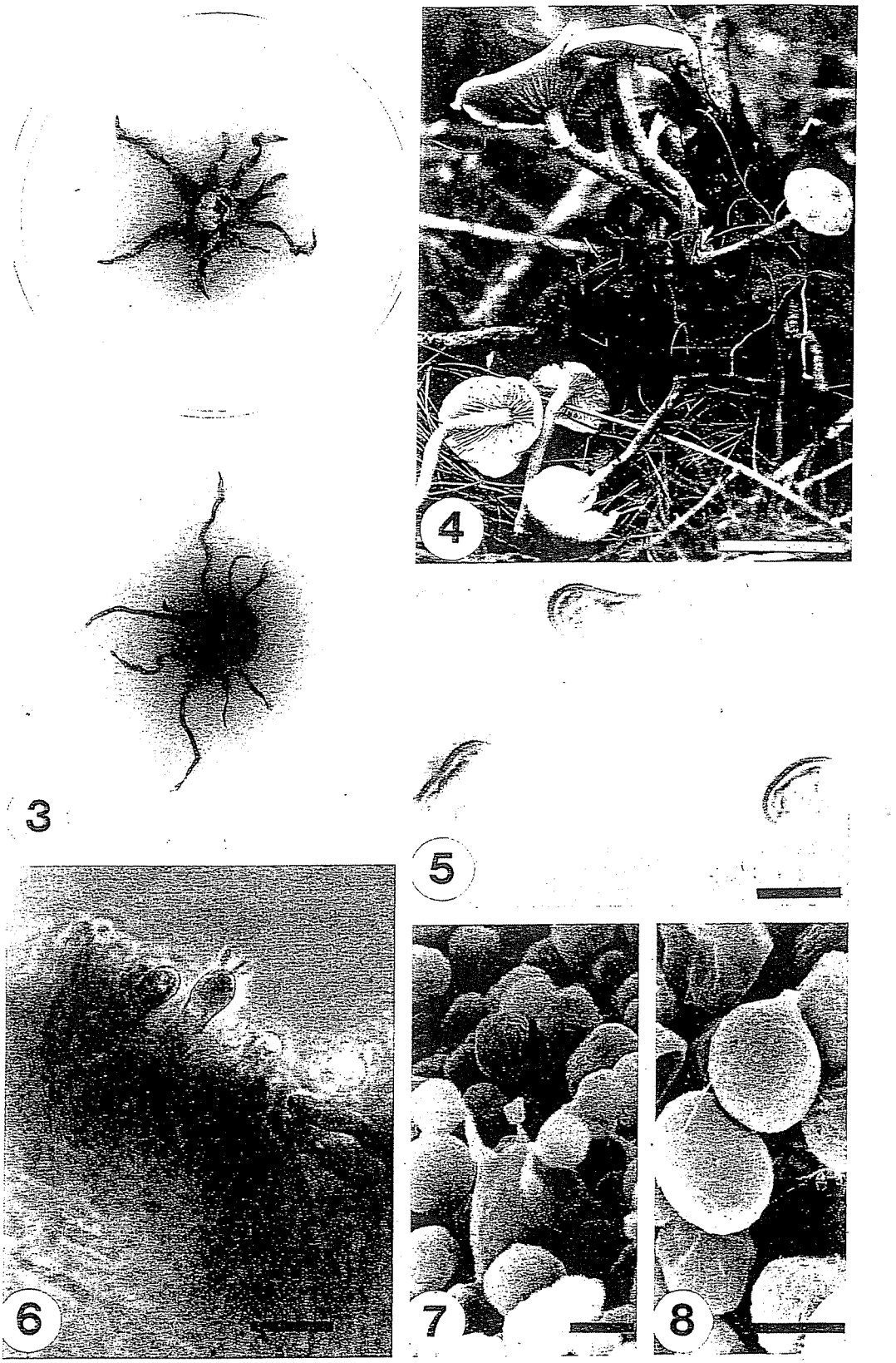
DNA amplification and PCR-RFLPs.—Using the primer set P-1 and 5S–2B, two different fragment sizes were obtained for the different isolates. PCR fragments approx 1200 bp in size were obtained for field isolates as well as for basidiospores from spore prints of *Armillaria* from South Africa. Isolates from Zambia (CMW3173), Cameroon (CMW3152) and from Zimbabwe (CMW3955 and CMW3952) had DNA amplification fragment sizes of approx 900 bp in size.

DNA fragments of different size were obtained after digestion with the restriction endonuclease *Alu*I (FIG. 9). Fragment sizes were scored against a 100-bp ladder to determine the length of the individual fragments. Fragments smaller than 100 bp were not determined because of the difficulty in visualizing these bands.

The South African isolates used in this study had similar RFLP profiles with fragments of approx 365, 245, and 135 bp, respectively. Isolates obtained from Zimbabwe had RFLP profiles with fragment sizes of approx 530, 220, and 175 bp, respectively. Determination of the RFLP profiles of the isolates thought to represent *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 Reunion (CMW3164) was similar to that of the South African *Armillaria* isolates. *Armillaria heimii* from Zambia (CMW3173) and the Cameroon (CMW3152) had the same RFLP profiles with DNA fragments of approx 520, 220, and 175 bp.

DNA sequence analysis.—DNA sequences were aligned by inserting gaps, resulting in the total length of 1241 bp. The absolute length, however, ranged from 866 to 1107 bp. The absolute length of the IGS-1 for the isolate of *A. heimii* from the La Reunion (CMW3164) was 1106 bp which is the same size as that observed for the South African isolates of *Armillaria* (CMW2717, CMW2740 and CMW3167). The remaining isolates from the Cameroon (CMW3152) and Zambia (CMW3173) identified by other researchers as *A. heimii* as well as the isolate of *Armillaria* from Zimbabwe had absolute lengths of 866, 879, and 896 bp, respectively. Indels were excluded from the data matrix leading to 730 characters used in the final analysis.

Isolates used in this study grouped in two major clusters (FIG. 10) when subjected to neigbor-joining



FIGS. 3–8. *Armillaria* sp. found in South Africa. 3. Culture. An *Armillaria* isolate from *P. elliotii* in a forestry plantation, grown 4 wk on MYA, seen from the top and from the bottom of the culture. 4. Basidiocarps obtained in Frankfort pine plantation, Sabie. Bar = 10 cm. 5. Light micrograph of the basidiospores. Bar = 10 μm. 6. Light micrograph of basidia. Bar = 25 μm. 7. SEM of basidia. Bar = 5 μm. 8. SEM of basidiosprores. Bar = 5 μm.

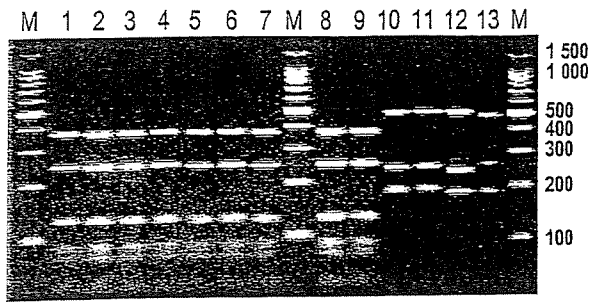


FIG. 9. A 3% agarose gel stained with ethidium bromide showing *AluI* restriction fragments for isolates of *Armillaria* species. Lanes 1–8 show profiles of the South African collections as follows: CMW2717 (1), CMW2740 (2), CMW7362 (3), CMW3950 (4), CMW3167 (5), basidiospores from one basidiocarp (6), basidiospores from a different basidiocarp (7) and basidiocarp tissue (8). Lane 9 shows the RFLP profile of isolates CMW3164 from La Reunion. Lanes 10 and 11 show fragments for isolates CMW3955 and CMW3952 from Zimbabwe. Lane 12 represents CMW3173 from Zambia and lane 13 CMW3152 from Cameroon. Lanes labelled M show a 100 bp ladder (band sizes indicated in base pairs).

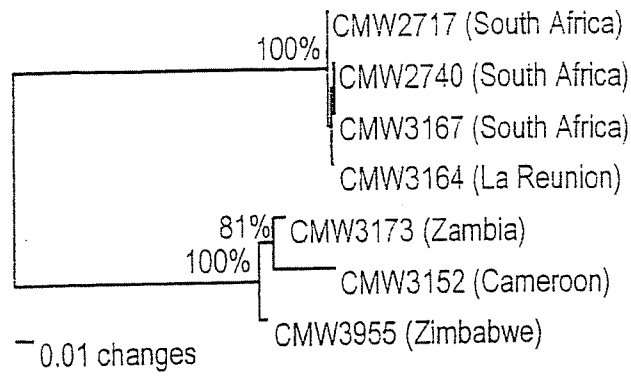


FIG. 10. Phylogram generated from a neighbor-joining search of the IGS-1 sequence data for the isolates used in this study. Scale represents total nucleotide changes. Bootstrap values (1000 replicates) are indicated above the branches.

analysis. *Armillaria* isolates from South Africa and La Reunion grouped together but were separate from *A. heimii* isolates originating in Zimbabwe, Zambia, and Cameroon. Isolates from Zimbabwe and the *A. heimii* isolate from Zambia grouped together and were more closely related to each other than to the *A. heimii* isolate from the Cameroon.

DISCUSSION

Armillaria root rot has been problematic since the earliest days of South African forestry (Bottomley 1937). Despite this, almost no attention has been afforded to identify 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 not exist (Wingfield and Knox-Davies 1980). Thus, the disease attributed to *Armillaria* was based on typical symptoms of Armillaria root rot (Lundquist 1987). Absence of rhizomorphs in the field is also a well recognized phenomena and thought to be related to environmental conditions such as soil composition, temperature and moisture (Bottomley 1937, Lückhoff 1964, Swift 1968, Rishbeth 1978). We were fortunate to be able to collect basidiocarps of the *Armillaria* sp. responsible for root rot on numerous occasions and to consider its morphology in some detail. Based on our observations we are convinced that the name *A. heimii*, used for this fungus in recent literature (Ivory 1987), is not appropriate.

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

The macromorphological features of the basidiocarps collected in the Frankfort forestry plantation showed similarities with that of *A. heimii* and *A. fuscipes*. It was, however, notable that the stipes and caps were much larger than those described for *A. heimii* basidiomes. Basidiocarps of *A. heimii* have a pileus of up to 3 cm diam, whereas the pileus of the basidiocarps collected in Frankfort were up to 5 cm diam and that of *A. fuscipes* up to 6 cm diam. Stipe lengths also differed considerably with stipe lengths of 6.4–8.7 cm noted for the basidiocarps in South Africa and up to 10 cm for *A. fuscipes*, which is in contrast to the shorter, 2.5–4.5-cm stipe length of *A. heimii*. More striking, was the prominent blackish (olive gray) color and the presence of the grayish (pale olive gray) flocci on the stipes of both the basidiocarps collected in South Africa and on the type material of *A. fuscipes*. This is in contrast to the darker ochraceous color described for *A. heimii*.

At the micromorphological level few differences, with the exception of basidiospore and basidium size, have been observed between the basidiocarps collected in South Africa, *A. heimii* and *A. fuscipes*. The

basidiospores and the basidia of the basidiocarps from South Africa were larger than those of *A. heimii* and *A. fuscipes*. These micro- and macromorphological differences suggest that the basidiocarps collected in South Africa are not representative of *A. heimii*.

IGS-1 amplicons were obtained by both Anderson and Stasovski (1992) and Harrington and Wingfield (1995) using primer set CL12R and O-1. Primer CL12R binds to the 3′ end of the LSU and O-1 on the 5′ end of the 5S gene. Amplifying the IGS-1 region of the South African *Armillaria* isolates thought to represent *A. heimii* was impossible using these primers. The highly conserved nature of the 5S gene (Hori et al 1977) and the specificity of primer O-1 for Basidiomycetes (Anderson and Stasovski 1992), made these results difficult to interpret. This phenomenon was also observed in amplifying the IGS-1 region of *Heterobasidion* species in which the 5S gene is inverted (TC Harrington pers comm).

Using primer 5S–2B that binds to the 3′ end of the 5S gene, led to successful amplification of the IGS-1 region. This indicated to us that the 5S gene in *A. heimii* and the South African *Armillaria* isolates must be in an opposite orientation relative to the other genes in the rRNA operon. This observation was later confirmed with sequencing data of the IGS-1 region.

RFLP profiles from the amplified IGS-1 region were the same for the South African *Armillaria* isolates, indicating that these belong to the same species. Zimbabwe is geographically close to the northern parts of South Africa, where our isolates were collected. It was thus expected 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 from 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 would be needed to determine the relationship between *Armillaria* species in forestry plantations in South Africa and those present in Zimbabwe.

Unexpectedly, the RFLP profiles of the isolates, identified by the collectors (TABLE I) based on their basidiocarp morphology as *A. heimii*, were dissimilar to those for the South African *Armillaria* species. Isolates thought to represent *A. heimii* from Zambia and Cameroon had the same RFLP profiles, indicating that these are the same species. This is in contrast to the RFLP pattern of the La Reunion *A. heimii* isolate, which differed from the other *A. heimii* isolates. This indicates that the *A. heimii* from La Reunion is a different species. RFLP profiles of the Zimbabwean *Armillaria* and those of *A. heimii* from Zambia and Cameroon were similar. Similar RFLP profiles were observed for the South African *Armillaria* isolates and the *A. heimii* isolate from La Reunion, 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 Africa and the isolate from La Reunion belong to the same species, but are not *A. heimii*.

The phylogram generated from DNA sequences for the LSU, 5S gene and the whole of the IGS-1 confirmed RFLP results. Representative isolates grouped into two major clusters, separated from each other by a long branch. The first group included all of the South African isolates as well as the isolate from La Reunion. *Armillaria* isolates from South Africa are, therefore, the same species and the same as the isolate that has been called *A. heimii* from La Reunion. The correct identification of the La Reunion isolate must now be resolved as it is clearly not the same as isolates labelled *A. heimii* from Zambia and Cameroon.

The second group in the phylogram included isolates labelled as *A. heimii* from Zambia and Cameroon, as well as the *Armillaria* isolate from Zimbabwe. As might be expected, the *Armillaria* isolates from Zimbabwe showed a closer 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*, which is the same as *A. heimii*. Based on the RFLP profile and sequence data, and the fact that *A. heimii* was originally described from Cameroon, we expect that the *Armillaria* isolates from the Cameroon, Zimbabwe and Zambia used in this study probably represents this species.

The type specimen of *A. heimii* is represented by very limited material and our attempts to obtain a PCR product and DNA sequence data without causing any damage to it were not successful. Given the limited availability of the material, it is unlikely that we will succeed in this quest. It is thus not possible to be certain whether isolates available to us and suggested to be of *A. heimii* actually represents this species. At present, all we can be sure of is that most of these isolates are different to the South African isolates. The exception is the isolate from the La Reunion which we believe is the same as the fungus that occurs throughout forest plantations of South Africa.

We have shown in this study that the *Armillaria* sp. common in pine plantations of South Africa, probably does not represent *A. heimii*. Examination of the type specimens of *A. fuscipes* and *A. heimii*, however,

show that the fungus in South Africa is very similar to *A. fuscipes*. Further study, using molecular techniques, of isolates from other parts of Africa will help to clarify this question. However, collection of isolates linked to sporophores will be needed to resolve the identity of the South African *Armillaria* sp. with absolute certainty.

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