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Armillaria species on tea in Kenya identified using isozyme and DNA sequence comparisons

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The aim of this study was to identify seven *Armillaria* isolates obtained from diseased tea bushes in Kenya using pectic enzyme profiles, PCR-RFLP and IGS-I DNA sequence data. The combination of these identification methods confirmed the presence of three distinct *Armillaria* groups. One of these groups resembled Zimbabwean group I (*A. fuscipes*). The second group was phylogenetically closely related to *A. mellea* ssp. *nipponica*. The third group was different from all other African isolates examined, but had isozyme patterns, especially of pectin methylesterases (PMEs), similar to those of isolates related to *A. mellea* ssp. *nipponica*. Analyses of sequence data suggested that this group is phylogenetically closely related to *A. hinnulea* from Australia and New Zealand.

Keywords: armillaria root rot, *Camellia sinensis*, IGS-1 rRNA, isozymes, taxonomy

Introduction

Records of armillaria root rot on tea (*Camellia sinensis*) from the African continent date back to the 1930s when Leach (1939) reported problems associated with this disease in Nyasaland. *Armillaria* is known to affect many plant species in the highlands of Kenya (Mwangi *et al*., 1989) and has limited the areas planted to tea. Losses of up to 50% have been recorded on smallholder farms, and armillaria root rot is a disease of major economic concern (Onsando *et al*., 1997).

Armillaria root rot on tea in Kenya is particularly common where the crop is established shortly after deforestation, suggesting that the *Armillaria* spp. responsible for the disease are native to the country (Goodchild, 1960). Symptoms on tea bushes infected by *Armillaria* include chlorotic foliage and somewhat stunted plants. These bushes also tend to flower prematurely (Onsando, 1986). Gaps resulting from death of infected plants invariably appear as the principal indicators of infection. Infection foci are often associated with stumps or remnants of roots of trees from the previous forest vegetation.

Several strategies have been applied in attempts to limit the impact of armillaria root rot disease on tea (Leach, 1939; Onsando, 1989; Otieno *et al*., 2003a,c), but these have had minimal success. Despite the severity of armillaria root rot in tea plantations, little has been done to determine the identity or diversity of the fungus causing the disease. The name most commonly attributed to the causal agent is *Armillaria mellea sensu lato*, but this species is known to encompass various distinct biological species (Korhonen, 1978; Anderson & Ullrich, 1979).

Various taxonomic studies have been conducted in the past to identify *Armillaria* spp. in Africa. Prior to the use of molecular methods, *A. camerunensis*, *A. heimii* and *A. mellea* were recognized (Pegler, 1977; Mohammed *et al*., 1989; Volk & Burdsall, 1995). Taxonomic studies based on basidiocarp morphology are hampered by the rarity and seasonal occurrence of these structures. This has motivated the use of molecular-based studies to identify and classify isolates. Recent studies using analyses of pectic enzymes, DNA sequences, and RFLP and AFLP profiles have suggested that several species other than those previously recognized may be present in Africa (Mwenje & Ride, 1996, 1997; Coetzee *et al*., 2000a; Mwenje *et al*., 2003; Otieno *et al*., 2003b; Pérez-Sierra *et al*., 2004).

Variation in mycelium and rhizomorph morphology has been observed amongst some Kenyan *Armillaria* isolates (Gibson, 1960). Likewise, Mwangi *et al*. (1989) reported three *Armillaria* groups from Kenya based on differences in cultural characteristics, interfertility tests and isozyme profiles. These groups were shown to differ from European *Armillaria* species. Isolates reported as *A. heimii* [SIG (somatic incompatibility group) II], *A. mellea* ssp. *africana* (SIG II) and two unidentified groups, SIG III and SIG

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IV, were described from Kenya (Mohammed *et al*., 1994; Mwangi *et al*., 1994). Most recently, studies characterizing isolates of *Armillaria* from tea, based on both morphology and DNA sequence data, identified two distinct taxa on this crop in Kenya. These were designated *Armillaria* group I, which resembles *A. heimii* in basidiocarp morphology, and *Armillaria* group II (Otieno *et al*., 2003b). Collectively, these studies showed that at least four taxa are present in Kenya: *A. mellea* ssp. *africana*, *A. heimii* and two as yet unnamed species [SIG III (*Armillaria* group II) and SIG IV].

Recent studies on *Armillaria* in South Africa, Zimbabwe and Ethiopia (Coetzee *et al*., 2000a; Mwenje *et al*., 2003; Gezahgne *et al*., 2004) suggested that isolates from these and other countries in Africa represent three phylogenetic lineages: *A. fuscipes*, *A. heimii* and Zimbabwean group III. *Armillaria fuscipes* encompassed isolates previously characterized and referred to as *A. heimii* (Coetzee *et al*., 2000a). The separation of the lineages, reflecting *A. fuscipes* and *A. heimii*, into species, and the application of the names to identify them subsequently evoked considerable debate (Otieno *et al*., 2003b; Pérez-Sierra *et al*., 2004). Recently, Coetzee *et al*. (2005) considered *A. fuscipes* and *A. heimii* to be synonymous based on their similarity in morphology and gave preference to the name *A. fuscipes*, as this is the older name. The authors showed that there are two major lineages in Africa. The first represents *A. fuscipes* and includes some isolates previously referred to as *A. heimii*. The second lineage is considered an unnamed species and represents the group earlier thought to be *A. heimii* by Coetzee *et al*. (2000a). In the present study, the identity of isolates from tea in Kenya was re-evaluated by comparing representative isolates with groups already described elsewhere in Africa. Identification of available isolates was based on isozyme comparisons as well as PCR-RFLP and comparisons of sequence data for the IGS-1 region of the rRNA operon.

Materials and methods

Origin of isolates

This study included seven isolates obtained from infected tea bushes in Kenya (Table 1). Six of these isolates (CMW15580, CMW15581, CMW15582, CMW15583, CMW15584 and CMW15585) were from the Tea Research Foundation of Kenya and were used recently by Otieno *et al*. (2003b) in their study of *Armillaria* isolates from tea (Table 1). Zimbabwean isolates previously characterized based on morphological and biochemical characteristics (Mwenje & Ride, 1996), PCR-RFLP profiles and comparisons of IGS-1 sequence data (Mwenje *et al*., 2003) were also included. A South African isolate previously identified as representing *A. fuscipes* (Coetzee *et al*., 2000a), isolates of *A. mellea* from Japan and South Korea (Coetzee *et al*., 2000b) and *A. hinnulea* from Australia (Coetzee *et al*., 2003a) were included for comparative purposes in RFLP analyses. Other collections used in the DNA sequence comparisons are listed in Table 2. All

Table 1 *Armillaria* isolates used in this study

Isolate code	Alternative code	Group/species designation	Host	Country of isolation	GenBank accession number	Sequence reference	RFLP fragment sizes (bp)
CMW15580	4EN1	Kenyan group I	*Camellia sinensis*	Kenya, Nandi	AY773968		380, 255, 130
CMW15581	10G1	Kenyan group I	*C. sinensis*	Kenya, Kirinyaga			380, 255, 130
CMW15582	13T1	Kenyan group I	*C. sinensis*	Kenya, Nyambene	AY773969		380, 255, 130
CMW15583	1BE1	Kenyan group III	*C. sinensis*	Kenya, Kericho			220, 140, 100
CMW15584	6M1	Kenyan group III	*C. sinensis*	Kenya, Nakuru	AF513024	Otieno *et al*. (2003b)	220, 140, 100
CMW15585	13T2	Kenyan group III	*C. sinensis*	Kenya, Nyambene	AF513022	Otieno *et al*. (2003b)	220, 140, 100
CMW15586	K6	Kenyan group II	*C. sinensis*	Kenya, Kericho	AY524982		220, 170
CMW15587	K7	Kenyan group II	*Saccharum officinarum*	Kenya	AY524981		220, 170
CMW10165	P7	*A. fuscipes* Zimbabwean group I	*Prunus persica*	Zimbabwe	AF489482	Mwenje *et al*. (2003)	380, 255, 130
CMW4456	Z1	Zimbabwean group II	*Brachystegia utilis*	Zimbabwe	AF489485	Mwenje *et al*. (2003)	480, 255, 175
CMW10116	56	Zimbabwean group III	*Newtonia buchananii*	Zimbabwe	AF489484	Mwenje *et al*. (2003)	480, 230, 175
CMW2740	B07-SA	*A. fuscipes*	*Pinus elliottii*	South Africa	AF204822	Coetzee *et al*. (2000a)	380, 255, 130
CMW4980	119 CBS164.94	*A. hinnulea*	*Eucalyptus obliqua*	Australia, Tasmania	AF445077	Coetzee *et al*. (2003a)	220, 170, 140, 100
CMW4990	3512/13	*A. hinnulea*	*Nothofagus* sp.	New Zealand	AF445078	Coetzee *et al*. (2003a)	220, 170, 140, 100
CMW3966	B608	*A. mellea*	unknown	South Korea	AF163611	Coetzee *et al*. (2000b)	300, 170
CMW3967	B731	*A. mellea*	*Chamaecyparis* sp.	Japan	AF163610	Coetzee *et al*. (2000b)	220, 170
CMW3961	B730	*A. mellea*	Unknown	Japan			220, 170

Table 2 Additional IGS-1 sequences for *Armillaria* spp. obtained from GenBank

Species/group designation	Isolate numbers	Country of origin	GenBank number	Sequence reference
A. fuscipes	CMW4874, Z2	Zimbabwe	AF489481	Mwenje *et al.* (2003)
Zimbabwean group I				
Zimbabwean group II	CWM4455, 40	Zimbabwe	AF489486	Mwenje *et al.* (2003)
Zimbabwean group III	CMW10115, 55	Zimbabwe	AF489483	Mwenje *et al.* (2003)
A. heimii	CMW3152, CA1, B935	Cameroon	AF204826	Coetzee *et al.* (2000a)
A. heimii	CMW3173, ZM1	Zambia	AF204825	Coetzee *et al.* (2000a)
A. heimii	CMW3164, LR3	Reunion	AF204824	Coetzee *et al.* (2000a)
A. fuscipes	CMW2717, A04-SA	South Africa	AF204821	Coetzee *et al.* (2000a)
A. fuscipes	CMW5844, WG11	Ethiopia	AY172032	Gezahgne *et al.* (2004)
A. fuscipes	CMW5846, WG2E	Ethiopia	AY172030	Gezahgne *et al.* (2004)
A. cepistipes	316	Finland	AF243068	Anderson & Stasovski (1992)
A. cepistipes	311	France	AF243067	Anderson & Stasovski (1992)
A. ostoyae	300	Germany	AF243049	Anderson & Stasovski (1992)
A. ostoyae	337	Denmark	AF243051	Anderson & Stasovski (1992)
A. mellea	CMW3964, B927	USA	AF163608	Coetzee *et al.* (2000b)
A. mellea	CMW4619, B1217	USA	AF163609	Coetzee *et al.* (2000b)
A. mellea	CMW4611, B917	South Korea	AF163613	Coetzee *et al.* (2000b)
A. mellea ssp. *nipponica*	HUA93110	Japan	D89922	Terashima *et al.* (1998)
A. tabescens	33	Italy	AF451835	Sicoli *et al.* (2003)
A. tabescens	48	Italy	AF451834	Sicoli *et al.* (2003)

isolates used in this study are maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI, University of Pretoria, South Africa) on malt extract agar (MEA) 20 g L^{-1} malt extract, 15 g L^{-1} agar, Biolab slants and stored at 4°C.

Pectin lyase and pectin methylesterase isozymes

Culture filtrates from the *Armillaria* isolates were produced and collected as described by Mwenje & Ride (1996), except that cell walls of msasa (*Brachystegia spiciformis*) were used in place of those of hazel (*Corylus avellana*). Mycelium was harvested by filtration through Whatman no. 1 filter paper and the culture filtrates dialysed and concentrated against polyethylene glycol (PEG, 12·5 g L^{-1}). After electrophoresis, gels were incubated briefly in 10 mM $CaCl_2$ and pectin lyases (PLs) and pectin methylesterases (PMEs) detected by incubation in 0·02 M Tris-HCl (pH 8·5) at room (24°C) temperature (Mwenje & Ride, 1996, 1997). The pectin-containing gels were stained overnight in ruthenium red (0·03 g L^{-1}) in water. PL bands appeared as clear white zones, whereas PMEs appeared as dark, red-purple bands (Cruickshank & Wade, 1980).

IGS-1 amplification and RFLP analyses

Cultures were grown in the dark under stationary conditions at 26°C in 250 mL conical flasks containing 50 mL liquid MY medium (malt extract 30 g L^{-1}, yeast extract 3 g L^{-1}, Biolab). Mycelium was harvested after 3 weeks by filtration using Whatman no. 1 filter paper. The mycelium was freeze-dried and ground to a fine powder. DNA was extracted from the powdered mycelium using the method described by Coetzee *et al.* (2000a). The DNA pellets were rehydrated in 50 μL of H_2O and stored at –20°C.

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 all *Armillaria* isolates. Two sets of primers were used to amplify DNA. The first primer pair included P-1 (5′-TTGCAGACGACTTGAATGG-3′) (Hsiau, 1996) and 5S-2B (5′-CACCGCATCCCGTCTGATCTGCG-3′). The second primer pair included P-1 and O-1 (5′-AGTCCTATGGCCGTGGAT-3′) (Duchesne & Anderson, 1990). The PCR reaction mixture included *Taq* polymerase (1·75 U) (Roche Diagnostics), PCR buffer with $MgCl_2$, additional $MgCl_2$ (2·5 mM), dNTPs (1 mM), primers (0·01 μM of each) and approximately 80 ng of template DNA. The PCR conditions included an initial denaturation at 94°C for 3 min followed by 35 cycles of denaturation (30 s, 94°C), primer annealing (30 s, 58°C) and elongation (1 min, 72°C). A final step of 7 min at 72°C was allowed for complete elongation of the amplicons. After electrophoresis on an ethidium bromide-stained agarose gel (10 g L^{-1}), PCR products were visualized under UV illumination.

RFLP analyses

IGS-1 amplicons obtained for the isolates were digested without prior purification as described by Harrington & Wingfield (1995). Restriction profiles were obtained by digesting 18 μL of the PCR product with the restriction endonuclease *Alu*I (20 U) for 5–7 h at 37°C. The resulting PCR-RFLP fragments were separated on an agarose gel (30 g L^{-1}) stained with ethidium bromide and visualized under UV illumination. Fragments smaller than 100 bp were not considered because of lack of resolution. RFLP fragment sizes were determined and compared with representative isolates of known groups (Coetzee *et al.*, 2000a; Mwenje *et al.*, 2003) or species included in this study.

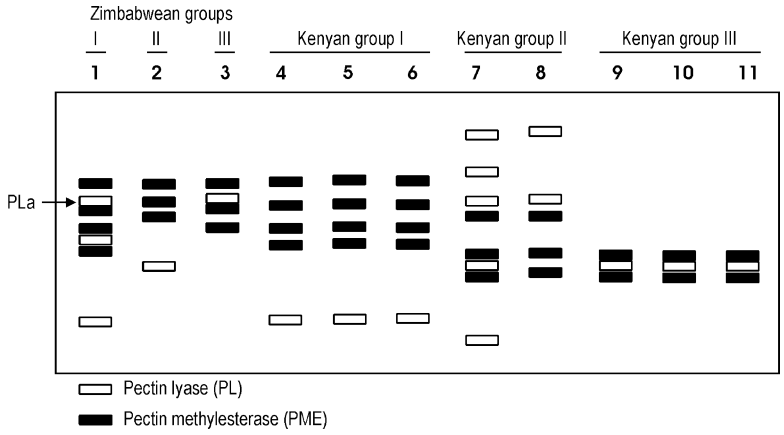

Figure 1 Pectin lyase and pectin methylesterases zymograms after electrophoresis in polyacrylamide gels containing pectin, showing grouping of African isolates of *Armillaria*. Lanes: CMW10165 (1), CMW4456 (2), CMW10116 (3), CMW15580 (4), CMW15581 (5), CMW15582 (6), CMW15586 (7), CMW15587 (8), CMW15583 (9), CMW15584 (10) and CMW15585 (11). PLa is a pectin lyase band characteristic of Zimbabwean groups I and II.

DNA sequence comparisons

IGS-1 sequences of four *Armillaria* isolates (CMW15580, CMW15582, CMW15586 and CMW15587) from Kenya (Table 1) were determined using an ABI PRISM 377 automated DNA sequencer. Sequence reactions were carried out using an ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit with AmpliTaq DNA polymerase FS (Applied Biosystems) according to the specifications of the manufacturers. The IGS-1 region of isolates CMW15586 and CMW15587 were sequenced in both directions using primers P-1, O-1, MCO-2 and MCO-2R (Coetzee *et al*., 2000b). Isolates CMW15580 and CMW15582 were sequenced in both directions using primers P-1, 5S-2B, MCP-2, MCP-2R, MCP-3, MCP-3R, 5S-3MC, 5S-3MCR, 5S-5MC and 5S-5MCR (Coetzee *et al*., 2000a).

Sequence data for the four *Armillaria* isolates obtained in this study were compared with sequences available on GenBank by means of a BLAST search. Details of isolates used in this comparison and obtained from GenBank are given in Table 2. IGS-1 sequences for isolates CMW15580 and CMW15582 were compared with the alignments of Coetzee *et al*. (2000a) available in TreeBase (matrix number M859, study number S566). IGS-1 sequence data for isolates CMW15584 and CMW15585, used in this study, were previously published by Otieno *et al*. (2003b). These sequences, together with those for CMW15586 and CMW15587 obtained in this study, plus sequences obtained from GenBank for a variety of *Armillaria* spp., were included in a separate data matrix for phylogenetic analysis. Sequences were manually aligned by including gaps.

Phylogenetic relationships between the Kenyan isolates and other *Armillaria* spp. were determined based on distance analyses using a Hasegawa, Kishino & Yano (HKY85) nucleotide substitution model in PAUP* version 4·0b10 (Swofford, 2000). Phylograms were generated from the distance matrices by means of a neighbour-joining clustering algorithm (Saitou & Nei, 1987). Bootstrap analyses (Felsenstein, 1985) with 1000 replicates were used to obtain confidence intervals for the clusters on the phylograms.

Results

Pectin lyase and pectin methylesterase isozymes

Armillaria isolates from tea resided in three groups based on their PL and PME patterns (Fig. 1). These groups are referred to as Kenyan groups I, II and III. Isolates CMW15580, CMW15581 and CMW15582 had almost identical PL and PME patterns, forming the first group, and resembled Zimbabwean group I isolate CMW10165, previously identified as *A. fuscipes* (Mwenje *et al*., 2003). These isolates had low activity or lacked a PL band (PLa), which is characteristic of Zimbabwean isolates residing in Zimbabwean groups I and III (Fig. 1). Isolates CMW15586 and CMW15587, previously identified as *A. mellea* ssp. *africana* (Mohammed *et al*., 1994; Mwangi *et al*., 1994), had unique and distinct bands forming a distinct group. Isolates CMW15583, CMW15584 and CMW15585 shared many common PME and PLa bands and formed a third group. These isolates had two PME bands in common with isolates CMW15586 and CMW15587. Isolates CMW15580, CMW15581 and CMW15582 showed faint PL bands compared with other isolates, but produced intense PME bands.

IGS-1 amplification and RFLP analyses

Primer set P1/5S-2B successfully amplified the IGS-1 region from isolates CMW2740, CMW4456, CMW10116, CMW10165, CMW15580, CMW15581 and CMW15582. Isolates CMW15580, CMW15581 and CMW15582 from tea yielded single fragments of approximately 1200 bp in size. The two isolates representing *A. fuscipes* (CMW10165 and CMW2740 from Zimbabwe and South Africa, respectively) had band sizes of approximately 1200 bp. Isolates CMW4456 and CMW10116, representing Zimbabwean groups II and III, respectively, resulted in an amplified fragment of approximately 900 bp.

RFLP profiles after digesting the amplicons with the restriction enzyme *Alu*I were the same for Kenyan group I isolates (CMW15580, CMW15581 and CMW15582) and two representative isolates of *A. fuscipes* from

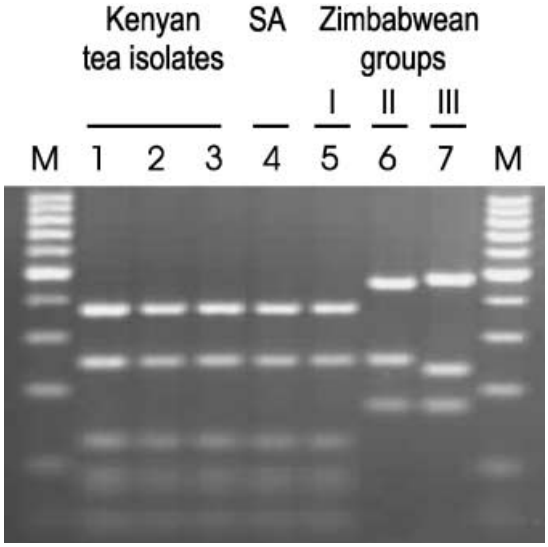

Figure 2 Agarose gel (3%) stained with ethidium bromide showing *Alu*I restriction fragments for *Armillaria* isolates. Lanes: CMW15581 (1), CMW15582 (2), CMW15580 (3), CMW2740 (4), CMW10165 (5), CMW4456 (6) and CMW10116 (7). Lanes labelled M: 100-bp ladder.

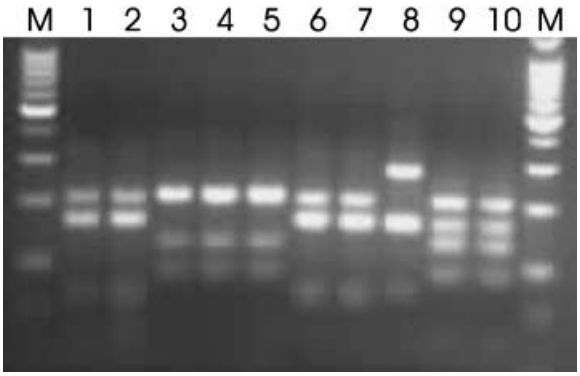

Figure 3 Agarose gel (3%) stained with ethidium bromide showing *Alu*I restriction fragments for isolates of *Armillaria*. Lanes: CMW15586 (1), CMW15587 (2), CMW15584 (3), CMW15585 (4), CMW15583 (5), CMW3961 (6), CMW3967 (7), CMW3966 (8), CMW4980 (9) and CMW4990 (10). Lanes labelled M: 100-bp molecular marker.

Zimbabwe and South Africa (Fig. 2). RFLP fragments of approximately 380, 255 and 130 bp in size, were obtained for these isolates. Isolates representing Zimbabwean group II (CMW4456) and group III (CMW10116) had different RFLP patterns from those of the tea isolates.

Amplification of the IGS-1 region for isolates CMW3961, CMW3967, CMW3966, CMW4980, CMW4990, CMW15583, CMW15584, CMW15585, CMW15586 and CMW15587 was successful with the primer set P-1/O-1. These isolates produced PCR fragments of approximately 690 bp with primers P-1 and O-1. Isolates representing *A. hinnulea* (CMW4980 and CMW4990) produced a PCR fragment of similar size using the same primer set.

RFLP patterns obtained after digestion with *Alu*I were the same for Kenyan group II isolates (CMW15586 and CMW15587) and the *A. mellea* isolates (CMW3961 and CMW3967) from Japan (Fig. 3). The amplicons digested with *Alu*I for these isolates produced fragment sizes of approximately 220 and 170 bp. Kenyan group III isolates (CMW15583, CMW15584 and CMW15585) produced identical RFLP profiles, with fragments of 220, 140 and 100 bp in size. Isolates representing *A. hinnulea* (CMW4980 and CMW4990) yielded fragment sizes of 220, 170, 140 and 100 bp (Fig. 3).

DNA sequence comparisons

Isolates CMW15586 and CMW15587 were identical to *A. mellea* from Japan (AF163610), as revealed by alignment against sequence data previously published by Coetzee *et al*. (2000b). Isolates CMW15584 and CMW15585 from tea were identical to each other, but differed from sequences obtained from isolates CMW15586 and CMW15587. A BLAST search using sequences of isolate CMW15585 gave the highest similarity to *A. hinnulea* (96%).

Neighbour-joining trees generated from the distance matrix grouped isolates CMW15580 and CMW15582 from tea in Kenya within a cluster representing *A. fuscipes* (Fig. 4). The phylogram generated from the separate matrix indicated that isolates CMW15586 and CMW15587 are most closely related to *A. mellea* (AF163610) from Japan, sharing the same terminal node (92% bootstrap support) (Fig. 5). Furthermore, these isolates, together with AF163610, grouped within a cluster that included *A. mellea* (AF163613 and AF163611) from South Korea and *A. mellea* ssp. *nipponica* (D89922) with 100% bootstrap support (Fig. 5). Isolates CMW15584 and CMW15585 were grouped within a cluster that included *A. hinnulea* (AF445077 and AF445078) with 100% bootstrap support (Fig. 5). Isolates CMW15584 and CMW15585, however, formed a subcluster within this group.

Discussion

Results of this study using isozyme analyses, RFLP patterns and comparisons of DNA sequence data showed that *Armillaria* from diseased tea bushes in Kenya represent three distinct groups. These results are in agreement with previous investigations that have found a variety of *Armillaria* groups in Kenya (Mwangi *et al*., 1989, 1994; Mohammed *et al*., 1994; Otieno *et al*., 2003b; Pérez-Sierra *et al*., 2004). In the present study, these groups are referred to as Kenyan groups I, II and III.

Based on isozyme, RFLP and DNA sequence similarities with those of isolates identified as *A. fuscipes* (Coetzee *et al*., 2000a, 2005), Kenyan group I isolates are the same as Zimbabwean group I isolates and represent *A. fuscipes*. Isolates residing in Kenyan group I were previously characterized by Otieno *et al*. (2003b). These authors showed that the isolates had RFLP profiles similar to

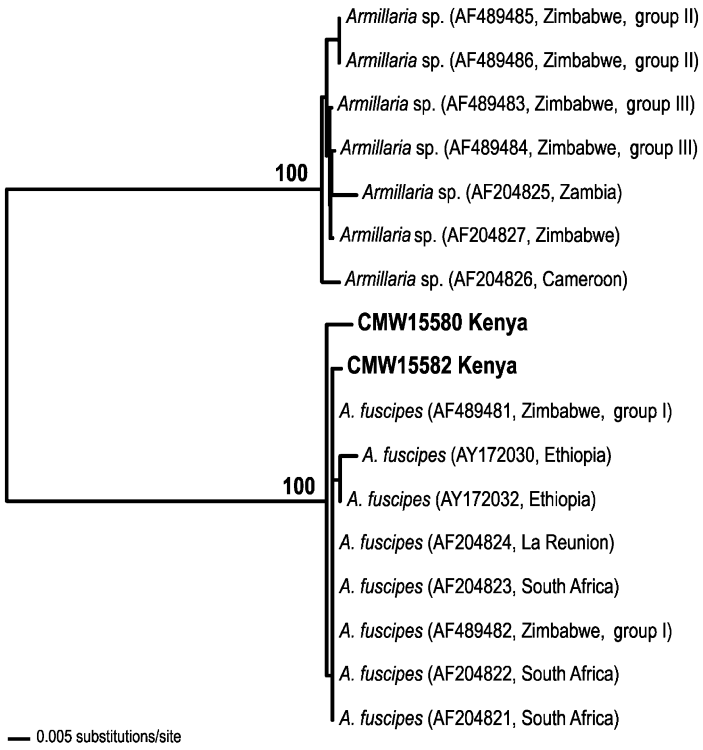

Figure 4 Phylogram generated with a neighbour-joining tree-building algorithm from IGS-1 sequence data. Scale bar: total nucleotide changes between taxa. Bootstrap values are indicated above tree branches.

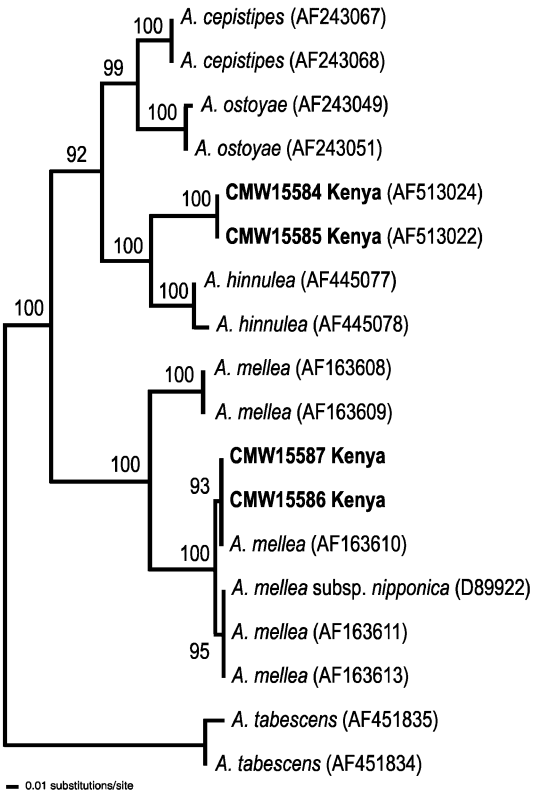

Figure 5 Neighbour-joining tree generated from IGS-1 sequence data for *Armillaria* spp. Scale bar: total nucleotide changes between taxa. Bootstrap values are indicated above tree branches.

those reported by Coetzee *et al*. (2000a) for isolates identified as *A. fuscipes* from South Africa. Otieno *et al*. (2003b), however, did not use the name *A. fuscipes*, referring instead to *A. heimii*. The name *A. fuscipes* has historic precedence and *A. heimii* should be considered a synonym of *A. fuscipes*.

Pérez-Sierra *et al*. (2004) showed that isolates that they referred to as *A. heimii* from Africa, which also included various samples from Kenya, can be separated into two groups based on PCR amplicon size and RFLP profiles. They suggested, however, that these groups represent two populations of *A. heimii*, rather than different taxa. The significant differences in DNA sequence of the IGS-1 region strongly suggest that the isolates represent at least two different taxa. One group is the same as Kenyan group I and *A. fuscipes*. The other group of isolates in the study by Pérez-Sierra *et al*. (2004) is apparently most closely related to Zimbabwean groups II and III and the isolates probably represent an undescribed species of *Armillaria*.

Armillaria fuscipes is a well-known pathogen of native plant and exotic *Pinus* species in various African countries. The recent study of Otieno *et al*. (2003b) treated this fungus from tea bushes in Kenya as *A. heimii*. This species has a wider distribution than Kenyan groups II and III and occurs at altitudes ranging from sea level to above 2000 m (Mwangi *et al*., 1994; Otieno *et al*., 2003b). It is concluded that this fungus represents *A. fuscipes* and that it is the major cause of armillaria root rot on tea in Kenya.

The group of *Armillaria* isolates from Kenya that have been assigned the name *A. mellea* ssp. *africana* by previous

investigators (Mohammed *et al*., 1994; Mwangi *et al*., 1994), referred to as Kenyan group II in this study, was also well defined. The IGS-1 region of these isolates could be amplified using primer pair P-1/O-1. This reflects a fundamental difference from most isolates originating in Africa and assigned variously to the species *A. fuscipes* and *A. heimii*, where the 5S gene is inverted (Coetzee *et al*., 2000a). Consistent with this result, the present study was further able to show that these isolates have IGS-1 sequences identical to *A. mellea* from Japan. These isolates also grouped with isolates including *A. mellea* from South Korea and *A. mellea* ssp. *nipponica* from Japan.

The presence of a fungus very closely related to or perhaps the same as *A. mellea* ssp. *nipponica* suggests that it has been introduced into Africa, most likely from Asia with tea plants. Although soilborne fungi such as *Armillaria* spp., which do not sporulate regularly, might seem unlikely candidates for intercontinental introductions, there have been two recent studies illustrating such spread. Thus, *A. mellea* is thought to have been introduced into Cape Town, South Africa, by early European settlers, perhaps on *Citrus* trees (Coetzee *et al*., 2001), and *A. gallica* appears to have been introduced into the Kirstenbosch Gardens on ornamental plants (Coetzee *et al*., 2003b). It appears that the fungus closely related to *A. mellea* ssp. *nipponica* on tea in Kenya is another example of such an introduction. The fact that this fungus does not have an inverted 5S gene also makes it very different from all other African *Armillaria* spp.

The Kenyan group III isolates from tea could not be clearly assigned to a species. These isolates have pectic isozyme patterns similar to those of isolates closely related to *A. mellea* ssp. *nipponica*. Comparisons of their IGS-1 DNA sequences suggest that they are most similar to *A. hinnulea*. Phylogenetic analysis indicated that the isolates belonging to this group form a highly supported cluster, but one separate from that representing *A. hinnulea*. The two groups are, however, phylogenetically most closely related to each other by virtue of branches connecting them to a shared internal node. Clearly this group of isolates represents a distinct species, separate from *A. mellea* ssp. *nipponica* and *A. fuscipes* in Kenya. Results of this study have shown that the group differs from other known *Armillaria* spp. based on DNA comparisons and cluster analyses. The taxonomic status of this fungus needs to be assessed once basidiocarps linked to the isolates are obtained and a description can be made.

Kenyan group III in this study is equivalent to SIG III and group II of Mwangi *et al*. (1994) and Otieno *et al*. (2003b), respectively. The fungus representing this group is sparsely dispersed in Kenyan tea plantations (Otieno *et al*., 2003b). It occurs only at higher altitudes (> 2000 m asl) and favours lower growth temperatures than the other two taxa from Kenya (Mwangi *et al*., 1994). Pathogenicity studies have shown that isolates in this group are not pathogenic (Mwangi *et al*., 1994). This result, however, was obtained based on the reaction of one isolate on seedlings of five tree species and should be considered as preliminary. Nonetheless, it appears that this fungus does not contribute significantly to armillaria root rot in Kenyan tea plantations.

The results of the present study have added to the characterization of *Armillaria* spp. occurring in Africa and especially those on tea in Kenya. The results of previous studies showing the presence of at least three different *Armillaria* spp. associated with root rot of tea in Kenya have been confirmed. In addition, it has been shown that it is most likely that only one of these fungi is native to Africa. This is the fungus referred to here as *A. fuscipes*, which appears to have a wide distribution in Africa, occurring in South Africa and as far north as Ethiopia (Coetzee *et al*., 2000a; Gezahgne *et al*., 2004). Outside the African continent, it has been reported only from Ceylon (Sri Lanka) (Petch, 1909). The other two fungi appear to be exotic. One of these is very closely related to *A. mellea* ssp. *nipponica* and the other to *A. hinnulea*. Population genetic studies on these fungi, and especially comparisons with populations from their suggested areas of origin, could provide valuable insights into the international movement of *Armillaria* spp.

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