Mycologia, 95(1), 2003, pp. 160–175.

Characterization of *Armillaria* isolates from tea (*Camellia sinensis*) in Kenya

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Abstract: *Armillaria* is a primary root rot pathogen of tea (*Camellia sinensis*) in Kenya. The main species presently described in this country are *A. mellea* and *A. heimii*. A survey covering fourteen districts of Kenya was carried out and forty-seven isolates of *Armillaria* collected. Cultural morphology, rhizomorph characteristics, somatic incompatibility and features of basidiomata were used to characterize the isolates, together with molecular analysis based on randomly amplified polymorphic DNA (RAPD), inter-simple sequence repeat (ISSR), restriction fragment length polymorphism (RFLP) of the internal transcribed spacer (ITS) and the intergenic spacer (IGS) regions and sequence of the IGS region. It can be concluded that two *Armillaria* species were present and they were different from *A. mellea*. The first group was morphologically similar to *A. heimii* but this was contradicted by the molecular data, suggesting that *A. heimii* could be a complex of several species. The second group was different from the first and morphological and molecular data strongly suggest that it could be a new *Armillaria* species.

Key Words: *Armillaria*, IGS, ISSR, ITS, RAPD, RFLPs, phylogeny

INTRODUCTION

Armillaria is a primary pathogen causing root rot of tea (*Camellia sinensis* (L) O Kuntze) in Kenya. The disease occurs wherever tea is grown in Kenya causing losses as high as 50% in small holder farms (Onsando et al 1997). Identification of *Armillaria* to species level in Africa is limited by the fact that basidiomata and rhizomorphs are usually absent in tropical regions (Gibson 1960, Swift 1968) and that it is frequently detected only as mycelium beneath the bark of infected plants. Rhizomorphs have been observed at high altitudes in Africa and occasionally basidiomata of *Armillaria* have been found in parts of East and Central Africa. These have been identified as African forms of *A. mellea* (Vahl : Fr.) Kummer and *A. heimii* Pegler (syn. *Clitocybe elegans* Heim). This was supported by Mohammed and Guillaumin (1994) who found these two species during a survey in different African countries. They also reported two additional groups of isolates from high altitude regions in Kenya, which were clearly distinct from the two species. One of these groups showed intra-specific somatic incompatibility typical of the pairings between diploids belonging to different clones.

The African *A. mellea*, unlike the species in Europe and North America, is homothallic (Mohammed et al 1994, Abomo-Ndongo et al 1997). Homothallism has also been reported in *A. mellea* isolates from Japan (Cha et al 1995, Ota et al 1998). Ota et al (2000) confirmed by isozyme and RAPD analysis that *A. mellea* isolates from Africa were identical to *A. mellea* from Japan. *A. heimii* has been described as a variable species having both homothallic and unifactorial heterothallic forms (Abomo-Ndongo et al 1997) associated with various hosts in different regions of tropical Africa. This unifactorial heterothallism seems to be unique to *A. heimii*.

The homothallic nature of some of these *Armillaria* spp. together with the rare occurrence of their basidiomata have restricted the use of mating tests (Korhonen 1978) and basidiomata morphology for the identification of African species. In most basidiomycetes, at least in tetrapolar species (for instance in *Armillaria* of the temperate regions), somatic incompatibility is used for the study of intraspecific variability and the distinction of species is based on sexual compatibility/incompatibility. Somatic incompatibility has been used to distinguish isolates of African *Armillaria* (Abomo-Ndongo and Guillaumin 1997). However, most attempts to characterize these have tended to employ methods that do not depend on the presence of basidiomata or haploid forms; e.g.,

Accepted for publication July 9, 2002.

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techniques based on the use of isozyme electrophoresis (Mwangi et al 1989, Agustian et al 1994, Mwenje and Ride 1996, 1997), molecular markers such as DNA restriction fragment polymorphisms (Anderson et al 1987, Smith and Anderson 1989), RAPD (Mohammed 1994), nuclear DNA-DNA homology (Jahnke et al 1987), and DNA sequence analysis (Anderson and Stasovski 1992). Analysis of the ribosomal DNA spacers, ITS and IGS, from *Armillaria* isolates collected from various geographical areas in tropical Africa discriminated *A. mellea*, *A. heimii* and a possible new species (Chillalli et al 1997). RFLP and nucleotide sequence data of the IGS region of ribosomal DNA operon were recently used to distinguish South African isolates (Coetzee et al 1998, 2000). They showed that both nuclear and organelle DNA-based molecular markers provide an alternative to mating tests and basidiomata morphology that can aid systematics of *Armillaria* in Africa.

Identification of *Armillaria* species in some African countries remains unresolved as highlighted by Coetzee et al (2000) who studied *Armillaria* in pine plantations in South Africa where *A. mellea* and *A. heimii* have been described. They concluded that isolates identified as *A. heimii* were either an undescribed *Armillaria* sp. or *A. fuscipes* (Petch 1909).

The objective of the present study was to characterize the *Armillaria* species causing root rot of tea in Kenya by different methods based on morphology, somatic incompatibility and DNA profiles generated by PCR with RAPD, ISSR, RFLPs of the ITS and IGS region and sequence of the IGS region.

MATERIALS AND METHODS

Origin of the isolates and culture.—*Armillaria* isolates were collected in the main tea growing districts in Kenya (FIG. 1) during a survey carried out between October and December 1997. These are listed in TABLE I. The fungus was isolated by removing mycelial fans from beneath the bark of roots and plating them on 3% MEA (3% Oxoid malt extract, 2% Lab M agar No. 1, in distilled water) containing 30 ppm of rose bengal (Sigma, USA) and 120 ppm of streptomycin (Sigma, USA). Isolations from basidiomata found in nature were carried out from both basidiospores and the pileus on 3% MEA. Six reference isolates were donated by Dr J-J Guillaumin (INRA Clermont-Ferrand, France) for purposes of comparison (TABLE II).

Somatic incompatibility.—Agar discs bearing undifferentiated mycelia without crust or rhizomorphs were cut from margins of two week old colonies using a 4 mm diameter cork borer. The discs were placed 5–10 mm apart on the surface of 3% MEA. Self pairings were done as controls. The cultures were incubated in the dark at room temperature (approx 22 C) and observed over a period of 2 to 3 wk for compatibility reactions.

Somatic incompatibility among some of the isolates was also studied using the method described by Hopkin et al (1989). The isolates were paired by placing small discs (3 mm diameter) from the edge of the colonies 5 mm apart on the surface of sterile cellophane on 2% MEA (2% Merck malt extract, 1.5% agar, in distilled water) as described by Abomo-Ndongo and Guillaumin (1997). Two replicates of each pairing were prepared. The plates were incubated in the dark at 20 C. After 21 days, 2 cm^2 of agar was cut around the paired isolates and immersed in a freshly prepared solution of 0.05% of L-Dopa (Sigma, UK) in a pH 7 potassium phosphate buffer (Sambrook et al 1989). The squares were incubated at 37 C for 1 h and then examined under the stereomicroscope for the presence of a black line between the thalli.

Cultural morphology.—Morphological characteristics of all isolates were studied on 2% MEA and 3% MEA containing 0.06% peptone (Oxoid) (MEA + P). Monospore and somatic cultures were studied on 3% MEA. These were incubated in the dark at room temperature (approx 22 C). Colony growth and morphological characteristics were observed over a period of 4 wk.

Rhizomorphs produced by woody inocula.—Stems of cassava (*Manihot esculenta*) 2.0–2.5 cm diameter were cut into segments 6 cm long. Ten of these were placed in 1-L Kilner jars containing 300 mL tap water and autoclaved for 15 min at 121 C then left to cool. The stems were inoculated by aseptically placing 4 mm diameter discs of 1-wk-old agar cultures on their upper ends. Inoculated stems were incubated in the dark at room temperature (approx 22 C) for approximately 12 wk. Such colonized stems were used as inocula. For each isolate three replicate inocula were buried singly in 800 cc vermiculite in a 1 L plastic pot. The pots were placed under a greenhouse bench at a temperature of about 18 C. The inocula were kept moist by adding 200 mL tap water to the surface of the vermiculite in each pot once every week. After 12 wk the inocula were removed, vermiculite gently washed off and observations made of the growth patterns of any rhizomorphs.

Basidiomata.—Gross morphological features of basidiomata were described and measurements of pileus, stipe, basidia, and basidiospores were recorded. They were examined for the presence of clamp connections.

Production of basidiomata *in vitro* was attempted in 1 L flasks. The medium consisted of 50 g milled beech (*Fagus sylvatica*) wood sawdust, 20 g whole blended orange, 60 g whole grain rice, 10 g peptone (Oxoid), and 5 g agar. The mixture was made up to 400 mL with water and autoclaved for 1 h at 121 C (Tirrò 1991). The flasks were inoculated and incubated at 25 C for 4 wk in the dark. After this period, the temperature was adjusted to 20 C with a 12-h photoperiod.

Extraction and purification of DNA.—Two different methods were used for the DNA extraction.

Method A: A modified CTAB method was used to extract DNA for RAPD and ISSR (Wachira et al 1997). The freeze-dried mycelium harvested from one Petri dish was ground up in CTAB buffer and the DNA extracted using chloro-

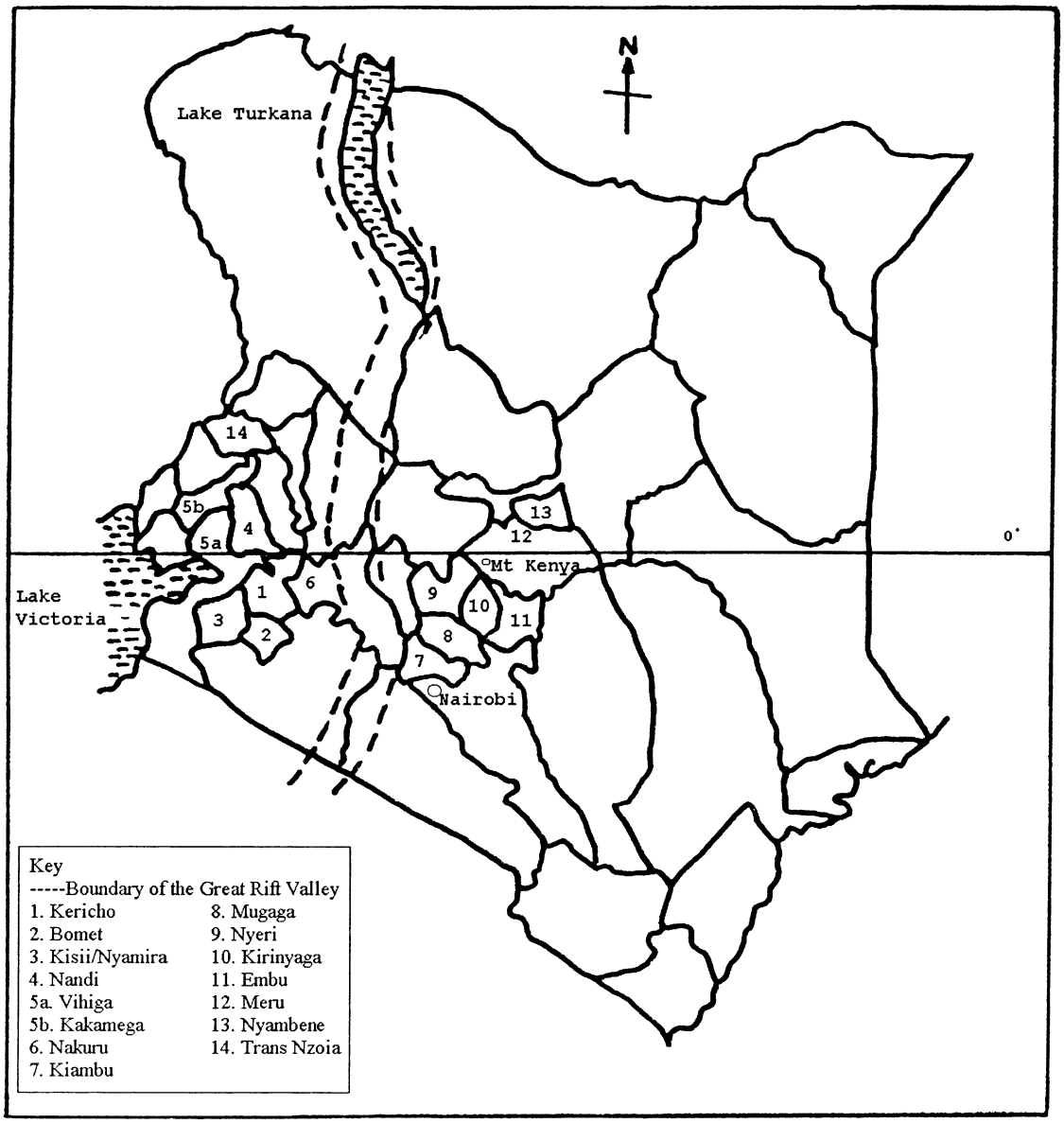


FIG. 1. Map of Kenya showing the districts where tea is grown and where the *Armillaria* isolates were collected.

form/isoamyl alcohol. The resulting nucleic acid was treated with RNAse overnight and the DNA was purified and precipitated.

Method B: This method was used to extract DNA for PCR-RFLPs. The isolates were grown in liquid media (1% malt extract, 0.5% yeast extract and 1% glucose). The use of 100-ppm oxytetracyclin (Sigma, UK) and 200-ppm streptomycin (Sigma, UK) was needed to avoid bacterial contamination. The flasks were incubated unshaken in the dark at 20 C for three weeks. The mycelium was harvested, rinsed with distilled water, frozen in liquid nitrogen and stored at −80 C. The total DNA of the isolates was extracted from frozen mycelium using the Dneasy™ Plant Mini Kit (Qiagen, Germany). The mycelium was first mechanically disrupted by grinding it to a fine powder under liquid nitrogen using a mortar and pestle. Then the kit protocol was followed.

Polymerase chain reaction (PCR) amplification.—Two different methods were used for the amplification. For DNA obtained with method A, each DNA sample (0.5 μL) was added to an amplification reaction solution consisting of 1.0 μL reaction buffer (×10), 1.0 μL $MgCl_2$, 0.5 μL dNTPS, 1.0 μL primer, 0.1 μL Taq polymerase (Applied Biotechnologies, U.K) and sterile distilled water to a volume of 10 μL. PCRs were performed for eleven RAPD primers: OPB-07, OPC-02, OPD-05, OPD-20, OPI-13, OPU-05, OPV-17, OPW-02, OPW-06, OPG-06, and OPM-04 (Operon Technologies Inc. USA) with the conditions at 94 C for 5 min (hot-start) then 93 C for 1 min, 42 C for 1.5 min, and 72 C for 1 min for 40 cycles, and a final extension phase of 5 min at 72 C. Six ISSR primers: dinucleotide repeats (GA)8T, (CT)8T, (GT)8CG, (AT)8T, (AG)8C and trinucleotide repeats (ACC)6 (International Livestock Research Institute,

TABLE I. Isolates of Kenyan *Armillaria* collected by Washington Otieno

District	Division	Isolate	Host	Altitude (m)	Group	GenBank
Kericho	Buret	$1BU_1$	*Camellia sinensis*	1800–2000	I	
	Ainamoi	$1AI_1$	*Dombeya* sp.	>2000	I	
	Ainamoi	$1AI_{2\text{-}3}$ (11)[a]	*Camellia sinensis*	>2000	II	AF513025/AF513030
	Belgut	$1BE_1$	*Camellia sinensis*	1800–2000	II	AF513023
Bomet	Konoin	2K (2)	*Camellia sinensis*	>2000	I	
Nandi	E. Nandi	$4EN_1$	*Camellia sinensis*	1800–2000	I	
Vihiga	Hamisi	$5H_1$	*Camellia sinensis*	<1800	I	
Nakuru	Molo	$6M_1$	*Camellia sinensis*	>2400	II	AF513024
Kiambu	Githunguri	7GI (6)	*Camellia sinensis*	1800–2000	I	
	Gatundu	$7GU_3$	*Camellia sinensis*	1800–2000	I	
	Lari	$7L_1$	*Camellia sinensis*	1800–2000	I	
Muranga	Kangema	$8KA_1$	*Camellia sinensis*	1800–2000	I	
Nyeri	Tetu	$9T_1$	*Camellia sinensis*	1800–2000	I	
	Tetu	$9T_2$	*Camellia sinensis*	1800–2000	II	AF513021
Kirinyaga	Gichugu	10G (4)	*Camellia sinensis*	1800–2000	I	
	Gichugu	$10G_3$	*Eucalyptus* sp.	1800–2000	I	
	Gichugu	$10G_5$	*Coffea arabica*	1800–2000	I	
	Ndia	$10N_1$	*Camellia sinensis*	1800–2000	I	
Embu	Manyatta	11MA (2)	*Camellia sinensis*	1800–2000	I	
Meru	S. Imenti	$12SI_1$	*Dioscorea* sp.	1800–2000	I	
	S. Imenti	12SI (2)	*Camellia sinensis*	1800–2000	I	
	S. Imenti	$12SI_3$	*Coffea arabica*	1800–2000	I	
	S. Imenti	$12SI_5$	*Musa acuminata*	1800–2000	I	
Nyambene	Tigania	$13T_1$	*Camellia sinensis*	1800–2000	I	
	Tigania	$13T_2$	*Camellia sinensis*	1800–2000	II	AF513022
Trans Nzoia	Cheranganyi	$14C_1$	*Camellia sinensis*	1800–2000	I	

[a] Number of isolates collected in the same plantation but from different plants.

Nairobi, Kenya) were also screened with the conditions at hotstart then 94 C for 30 s, 52 C for 45 s, and 72 C for 2 min in 40 cycles. The amplification was performed with a Techne: Model FGENO2TD (UK) thermocycler.

For DNA obtained with method B, the ITS was amplified as described by Chillali et al (1997) with the universal primers ITS1 and ITS4 (White et al 1990). The IGS of the ribosomal DNA between the 26S and 5S gene of the isolates was amplified with two different sets of primers. The first set included the primers LR12R and O-1 (Harrington and Wingfield 1995). The second set included the primers P-1 and 5S–2B (Coetzee et al 1998). The PCR program to amplify the IGS was described by Sierra et al (1999). Ready-To-Go PCR beads (Amersham Pharmacia Biotech) were used for the PCR amplification. Individual reactions were brought to a final volume of 25 μL. Each reaction contained 1.5 units of *Taq* DNA polymerase, 10 mM Tris-HCl, 50 mM KCl, stabilizers including BSA, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 0.1 μM of each primer and purified water (Sigma Chemical Co). The amplifications were performed on a Progene (Techne, UK) thermocycler.

Electrophoresis, data analysis, and RFLPs.—Following method A, electrophoresis was performed using 1.5% agarose (ABgene, UK) gel in Tris-boric acid-EDTA (TBE). Afterwards it was stained in ethidium bromide (Sigma, UK) (10 μL/L of water).

The data were scored and entered in a computer as binary matrices where 0 coded for absence and 1 for presence of a band. Bands produced consistently over three amplifications from the same DNA extraction were scored. Estimates of similarity were based on the total number of

TABLE II. Additional isolates for comparison purposes

Isolates	Host	Country	Altitude (m)	Collector	Identity	GenBank
ST1	*Theobroma cacao*	São Thomé	1000–1500	Unknown	*A. mellea*	
K5	*Cupressus funebris*	Kenya	>2000	IAS Gibson	*A. mellea*	AF513029
K8	*Grevillea robusta*	Kenya	Unknown	M. Ivory	*A mellea*	
K10	*Pinus patula*	Kenya	>2000	Unknown	*Armillaria* sp	AF513027
K12	*Pinus patula*	Kenya	>2000	IAS Gibson	*Armillaria* sp	AF513026
K14	*Pinus radiata*	Kenya	>2000	IAS Gibson	*Armillaria* sp	AF513028

shared fragments (Nei and Li 1979). The principal component and the average linkage cluster analyses were performed using GENSTAT 5 Version 4.1. The unweighted pair group method with arithmetic mean (UPGMA) was used to construct the phenotypic similarity.

Following method B, the ITS and IGS amplified product were purified with a QIAquick™ Purification Kit (Qiagen, Germany). The ITS purified product was digested with the restriction enzymes *Alu* I, *Hinf* I and *Nde* II (Chillali et al 1997). The restriction enzyme analysis was performed separately by adding 5 units of enzyme (Amersham Pharmacia Biotech, UK) and incubating at 37 C for at least an hour. The IGS purified product was digested with the restriction enzyme *Alu* I (Harrington and Wingfield 1995). The digested fragments of the ITS and IGS regions were separated in a 3% agarose (Sigma, UK) gel containing 7.5 μL of ethidium bromide (Sigma, UK).

IGS sequence and phylogenetic analyses.—Sequencing of the IGS region between the 26S and 5S of some of the isolates was done using the primers LR12R and O-1 by MWG BIOTECH AG Ebersberg (Germany). Additional sequences from the GenBank databases available through the National Center for Biotechnology Information (NCBI, Bethesda, Maryland) were obtained using the search facility Blast. The sequences were edited and aligned with the software for Macintosh, EditSeq and MegAlign of Lasergene (DNASTAR 2000) programs. The alignments were initially done with the CLUSTAL option in MegAlign and were manually adjusted. Insertions and deletions were coded using MacClade (Maddison and Maddison 1992). The alignment of the IGS was submitted to EMBL-Align database as ALIGN_000374. Phylogenetic analyses were performed using PAUP version 4.0b10 (Swofford 1998). Heuristic searches using maximum parsimony with TBR (Tree Bisection Reconnection) branch swapping were performed in PAUP. Clade support was evaluated using Jackknife analysis (in PAUP) with 30% deletion and fast stepwise addition was calculated with 10 000 replicates. Groups shown in 50% or more of the trees were retained.

RESULTS

Somatic incompatibility.—Two types of reactions were observed between colonies of paired isolates: the merging of mycelia at points of contact or failure of mycelia to merge. Isolates whose colonies failed to merge and which produced a pigmented line at their interfaces were recorded as incompatible. The presence of a dark line of demarcation at the interface of the colonies was noted as the principal indicator of isolates belonging to different groups. Isolates of Group I did not produce a dark demarcation line in pairings with themselves but were separated by a dark line from isolates of Group II. In pairings between isolates of Group II, the dark demarcation line was present. The same results were obtained using the method described by Hopkin et al (1989).

Cultural morphology and rhizomorph characteristics.—The appearance of colonies of individual isolates was similar in the two media tested. On the basis of their colony morphology, the isolates were placed in two groups (TABLE I). Group I consisted of isolates with flat, crustose, rhizomorphogenic colonies. The entire colony often turned into a network of rhizomorphs with only a small mycelial center. White/grey mycelium was observed at the colony centers and on rhizomorph surfaces. The rhizomorphs were compact or open in appearance (due to high or low frequency of branching), both submerged and aerial, cylindrical or flat. Group II consisted of isolates which had white, raised, typically mycelial colonies that became dark brown as they aged, some of them with thin submerged cylindrical rhizomorphs visible from the underside of colonies and some of them with no rhizomorphs (FIG. 2).

Isolates in Group I produced rhizomorphs in vermiculite with three different branching patterns: dichotomous, palmately branched and unbranched rhizomorphs. No rhizomorphs or very few unbranched rhizomorphs were formed by isolates in Group II.

Basidiomata and rhizomorphs in nature.—Basidiomata were infrequent and during the rainy season were found in only one tea plantation in Kericho (location: 0°22′S; 35°21′E; altitude >2180 m). The cultures obtained by direct isolation from the basidiomata flesh were placed in Group I by morphology and the occurrence of the dark demarcation line in pairings.

Basidiomata in nature occurred typically in clusters of 5–21, fused at the point of attachment to the base of infected plants (FIG. 3). The pileus was (8.5–)10–15(–16.5) mm in diameter, convex, applanate to umbonate, with a non-striate margin, light ochraceous but dark-brown at the disk center. The stipe was creamy white in color, 45–50 × 3–6 mm with a whitish, fugacious annulus attached to its upper quarter. Lamellae were white cream in color. Basidia were 30–35 × 6–7 μm, elongate clavate with 4 sterigmata. The basidiospores were (4.5–)5.0(–7.5) × (4.0–)5.0(–6.5) μm, sub-globose to ovoid. Clamp connections were absent.

Colonies that arose from basidiospores were characteristically fluffy with raised mycelium when young but became rhizomorphic, flat, and crustose as they aged. In contrast, colonies that arose from the pileus were consistently rhizomorphic, flat, and crustose as described for isolates in Group I. The majority of isolates had the colony type that was characteristically crustose and rhizomorphic with thin mycelium at the center. However, individual isolates showed minor differences. Characteristics of rhizomorphs in culture

FIG. 2. Typical culture morphology of isolates in Group I (left) and isolates in Group II (right) on 2% MEA.

FIG. 3. Basidiomata of *Armillaria* observed in Kericho at the base of tea plant.

varied slightly with the medium, production being more abundant and branching more frequent in 3% MEA + P than in 2% MEA.

The basidiomata formed *in vitro* were immature. Only one isolate from Group II (9T2) produced these. Pileus and stipe were differentiated but the lamellae edge was sterile and crowded with basidio-like cheilocystidia that were fusoid, clavate to ventricose in shape, hyaline and thin walled. No true basidia or basidiospores were found. Temperature was a key factor for the induction of growth for isolates in Group II. The fungus showed growth only when temperature was lowered from 25 C to 20 C.

In nature, very few, if any, rhizomorphs were found from the base of the stipe downwards but, if present, these were firmly in contact with the root cortex running over the surface of the bark. Rhizomorphs firmly in contact with the roots of infected plants were found for all isolates except isolates $1AI_2$ (8 isolates) and $13T_2$ from Group II and isolates $7GI_2$, $10G_4$ from Group I. An extensive subterranean network of rhizomorphs was found only for isolates $6M_1$ and $9T_2$ from Group II.

DNA amplification and polymorphism.—Following method A, the total cellular DNAs of *Armillaria* were used as templates and therefore the genomic origin of the amplified RAPD and ISSR fragments in the isolates could not be specified. Successful DNA amplification was, however, obtained with 10 RAPD and 3 ISSR primers that gave respectively a total of 181 and 39 fragments with an average of 20 and 13 fragments per primer. The amplified fragments ranged in sizes from <564 to 1977 bp for RAPD and <564 to 1685 for ISSR primers. The primers OPM-04, (AT)8T, (AG)8C and (ACC)6 failed to amplify fragments from the fungal DNA. Of the amplified fragments, 94.5% and 89.7%, respectively, were polymorphic for the RAPD and the ISSR primers but only 127 and 29 fragments, respectively, of these were considered for analysis to derive similarity values.

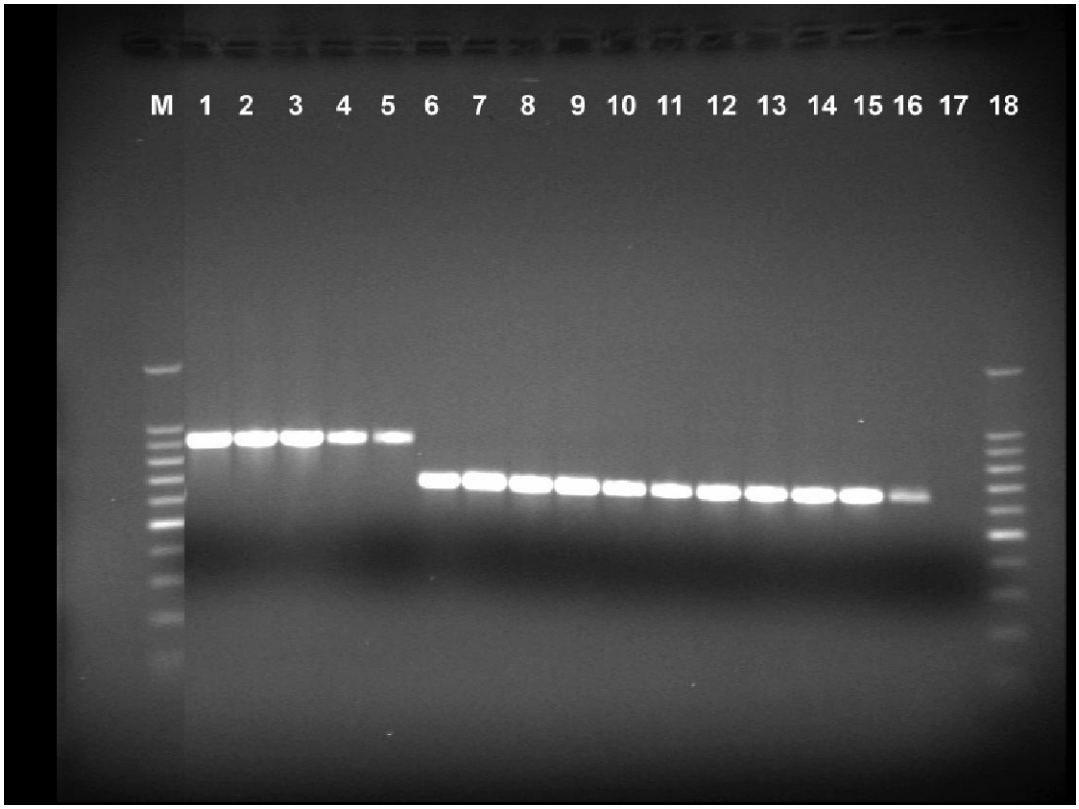
Following method B, amplification of the ITS with the primers ITS1 and ITS4 gave a single fragment of about 700 bp for the isolates in Group I and a single fragment of about 900 bp for the isolates in Group II (FIG. 4) and also for the reference isolates ST1, K5, K8, K10, and K12 (data not shown). A band of about 800 bp was obtained for the isolate K14 (data not shown).

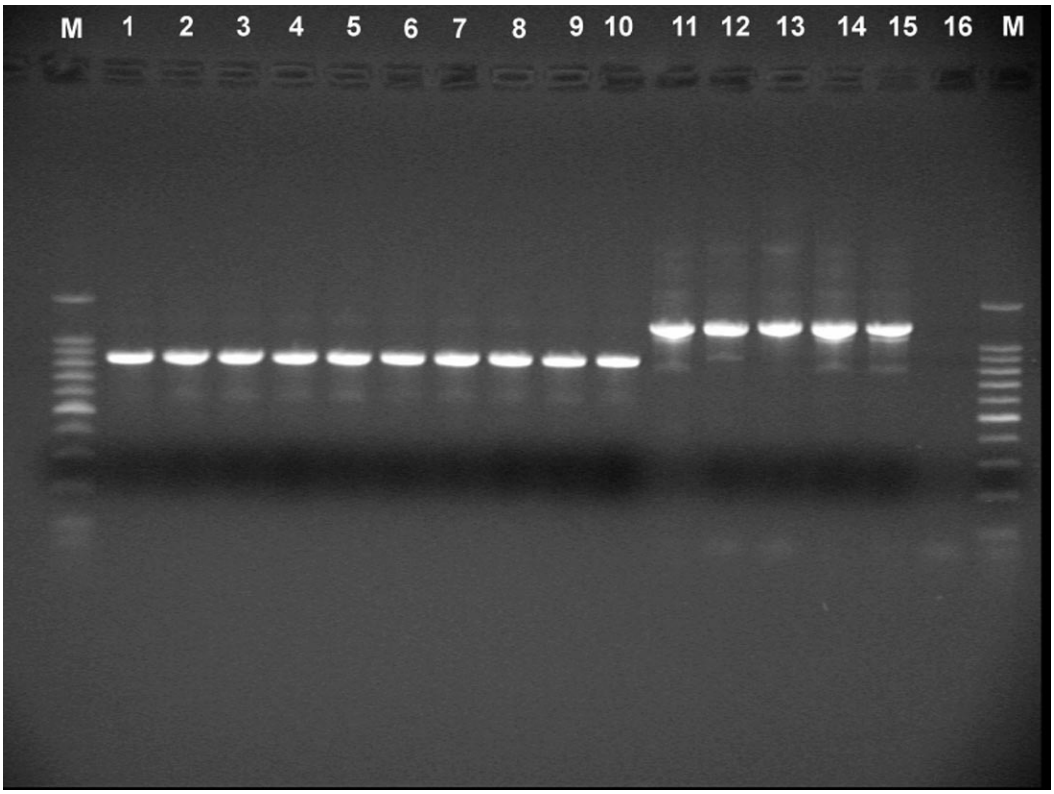
The amplification of the IGS region of the ribosomal DNA of the isolates in Group I with the primers P-1 and 5S-2B gave one single fragment over 1000 bp. No amplification bands were obtained with the primers LR12R and O-1, except for isolate $11MA_2$, which showed a very poor amplification band. Amplification of the isolates in Group II with the set of primers LR12R and O-1 gave a single fragment of approximately 900 bp. The same result was obtained for reference isolates ST1, K5, K8, K10, and K12 (FIG. 5). Isolate K14 gave a band of about 800 bp (data not shown). Amplification with the primers P-1 and 5S-2B from these isolates was very poor.

RFLPs.—The ITS restriction patterns obtained for isolates in Group I gave a pattern of about 480, 160, 85 bp with *Alu* I, a pattern of about 220, 190, 170, 72 bp with *Hinf* I (FIG. 6), and a pattern of about 390, 250 bp with *Nde* II. Isolates in Group II gave a pattern of about 510, 225, 95 bp with *Alu* I, a pattern of about 360, 230, 150, 100 bp with *Hinf* I (FIG. 6), and a pattern of about 590, 270 bp with *Nde* II. The restriction patterns of reference isolates K10 and K12 were identical to the patterns obtained for isolates in Group II. Fragments below 100 bp were only included when clearly visualized. Reference isolates ST1, K5, and K8 showed restriction patterns that differed from the above, approximately 320, 235, 190, 150 bp with *Alu* I, 280, 180, 170, 140, 100 bp with *Hinf* I, and 280, 240, 230, 150 bp with *Nde* II. Isolate K14 also gave different patterns from all the above (data not shown).

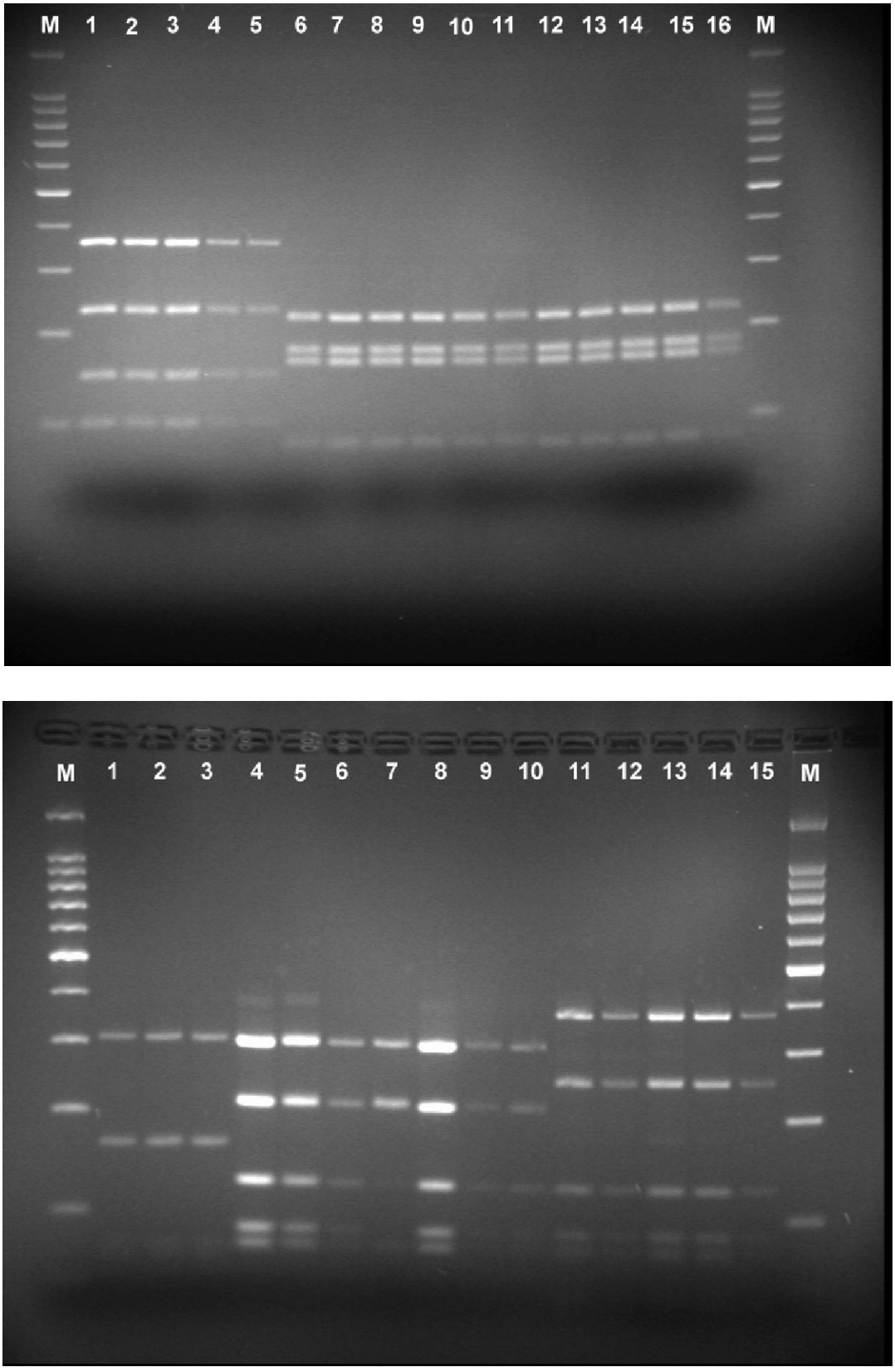
The digestion of the IGS amplification product with the restriction endonuclease *Alu* I (FIG. 7) showed restriction fragments of approximately 380, 245, and 135 bp for isolates in Group I and approximately 310, 220 and 135 bp for isolates in Group II. Reference isolates K10 and K12 showed identical pattern to isolates from Group II, whereas reference isolates ST1, K5, and K8 showed restriction fragments 310, 170 bp, respectively, and isolate K14 showed a different pattern from the above (not shown).

Phenotypic similarity among the isolates.—The matrix of similarity coefficient values based on shared fragments showed that similarities among the isolates ranged from 95% (between isolates $10G_5$ and $10G_6$) to 29% (between isolates $1BU_1$ and $10G_3$). A dendrogram based on average linkage cluster analysis resolved the 47 isolates into two major clusters (FIG. 8). The larger of these represented isolates from morphological Group I and showed differences that indicate greater variability among isolates than in those within the smaller cluster. The smaller cluster consisted of isolates in Group II. This variability is also evident in the principal co-ordinate plot (FIG. 9). The principal co-ordinate plot placed all the isolates in Group I together in a large cluster where three subgroups could be discerned. Isolates $1AI_1$, $1BU_1$, $2K_2$, $7GI_3$, $7GI_6$, $7GU_3$, $9T_1$, $10G_1$, $10G_4$, $10G_5$, $10G_6$, $10N_1$, $12SI_5$, $13T_1$, and $14C_1$ were placed together in a subgroup separate from ten other isolates $2K_1$, $4EN_1$,





FIGS. 4–5. Agarose gel (3%) showing the amplified PCR product for the ITS (Fig. 4) region for isolates $1AI_{2e}$, $1BE_1$, $6M_1$, $9T_2$, $13T_2$, $1BU_1$, $4EN_1$, $5H_1$, $7L_1$, $7GI_2$, $9T_1$, $10G_5$, $11MA_2$, $12SI_2$, $14C_1$, $1AI_1$, and negative control, and IGS region (Fig. 5) for isolates ST1, K5, K8, $1AI_{2e}$, $1BE_1$, $6M_1$, $9T_2$, $13T_2$, K10, K12, $1BU_1$, $7L_1$, $7GI_2$, $11MA_2$, $14C_1$, and negative control. A 100 bp ladder was used as a marker in the first and last lanes.



FIGS. 6–7. Agarose gel (3%) showing the *Hinf* I restriction pattern of the ITS region (Fig. 6) for the isolates $1AI_{2e}$, $1BE_1$, $6M_1$, $9T_2$,13T2, $1BU_1$, $4EN_1$, $5H_1$, $7L_1$, $7GI_2$, $9T_1$, $10G_5$, $11MA_2$, $12SI_2$, $14C_1$ and $1AI_1$, and the *Alu* I restriction pattern for the IGS (Fig. 7) for the isolates ST1, K5, K8, $1AI_{2f}$, $1BE_1$, $6M_1$, $9T_2$, $13T_2$, K10, K12, $10G_5$, $7L_1$, $7GI_2$, $5H_1$, and $14C_1$. A 100 bp ladder was used as a marker in the first and last lanes.

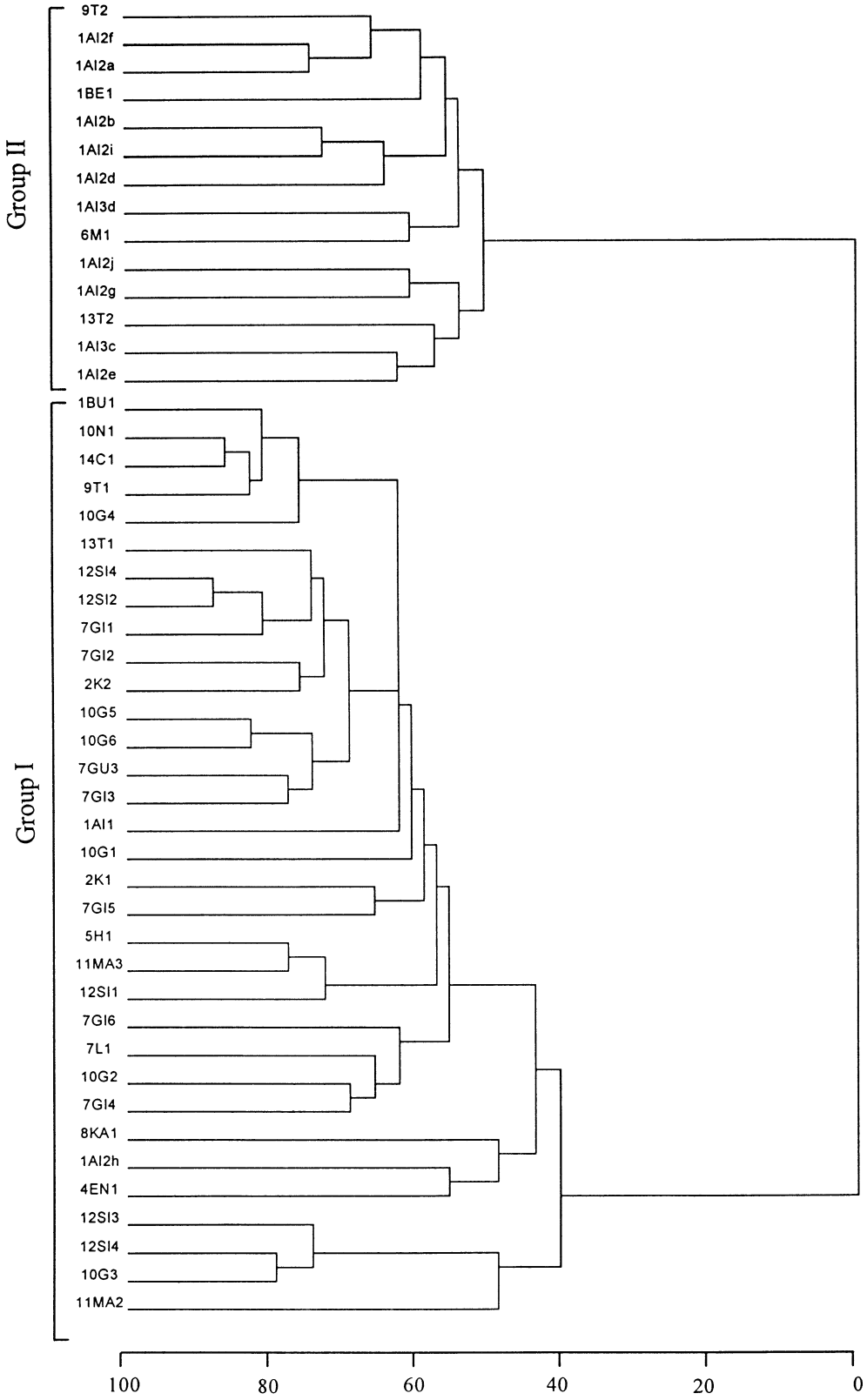


FIG. 8. Dendrogram of *Armillaria* isolates (listed in TABLE I) based on average linkage analysis.

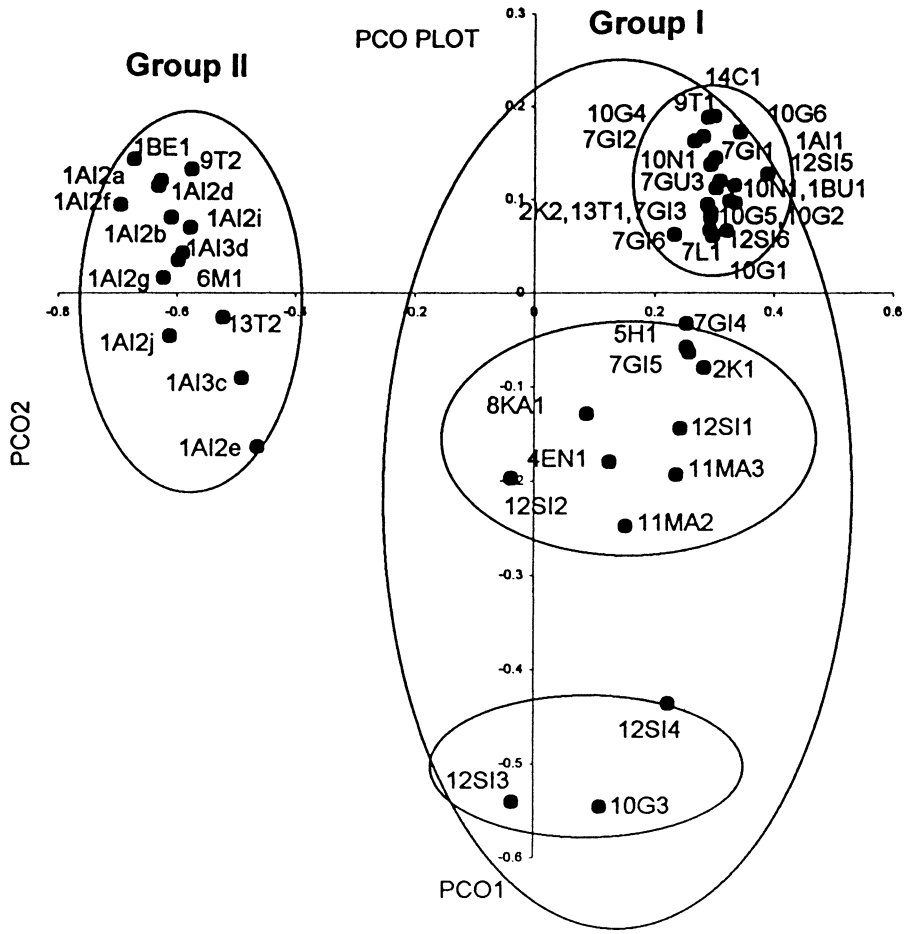


FIG. 9. Different clusters of 47 isolates of *Armillaria* (listed in TABLE I) using average linkage cluster analyses based on similarity data. The first two principal components accounted for 49% of the total variation revealed.

$5H_1$, $7GI_4$, $7GI_5$, $8KA_1$, $11MA_2$, $11MA_3$, $12SI_1$, and $12SI_2$ in another sub-group, and isolates $10G_3$, $12SI_3$, and $12SI_4$ forming the most distant sub-group. The principal co-ordinate plot placed all the isolates in Group II together in the smaller cluster and had no major sub-clusters.

Phylogeny.—The sequences of the IGS of the isolates in Group II were identical. The sequences were submitted to GenBank (National Center for Biotechnology Information) and their accession numbers appear in TABLE 1. The sequence obtained was compared with sequences published in GenBank using the Blast search facility, and five *Armillaria* sequences, *A. gemina* (AF243053/AF243054), and *A. ostoyae* (AF243048/AF243050/D89924) matched most closely to the sequence. Other *Armillaria* species selected for comparison were *A. borealis* (AF243056), *A. calvescens* (AF243070/AF243071), *A. cepistipes* (AF243068/AF243069), *A. lutea* (=*gallica*) (AF243066), *A. nabsnona* (A243060), *A.* sp. NABS X (AF243061), *A. sinapina* (D89925), *A. singula* (D80026), *A. jezoensis* (D89921), *A. cepistipes* (D80019), and *A. gallica* (=*lutea*) (D89920) (TABLE III). Because there was the possibility of this group's being a form of *A. mellea*, the sequences of the IGS of 20 *A. mellea* published in GenBank were included (TABLE III). Isolates from Group I could not be included in the analysis because they had less than 10% sequence similarity with any other *Armillaria* species and an alignment was not possible. *Lepiota castaneidisca* (AF391078), *Lepiota cristata* (AF391071), *Schizophyllum commune* (AF249462), and *Schizophyllum commune* (AF249532) were used as outgroups.

A heuristic search with all the isolates yielded 75 equally parsimonious trees. A strict consensus was calculated. The alignment generated 672 characters, 418 of which were parsimony-informative (62.2%). The tree generated (FIG. 10) showed four major clades (A–D). Clade A comprises the *Armillaria* isolates from Group II together with the reference isolates K10 and K12. This had 100% Jackknife support. Long branches (99 steps) suggested some distance in the relationship of this clade with the remaining clusters. Clade B comprises the closest matches from

TABLE III. List of isolates which IGS sequences were retrieved from GenBank

Name	Code	Alternative code	Host	Origin	Collector	GenBank
A. borealis	331-1		*Betula* sp.	Finland		AF243056
A. calvescens	21-2		*Acer saccharum*	USA		AF243070
A. calvescens	11-9		*Acer saccharum*	USA		AF243071
A. cepistipes	311-1		Lawn	Finland		AF243068
A. cepistipes	304-1		*Alnus glutinosa*	Finland		AF243069
A. cepistipes	F306		*Galeola septentrionalis*	Japan		D89919
A. gallica (=*lutea*)	HUA9125		*Prunus ssiori*	Japan		D89920
A. gemina	35-5		Unknown	USA		AF243053
A. gemina	160-8		Unknown	USA		AF243054
A. jezoensis	HUA9116		*Ulmus japonica*	Japan		D89921
A. lutea (=*gallica*)	90-4		*Fraxinus* sp.	USA		AF243066
A. mellea	B1245	CMW4627, D-5	*Prunus* sp.	France	Unknown	AF163598
A. mellea	B527	CMW4607, PM8	Unknown	France	Guillaumin, JJ	AF163599
A. mellea	B525	CMW3957, KD1	Unknown	France	Guillaumin, JJ	AF163600
A. mellea	B1247	CMW4628, P-5580	*Chamaecyparis*	England	Sierra, A-P	AF163601
A. mellea	B176	CMW3179, M1	*Rosa* sp.	England	Rishbeth, J	AF163602
A. mellea	B1240	CMW4624, AM1	Unknown	England	Sierra, A-P	AF163603
A. mellea	B186	CMW4603, BQ5F	Unknown	England	Gregory, S	AF163604
A. mellea	B1212	CMW4615, 94056/1	Unknown	Hungary	Szanto, M	AF163605
A. mellea	B1205	CMW4613, 86009/1	Unknown	Iran	Saber, M	AF163606
A. mellea	B929	CMW3962	*Mayten boria*	USA	Bruns, TD	AF163607
A. mellea	B927	CMW3964	*Quercus*	USA	Bruns, TD	AF163608
A. mellea	B1217	CMW4619, 216	Unknown	USA	Raabe, B	AF163609
A. mellea	B731	CMW3967, 1003″	*Chamaecyparis* sp.	Japan	Shaw, CG	AF163610
A. mellea	B608	CMW3966, AF	Unknown	South Korea	Sung, J-M	AF163611
A. mellea	B916	CMW4610, A-5	Unknown	South Korea	Sung, J-M	AF163612
A. mellea	B917	CMW4611, A-2	Unknown	South Korea	Sung, J-M	AF163613
A. mellea	B495	CMW3155, NABS VI, 49-5	*Acer saccharum*	USA	Anderson, JB	AF163614
A. mellea	B497	CMW3956, NABS VI, 97-1	*Acer rubrum*	USA	Anderson, JB	AF163615
A. mellea	B282	CMW4605, TCH-2-1	*Betula populifolia*	USA	Harrington, TC	AF163616
A. mellea	B623	CMW4609, PJZ-87-1A	Unknown	USA	Zambino, PJ	AF163617
A. mellea ssp. *nipponica*	HUA93110		*Fraxinus lanuginosa*	Japan		D89922
A. nabsnona	207-4		Hardwood	Canada		F243060
A. sp. NABS	206-1		Conifer	Canada		AF243061
A. ostoyae	300-2		*Pinus strobus*	Canada		AF243048
A. ostoyae	27-1		*Picea glauca*	USA		AF243050
A. ostoyae	HUA9242		*Gastrodia elata*	Japan		D89924
A. sinapina	HUA9124		*Ulmus japonica*	Japan		D89925
A. singula	HUA9101		*Fraxinus mandshurica*	Japan		D89926

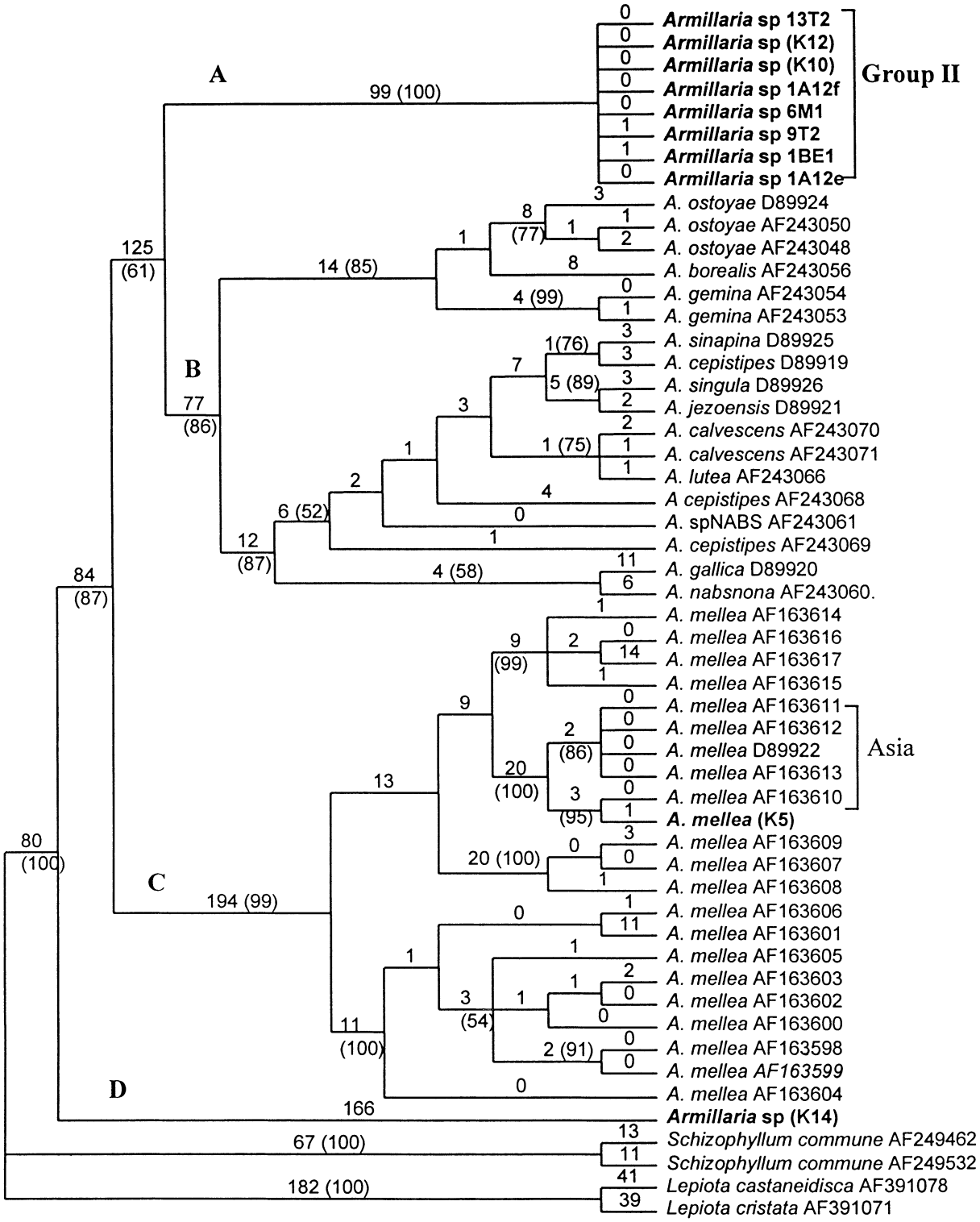


FIG. 10. One of the 75 equally parsimonious trees recovered using sequences of the IGS. *Lepiota castaneidisca* (AF391078), *Lepiota cristata* (AF391071), *Schizophyllum commune* (AF249462) and *Schizophyllum commune* (AF249532) were used as outgroups. Plain figures indicate branch lengths and figures in brackets show the Jackknife support value. Length = 1038, CI = 0.784, RI = 0.920.

Blast search and the other *Armillaria* selected for comparison (all mentioned above). Clade C comprises all the *A. mellea* isolates, including *A. mellea* from Kenya (reference isolate K5). This isolate was grouped with *A. mellea* from Japan. Clade D was formed by the reference isolate K14, which was different to the rest of the isolates from Kenya.

DISCUSSION

Somatic incompatibility and cultural morphology of the Kenyan *Armillaria* isolates separated them into two groups. Molecular data based on RAPD, ISSR, and RFLPs of the ITS and IGS regions separated the isolates into the same two clear and distinct groups. Except by the somatic incompatibility results, Group I showed higher variability among the isolates. This may suggest that Group I represents a more ancestral form of the fungus in Kenya, but further studies are required to be conclusive. Also, additional isolates from Group II are needed to see if they show any variability.

Basidiomata of *Armillaria* were found only in one tea plantation located at a high altitude (2180 m) in Kericho and they corresponded to Group I. This confirms that natural fructification by the fungus occurs rarely in Africa and may be limited to the cooler areas of the continent (Mohammed et al 1994, Mwangi et al 1994). The description of the basidiomata conforms to that of *A. heimii* (Heim 1963, Pegler 1977) except for the stipe size, which was slightly larger (4.5–5.2 × 3–6.5 mm) compared to the original description (2.5–4.5 × 2–3 mm). However, the generally small dimensions, the basidiomata colors, the fugacious veil, and the spore size point to *A. heimii.*

Monospore isolates of Group I had a light brown fluffy appearance in culture when young but turned crustose with age. The cultural morphology of crustose rhizomorphic rather than mycelial colonies of Group I also resembles *A. heimii,* according to Mwangi (pers comm 2000). Rhizomorph production has been reported to be limited in Africa, but in this case Group I showed presence of rhizomorphs firmly in contact with the root surface, particularly at high elevations, for almost all isolates.

The amplification of the ITS region of isolates from this group by PCR gave a product of about 700 bp that was similar to the band size described by Chillali et al (1997) for *A. heimii* from other African countries. The restriction pattern with the enzyme *Nde* II was also similar, but the restriction patterns with the enzymes *Alu* I and *Hinf* I were different from the ones obtained for *A. heimii* by Chillali et al (1997). The IGS region of Group I was amplified and a band of over 1000 bp was obtained. This PCR product was different in size from *A. heimii* from other African countries. The digestion with the restriction enzyme *Alu* I gave a different pattern to the *A. heimii* from other African countries but was similar to the one obtained by Coetzee et al (2000) and identified as an *Armillaria* sp. or *A. fuscipes.* There is strong evidence that Group I represent *A. heimii* but molecular data suggest that there are sub-groups within *A. heimii* or this is a complex of several species.

There were no basidiomata found that represented Group II. The cultures from mycelium found under the bark of infected plants had the appearance of a diploid: white mycelial colonies that became dark brown as they aged. Isolate $6M_1$ and isolate $9T_2$ showed an extensive network of naturally produced rhizomorphs in soil. In contrast, the production of branched and unbranched rhizomorphs *in vitro* was abundant in Group I, and rhizomorphs were thin and submerged or were not formed at all for Group II. Molecular data showed that this group was different from Group I and different from *A. mellea.* The phylogenetic analysis showed that the IGS region between the 26S and 5S of the isolates from Group II was identical to the IGS region of reference isolates K10 and K12 and different from *A. mellea* and other *Armillaria* species sequences published in GenBank. Reference isolates K10 and K12 have been previously described as potential new *Armillaria* species by Chillali et al (1997). There is strong morphological and molecular evidence to suggest that Group II could be a new *Armillaria* species but basidiomata are essential for its conclusive description and naming. So far, only very immature basidiomata have been produced in the *in vitro* attempts.

Somatic incompatibility is one of the methods that have been used for the identification of genotypes, and the reaction between different species of *Armillaria* is usually characterized by the presence of a black line along the demarcation zone (Guillaumin et al 1991). The melanized cellular contents of the hyphae constitute the black line and the mechanism that causes the hyphae to become melanized is unknown (Mallet and Hiratsuka 1986). The tests showed no dark demarcation lines in pairings within Group I, but they were present in pairings within Group II. This phenomenon was reported by Mohammed and Guillaumin (1994) in isolates from the Kenya highlands. Abomo-Ndongo and Guillaumin (1997) reported dubious reactions for isolates K10 and K12. A similar phenomenon has been observed on other Basidiomycetes such as *Ganoderma* in oil palms where most isolates, even when taken from the same plant, were somatically incompatible with one another (Miller et al 1999). Group II seems to be an exception among *Armillaria* species, with the occur-

rence of the dark demarcation line in intra-group pairings.

It can be concluded from this study that two different *Armillaria* species causing damage in tea were present during this survey. Isolates in Group I were widely distributed and were found in all locations where tea was grown in Kenya. Basidiomata of these were found only in one high-altitude location and they may represent *A. heimii*. The three subgroups found within Group I were not characterized by location, host, or altitude and therefore, no further ecologically functional subgroupings could be made based on our findings. Isolates in Group II were found at higher altitude and were not so widely distributed. Basidiomata of these were not found. Some of the isolates presented extensive networks of rhizomorphs in the soil, and based on the morphological and molecular data it could be a new species. It was surprising that no isolates conforming to *A. mellea* were found during this survey. Reference isolate K5 (*A. mellea* from Kenya) was grouped in the phylogeny tree with *A. mellea* from Japan. This supports the hypotheses by Ota et al (2000) that part of the Japanese and African *Armillaria* population are derived from the same origin and migration may have occurred from Japan or other Asian countries to Africa. Research is in progress to resolve the *A. heimii* complex, to elucidate the identity of Group II, and to determine their relationships to other *Armillaria* species.

ACKNOWLEDGMENTS

We thank Dr. J-J Guillaumin and Mr. P Desray of UMR INRA, Clermont-Ferrand, France, for their generous donation of isolates and for their helpful advice and support. We also thank Dr. C. Prior and Dr. R. Cook of the Royal Horticultural Society (RHS), UK; Dr. J. Nicklin and Prof. P. Bridge of the School of Biological and Chemical Sciences, Birkbeck College, London; and Dr. Francis N. Wachira of the Department of Botany, Tea Research Foundation of Kenya, for their support and helpful comments on the manuscript. Dr. B. Spooner and Prof. P. Bridge of the Royal Botanical Gardens at Kew, UK kindly gave us access to *Armillaria* material from the Herbarium. Dr. B. Henricot of the RHS helped with the phylogeny.

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