Characterisation of Zimbabwean *Armillaria* using IGS-1 sequences and AFLP analysis

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Armillaria root and butt rot disease is a common problem in peach orchards, tea and pine plantations in the Eastern Highlands of Zimbabwe. The species of *Armillaria* causing this disease have not been fully identified but it is believed that at least three species are involved. These included *A. fuscipes* (previously referred to as RFLP Group I) and two unnamed species known as RFLP Group II and RFLP Group III. The aim of the study was to use PCR-RFLP, sequences of the IGS-1 region of the rDNA operon and AFLP fingerprinting to characterize 27 Zimbabwean *Armillaria* isolates. PCR-RFLP tests showed that the isolates resided in five groups. Analysis of sequence data elucidated four groups, which were also supported by AFLP data. Thirteen isolates belonged to RFLP Group I, which is considered to represent *A. fuscipes*, four isolates were most similar to those previously referred to as Zimbabwean RFLP Group II and two isolates clustered most closely with RFLP Group III. The remaining isolates appear to represent *Armillaria* taxa not previously found in Zimbabwe.

Key words: Africa, basidiomycetes, fingerprinting, RFLP, species concepts.

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Introduction

Armillaria root rot disease is well known in the eastern highlands and northern parts of Zimbabwe (Swift, 1972; Mwenje and Ride, 1996). These areas are characterised by a high altitude that ranges from 1000-2000 m above sea level and the annual rainfall exceeds 1000 mm (http://geographie.unierlang.de/scamimi/podzol/Podzols.htm). Armillaria root rot has been a problem in Zimbabwean plantations for many years with the first studies on the disease dating back to 1972 (Swift, 1972). Since that time, substantial losses due to this disease have been reported in pine plantations and fruit orchards in the eastern highlands of Zimbabwe (Mwenje *et al*., 1998).

The identity of the *Armillaria* spp. causing root rot in Zimbabwe has been the subject of a number of studies (Mwenje and Ride, 1996; Mwenje *et al*., 2003). These have utilized various collections of isolates and have applied numerous techniques. Yet, the identity of these fungi remains to be fully resolved. At the present time, it is believed that at least three taxa occur in the country and these have been referred to as RFLP Groups I-III. Of these three groups, one (RFLP Group I) is believed to represent *A. fuscipes* (Coetzee *et al*. 2000b).

Several techniques have been applied to identify *Armillaria* species in various parts of the world. These include interfertility tests (Hintikka, 1973), DNA-based molecular techniques (Smith and Anderson, 1989; Anderson and Stasovski, 1992; Harrington and Wingfield, 1995; Volk *et al*., 1996; Coetzee *et al*., 2000a,b; Mwenje *et al*., 2003), isozyme and protein analysis (Morrison *et al*., 1985; Wahlström *et al*., 1991; Mwenje and Ride, 1996), immunological assays (Burdsall *et al*., 1990) and morphological characterisation (Watling *et al*., 1982; Bérubé and Dessureault,

1988). Morphological characterisation is easy to perform but its major draw back in Zimbabwe is the rare occurrence of basidiocarps (Swift, 1972) or rhizomorphs (Mwenje and Ride, 1996) in the field. Interfertility tests are time consuming and can give ambiguous results. This technique is also applicable only to heterothallic species and most African isolates are reported to be homothallic (Abomo-Ndongo *et al*., 1997). Isozyme patterns, protein patterns and immunological assays give reproducible results but their disadvantage is that they can be time consuming.

Of the available techniques for the identifycation of *Armillaria* spp, DNA based methods appear to give the most rapid and consistent results. Currently, sequences of the IGS-1 and ITS are most commonly used (Anderson and Stasovski, 1992; Chillali *et al*., 1998; Coetzee *et al*., 2000b). It is, however, important to recognise that the IGS-1 region of the African *Armillaria* species is not phylogenetically comparable with that of the other species as the result of an inversion of the 5S gene (Coetzee *et al*., 2000b). For rapid identification, RFLPs arising from restriction digests of IGS-1 can give relatively accurate and rapid results. This approach has been used for North American species of *Armillaria* (Harrington and Wingfield, 1995). It has also been used in preliminary studies to characterize species of *Armillaria* from Southern Africa including Zimbabwe (Coetzee *et al*., 2000b; Mwenje *et al*., 2003).

For many years, it was assumed that African *Armillaria* species included the two species *A. heimii* and *A. mellea* (Abomo-Ndongo *et al*. 1997). Mwenje and Ride (1996), using isozymes, showed that a collection of Zimbabwean *Armillaria* isolates consisted of three groups. Coetzee *et al*. (2000b) used basidiocarp morphology and DNA sequence comparisons to show that *A. fuscipes* is present in Southern Africa including Zimbabwe and this was later confirmed by Mwenje *et al*. (2003) using RFLP profiles and sequence data for the IGS-1 region of the RNA operon. Using DNA based techniques, both Coetzee *et al*. (2001) and Mwenje *et al*. (2006) showed that *A. mellea* is present in Africa. Both studies, however, concluded that this species is probably not native to the continent but has most likely been introduced a number of times on agronomically important tree crops. The phylogenetic relationships of the African *Armillaria* species in relation to other species in the genus has been well illustrated by both Coetzee *et al*. (2005) and Maphosa *et al*. (2006) using ITS and partial elongation factor 1-alpha DNA sequence data respectively.

Amplified Fragment Length Polymorphism (AFLP) represents a DNA fingerprinting method that is commonly used in population genetic studies. The method described by Vos *et al*. (1995) has thus been used to generate DNA fingerprints for many organisms. The technique provides a powerful tool to study genetic variability and relatedness in many groups of organisms. Pérez-Sierra *et al*. (2004) used the technique to consider the identity of African *Armillaria* isolates that they treated as *A. heimii*. These authors also showed that their isolates could be separated into two distinct groups, one of which they referred to as *A. heimii* and the other as an unknown species. In their study, isolates from West Africa grouped with an isolate from east Africa while southern and eastern Africa isolates grouped together. This was consistent with the findings of Coetzee *et al*. (2000b) who showed that isolates from South Africa represented a single taxon (*A. fuscipes*) that differed from isolates from Cameroon, Zambia and Zimbabwe.

The primary aim of this study was to characterize a collection of Zimbabwean *Armillaria* isolates from different hosts. This was achieved using techniques previously applied and also to compare these with the AFLP technique. Isolates previously characterized by Mwenje *et al*. (2003) were included in this study to provide a framework for the analyses.

Materials and methods

Origin of Isolates

All samples were collected from the eastern highlands and northern parts of Zimbabwe (Table 1) and are maintained in the Culture Collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

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

Isolate number	Alternative culture collection number	Host	Origin	RFLP group	IGS-1 RFLP size	Genbank number
CMW1	DFB	*Prunus persica*	Pondo	IV	485, 255, 170	EU380251
CMW3	FB1	*Pr. persica*	Pondo	IV	485, 255, 170	EU380250
CMW4456	Z1	*Brachystegia utilis*	Eastern Highlands	II	480, 255, 175	
CMW4457	40	*Camellia sinensis*	Nyanga	II	480, 255, 175	EU380252
CMW9950	D6	*Pr. persica*	Dombera	I	380, 255, 130	
CMW9953	P1	*Pr. persica*	Pondo	I	380, 255, 130	
CMW9954	P21	*Pr. persica*	Pondo	III	480, 230, 175	EU380246
CMW9955	P3	*Pr. persica*	Pondo	I	380, 255, 130	
CMW9956	P5	*Pr. persica*	Pondo	I	380, 255, 130	
CMW9957	P6	*Pr. persica*	Pondo	I	380, 255, 130	
CMW9958	NP1	Citrus	Pondo	I	380, 255, 130	
CMW9959	P4	*Pr. persica*	Pondo	I	380, 255, 130	
CMW9960	D1	*Pr. persica*	Dombera	I	380, 255, 130	
CMW9961	D2	*Pr. persica*	Dombera	I	380, 255, 130	
CMW9962	D3	*Pr. persica*	Dombera	I	380, 255, 130	
CMW9963	D4	*Pr. persica*	Dombera	I	380, 255, 130	EU380243
CMW9964	D5	*Pr. persica*	Dombera	IV	485, 255, 170	
CMW9966	D8	*Pr. persica*	Dombera	I	380, 255, 130	
CMW9967	D10	*Pr. persica*	Dombera	IV	485, 255, 170	EU380249
CMW10115	56	*Newtonia buchananii*	Harare	III	480, 230, 175	EU380245
CMW10165	P7	*Pr. persica*	Pondo	I	380, 255, 130	EU380244
CMW11589	P22	*Pr. persica*	Pondo	IV	485, 255, 170	
CMW11649	Z43	*C. sinensis*	Nyanga	II	480, 255, 175	EU380254
CMW11650	JM3	*Pinus kaseyi*	Staplefords	V	480, 300, 175	EU380248
CMW11653	Z45	*C. sinensis*	Nyanga	II	480, 255, 175	EU380253
CMW11662	JM2	*P. kaseyi*	Staplefords	V	480, 300, 175	EU380247
CMW11666	M37	*P. elliotti*	Martin Forest	IV	485, 255, 170	
CMW11672	P11	*Pr. persica*	Pondo	IV	485, 255, 170	

DNA extraction

Isolates were grown on MYA (1.5% Biolab Malt extract; 0.2% Biolab Yeast extract and 1.5% Biolab Agar) at 22 ºC in the dark for two weeks. The isolates were then transferred to liquid MY (1.5% Biolab Malt Extract and 0.2% Biolab Yeast Extract) and grown at 22 ºC for three weeks. Mycelium was harvested by filtration, freeze-dried and ground to a fine powder in liquid nitrogen. DNA was extracted as described in Coetzee *et al*. (2005) with the modification that proteins were removed using isoamyl alcohol:phenol:chloroform (1:24:25 v/v) in place of isoamyl alcohol:chloroform(1:24 v/v). DNA concentration was quantified by UV spectroscopy using a Beckman Du Series 7500 UV Spectrophotometer. The DNA samples were stored at –20°C until needed.

PCR amplification

DNA extracted from the isolates was used as template for PCR amplification of the IGS-1 region. The IGS-1 region between the 3' end of the large subunit (LSU) and the 5' end of the 5S gene of the RNA operon was amplified using the primer pair P-1 (5' TTG CAG ACG ACT TGA ATG G 3') (Coetzee *et al*. 2000a) and 5S-2B (5' CAC CGC ATC CCG TCT GAT CTG CG 3') (Coetzee *et al*., 2000b). The PCR reaction mixture included 1.75 U of *Taq* polymerase (Roche Diagnostics), 1 mM dNTPs, 0.01 µM of each primer, PCR buffer with $MgCl_2$, 0.25 mM of additional $MgCl_2$ and approximately 90 ng of template DNA. PCR conditions were an initial denaturation at 94ºC for one minute, followed by 35 cycles of denaturation (94 ºC, 30 seconds), primer annealing (60ºC, 20 seconds) and elongation (72ºC, 30

seconds). A final elongation step of five minutes at 72ºC was allowed for the complete elongation of the amplicons. The quality of PCR reaction products was determined through electrophoresis on a 1% ethidium bromide-stained agarose gel and visualized under UV illumination.

Restriction Fragment Length Polymorphism (RFLP) analysis

The IGS-1 amplicons were digested using the restriction endonuclease *Alu*I without prior purification as described by Harrington and Wingfield (1995). PCR reaction mix (18 µL) was subjected to digestion by 10 U of *Alu*I for three hours at 37ºC. The resultant RFLP fragments were separated through electrophoresis on a 3% agarose gel stained with ethidium bromide and visualized under UV illumination. Fragments less than 100 base pairs (bp) in size were not scored because of their low visibility. RFLP fragment sizes were determined using the molecular size standard marker and compared with those of other Zimbabwean isolates (Mwenje *et al*., 2003). Isolates CMW10165, CMW4457 and CMW10115 were used as control isolates representing Zimbabwean RFLP groups I, II and III respectively, which were characterized by Mwenje *et al*. (2003).

DNA sequencing

The PCR amplicons of the IGS-1 region of isolates CMW10165, CMW9963, CMW-9964, CMW9967, CMW4457, CMW11649, CMW11653, CMW3, CMW1, CMW11662 and CMW11650 were sequenced using an ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase FS (Perkin Elmer, Warrington, UK) following the protocol of the manufacturer. The sequences were determined on an ABI PRISM™ 3100 (Applied Biosystems/HITACHI, Foster City, California, USA) automated DNA sequencer after purification of the sequencing reaction products using QIAquick PCR purification kit (QIAGEN, Hilden, Germany). Primers P-1 and 5S-2B as well as internal primers MCP-2R, MCP-3, MCP-3R, 5S-3MC, 5S-3MCR, 5S-4MC, 5S-4MCR, MCP-2A, MCP-2AR, 5S-5MC and 5S-5MCR (Coetzee *et al*., 2000b) were used to sequence both DNA strands.

DNA sequence analysis

Analysis of raw sequences was done using Sequence Navigator version 1.01 (ABI PRISM^{TM}). Sequence data were compared by means of BlastN search with sequences already available on Genbank (http://www.ncbi.nlm.nih.gov). DNA sequence data were aligned using the program ClustalX version 1.8 (Thompson *et al*., 1997) and manually adjusted. Distance analysis was based on the HKY85 (Hasegawa *et al*., 1985) nucleotide substitution model with random addition of data. Phylogenetic trees were generated using the Neighbor-Joining tree building algorithm (Saitou and Nei, 1987) in PAUP* version 4.0b10 (Swofford, 1998). Missing and ambiguous data were excluded from the analysis. Two sets of bootstrap analyses (1000 replicates) (Felsenstein, 1985) were preformed to calculate the confidence intervals at the branch nodes. Isolates CMW9963 and CMW10165 were used as outgroup taxa in the first set and isolates CMW4456 and CMW11649 in the second set.

Amplified Fragment Length Polymorphism (AFLP) analysis

AFLP profiles were generated from the extracted DNA using the method described by Vos *et al*. (1995) with minor modifications. Genomic DNA was diluted to approximately 10 ng/µl and digested with *Eco*RI and *Mse*I (2 U each) (New England Biolabs, Beverly, Massachusetts) following the manufacturer's instructions. The generated fragments were ligated to the following adapter sequences: *Eco*RI: (5' CTC GTA GAC TGC GTA CC/CAT CTG ACG CAT GGT TAA 5') and *Mse*I: (5' GAC GAT GAG TCC TGA G/TAC TCA GGA CTC AT 5') (Vos *et al*., 1995) using T4 DNA ligase (New England Biolabs, Beverly, Massachusetts). These DNA fragments were subjected to an initial pre-amplification step. The reaction mixture included 0.3 µM *Eco*RI + A (5' GAC TGC GTA CCA ATT CA 3') primer, 0.3 µM *Mse*I + C (5' GAT GAG TCC TGA GTA AC 3') primer (Vos *et al*., 1995), 0.2 mM dNTPs, PCR buffer containing 1.5 mM $MgCl_2$, 0.6 U *Taq* Polymerase (Roche Diagnostics) and diluted restriction-ligation mix. Reaction conditions were 30 seconds at 72ºC followed by 25 cycles

of 30 seconds at 94ºC; 30 seconds at 56ºC; one minute + one second per cycle at 72ºC and a final cycle of two minutes at 72ºC. Product was diluted in low TE buffer and electrophoresed on 1% ethidium bromide stained agarose gel and visualized under UV.

The diluted product of the preamplification step was used as template for the subsequent final amplification step. Reaction conditions for the final amplification step were 13 cycles for 10 seconds at 94ºC; 30 seconds at 65ºC with temperature decreasing by 0.7ºC per cycle during subsequent cycles; one minute at 72ºC; followed by 23 cycles of 10 seconds at 94ºC; 30 seconds at 56ºC; one minute + one second per cycle at 72ºC and a final cycle of one minute at 72ºC. The reaction mixture included the diluted pre-amplification product, PCR buffer, 0.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.04 µM Infrared dye (IRD) labelled (Li-COR, Lincoln, NE, USA) *Eco*RI + 2 (5'GAC TGC GTA CCA ATT C ACT/AGC_3') primer, 0.25 µM *Mse*I + 2 (5' GAT GAG TCC TGA GTA A CTC/CGC 3') primer and 0.6 U *Taq* Polymerase (Roche Diagnostics). The three primer combinations used are *Eco*RI + ACT/*Mse*I + CTC, *Eco*RI + ACT/*Mse*I + CGC and *Eco*RI + AGC/ *Mse*I + CTC. Formamide loading dye was added after completion of the reactions. Product was denatured at 94ºC for three minutes prior to loading and run on 8% Long Ranger Polyacrylamide Gel (Cambrex Bioscience, Rockland. USA) on a LI-COR automated sequencer (LI-COR, Lincoln, NE, USA) and the results were analyzed using SAGA^{MX} software version 2.1 (Li-COR, Lincoln, NE, USA).

Bands were scored as present (1) or absent (0) and ambiguous bands were recorded as missing data. Data generated from the three primer combinations were combined to form a combined data matrix consisting of all the bands. Genetic pairwise distances between the isolates were calculated using the Nei and Li (1979) genetic distance model in PAUP* version 4.0b10 (Swofford, 1998). The distance matrix generated was then analyzed using a Neighbor - Joining clustering algorithm (Saitou and Nei, 1987) in PAUP* version 4.0b10 (Swofford, 1998). A bootstrap analysis (Felsenstein, 1985) with 1000 replicates was performed to determine support at the branch nodes. Bootstrap values greater than 50% were retained in the dendrogram. Isolates CMW9960 and CMW9955 representing *Armillaria* Group I were used as outgroups. A Dice-coefficient was also calculated to test the similarity between the isolates.

Results

DNA amplification sizes

All Zimbabwean isolates gave successful amplification of the IGS-1 region using primer pairs P-1 and 5S-2B. Three size groups of 1200 bp, 1000 bp and approximately 900 bp were observed.

Restriction fragment length polymorphisms

Five different RFLP patterns were observed and these were designated Groups I to V (Table 1). The restriction maps for these RFLP patterns were generated from the sequence data (Fig. 1). The fragment and amplicon sizes for these five RFLP Groups are summarised as follows. RFLP Group I isolates had a restriction pattern with fragment sizes of approximately 380, 255 and 130 bp and an amplicon size of 1200bp. Isolates of RFLP Group II had fragment sizes of approximately 480, 255 and 175 bp, those in RFLP Group III had fragment sizes of approximately 480, 230 and 175 and those in Group IV had fragment sizes of approximately 485, 255 and 170 bp. RFLP Groups II, III and IV had an uncut amplicon size of 900bp. Isolates in RFLP Group V had RFLP pattern with fragment sizes of approximately 480, 300 and 175 bp and amplicon size of 1000 bp.

DNA sequence data and analyses

IGS-1 sequence data were successfully obtained for a selection of isolates and deposited in GenBank (Table 1) and Tree Base (Study accession number = S2082 Matrix accession number = M3916). A total of 16 missing and ambiguous characters were excluded from the dataset before subsequent analysis. The phylogenetic tree generated from the DNA sequences grouped isolates into four well supported groups (Fig. 2). Isolates CMW9963 and CMW10165, representing RFLP Group I, had a 100% bootstrap support. Isolates CMW11662 and CMW11650

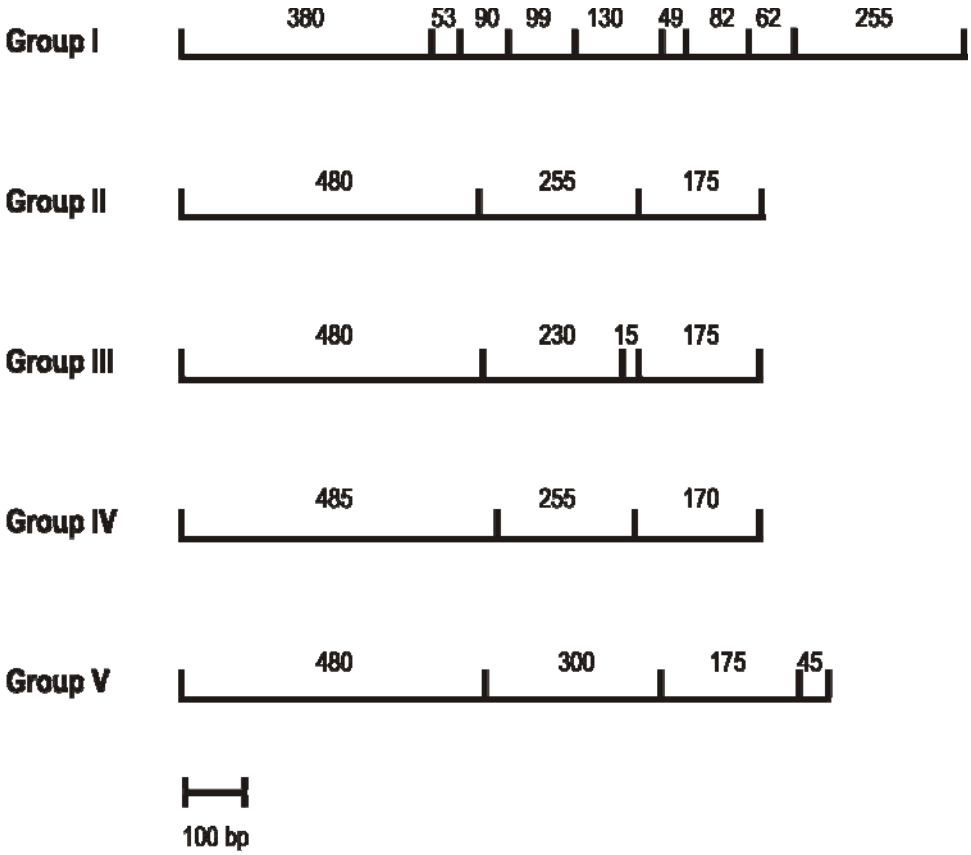

Fig. 1. IGS-1 restriction maps generated for the enzyme *Alu*I based on the restriction patterns indicated in Table 1 for RFLP groups I, II, III, IV and V. Numbers designate the approximate length (bp) of the fragments after digestion.

representing RFLP Group V formed a distinct group with a support value of 100%. Isolates CMW4456, CMW11653 and CMW11649 belonging to RFLP Group II formed a cluster with a bootstrap support value of 88%. Isolates CMW11653 and CMW11649 further formed a sub-group within this group (100% bootstrap support). Isolates belonging to RFLP Groups III and IV formed one group in the DNA sequence analyses, but with a low bootstrap support (54%).

AFLP analysis

A total of three primer combinations were employed and individual primer pairs produced different polymorphisms with bands either monomorphic or polymorphic. There were 413 bands and this represented an average of 137 bands per primer pair. The Neighbor-Joining tree generated from the distance matrix separated the isolates into two large clusters and one intermediate group between the larger clusters (Fig. 3). The first cluster included isolates belonging to RFLP Group I. The second significant cluster had isolates residing in RFLP Groups III, IV and V. The smaller and intermediate cluster comprised isolates belonging to RFLP Group II. RFLP Group I isolates grouped together with bootstrap support of 96%. Within this group, the isolates from Dombera (88% bootstrap support) and those from Pondo (71% bootstrap support) grouped in respective clusters. RFLP Group II isolates formed a cluster (98% bootstrap support) and were clearly separate from the other RFLP Groups. RFLP Group IV formed a cluster with 56% support and further formed two sub-groups. One sub-group had a support value of 65% with isolates CMW9964, CMW-9967, CMW11672, CMW11589 and CMW3. Isolates CMW1 and CMW11666 formed another sub-group with a 64% support value. Isolates of RFLP Groups III and V formed a cluster having 80% support, with RFLP Group V isolates further forming a sub-group with a 97% support and those of RFLP Group III forming a cluster with a 64% support value.

After calculating the Dice-similarity coefficient, isolates of Group I had similarity values that ranged from 61%-89%. Isolates of Group II had similarity values ranging from 68-75%, Group III isolates had a similarity value of 76%, Group IV isolates had similarity values that ranged from 60%-89% and Group

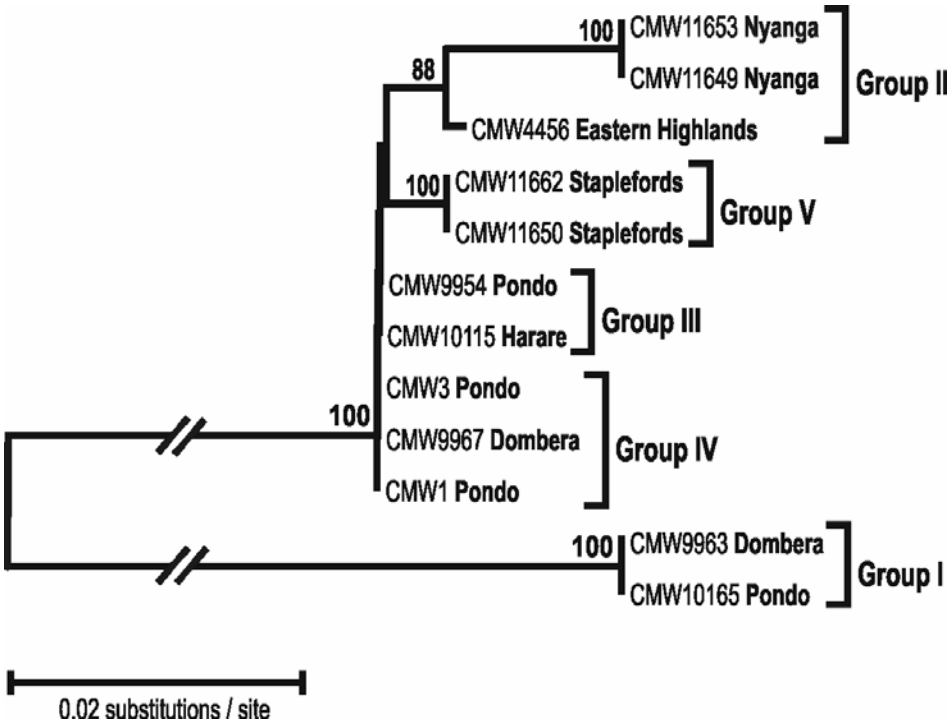

Fig. 2. Neighbour-Joining tree rooted to midpoint based on IGS-1 sequence data. Bootstrap values are indicated on the tree branches. Corresponding groups and areas of origin for the isolates are also indicated. The scale bar indicates the genetic distance between the isolates.

V isolates had a similarity value of 81%. With the exception of Groups III and V, the similarity values between the groups were lower than 50%. The similarity between Groups III and V was the same as the within group similarity.

Discussion

A collection of isolates from different parts of Zimbabwe were characterised using PCR - RFLP, sequencing and AFLP analysis. Amplification of the IGS-1 region demonstrated that there was variation in IGS-1 amplicon sizes. Furthermore, five different RFLP Groups were obtained. Most of these RFLP Groups correlated with the separations observed based on AFLP data and IGS-1 sequence data. Three of the RFLP Groups have previously been identified based on their isozyme profiles (Mwenje and Ride, 1996) and further confirmed using IGS-1 sequences (Mwenje *et al*., 2003).

RFLP Group I isolates were identical to a species from South Africa identified as *A. fuscipes* (Coetzee *et al*., 2000b). This species has also been reported in Zimbabwe (Mwenje *et al*., 2003) and Ethiopia (Gezahgne *et al*., 2004). *Armillaria fuscipes* was originally described in Sri Lanka by Petch (1909). Based on IGS-1 and AFLP data, RFLP Group I isolates were clearly separated from the other groups and formed a distinct group. The results provided conclusive evidence that this group represented at least one separate species and based on the DNA sequence data was quite distantly related to the fungi in the other RFLP Groups. RFLP Group II isolates were less frequently encountered in Zimbabwe than RFLP Groups I and III. Only four isolates of this fungus have thus far been collected. RFLP Group II isolates have a significantly unique IGS-1 sequence and RFLP profile. In addition, AFLP profiles showed that they were not closely related to the fungi in any of the other RFLP Groups. The data confirm the findings of Mwenje *et al*. (2003), who has suggested that these isolates represent an undescribed species.

Isolates representing RFLP Group III showed slight variation in their IGS-1 DNA sequences. Although significant sequence variation was not expected within the same species, the phenomenon was not without precedent and has also been reported in other fungi (Garbelotto *et al*., 1993). RFLP variation within a species has also been reported

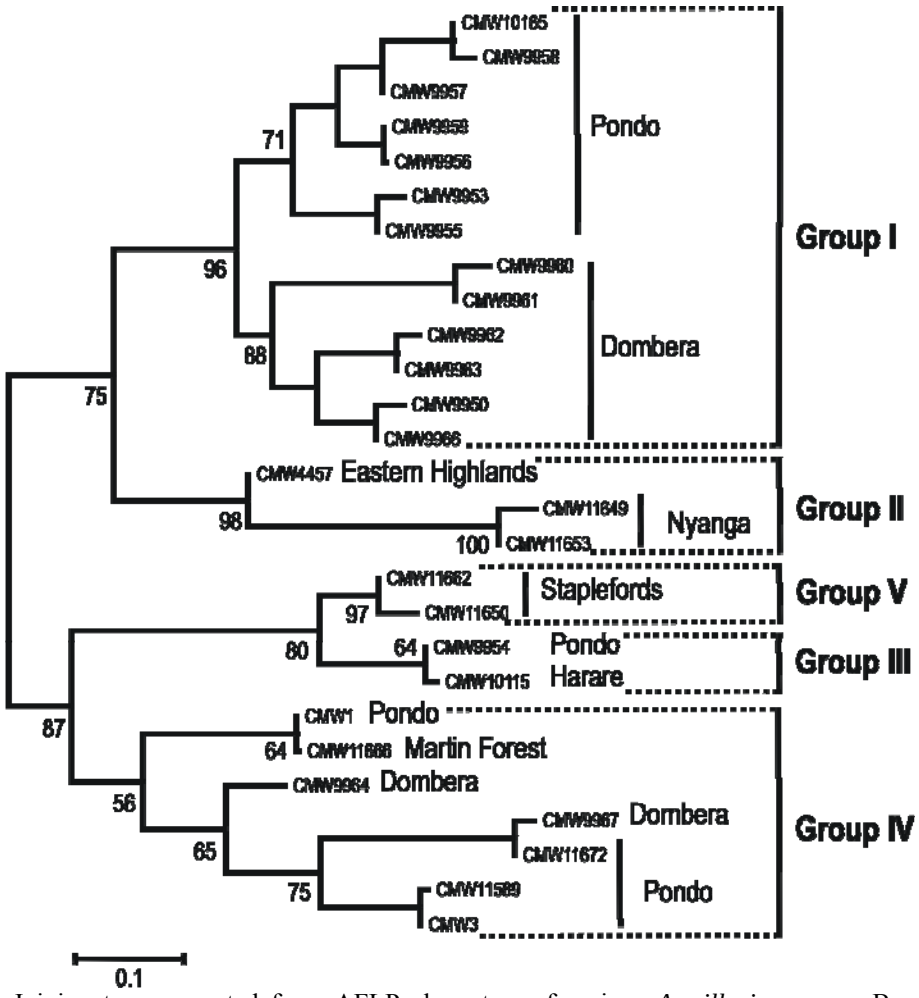

Fig. 3. Neighbor-Joining tree generated from AFLP characters of various *Armillaria* groups. Bootstrap values are shown on the branches and the scale bar corresponds to the distance between AFLP data for different isolates. The areas of origin of the isolates and corresponding RFLP groups are indicated next to the tree.

previously in *Armillaria* (Anderson and Stasovski, 1992; Harrington and Wingfield 1995; Pérez-Sierra *et al*., 1999; Coetzee *et al*., 2000a; Dunne *et al*., 2002) RFLP Group IV isolates had similar IGS-1 PCR amplicon sizes to those in RFLP Groups II and III. However, these groups differed in their RFLP patterns. The IGS-1 phylogram derived from DNA sequence data showed that RFLP Group IV isolates were most closely related to isolates of RFLP Group III. However, based on AFLP profiles, Group IV isolates seemed to be distinct and probably represented an undescribed species.

Isolates residing in RFLP Group V either represented an undescribed species or were variants of an existing species. BlastN searches showed that their IGS-1 sequences were most similar to those of Zimbabwean RFLP Group III. RFLP Groups III and V could represent the same species that exhibited high levels of intraspecies variation in its IGS-1 region of the rRNA operon. AFLP data showed that Groups III and V had similar dice similarity coefficients, providing additional evidence that they represent the same species.

AFLPs have previously been used to study *Armillaria* isolates both at the inter- and intraspecific levels (Kim *et al*., 2006; Terashima *et al*., 2001, 2006). This technique has, however, been used only once to characterise *Armillaria* spp. from Africa (Pérez-Sierra *et al*., 2004). With the exception of RFLP Groups III and V, the similarity values were high within the RFLP Groups and very low between groups. This supports the separation of the isolates into different groups based on RFLP. The AFLP results further supported the suggestion (Mwenje *et al*., 2003) that there were at least three species of *Armillaria* in Zimbabwe.

AFLP as well as RFLP and DNA sequences from the IGS-1 region were used to identify *Armillaria* isolates from different geographic areas and hosts in Zimbabwe. Similar

results obtained for the AFLP data and IGS-1 sequence comparisons supported the potential use of the AFLP technique in *Armillaria* characterisation. Overall results suggest that here were at least four distinct *Armillaria* taxa in Zimbabwe. These included the putative fungus *A. fuscipes*, the two groups previously characterised based on RFLP analyses (RFLP Group II and Group III). The fourth taxon had not previously been seen in collections from Zimbabwe, but was clearly present based on IGS-1 and AFLP data. In the future, a concerted effort will need to be made to obtain sporocarps of *Armillaria* species from Zimbabwe. These, together with data from the present and previous studies will be necessary to provide names for the undescribed species, thus completing the characterisation of *Armillaria* spp. from Zimbabwe.

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