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ORIGINAL PAPER

Mycorrhiza status of *Gnetum* spp. in Cameroon: evaluating diversity with a view to ameliorating domestication efforts

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Abstract A field survey was carried out to investigate the diversity of mycorrhizal fungi associated with *Gnetum* spp. in Cameroon. The extent and variation of ectomycorrhizal colonisation as well as the degree of host specificity were evaluated. *Gnetum* spp. were found to be almost always ectomycorrhizal in all sites visited. There were just two ectomycorrhizal morphotypes ('yellow' and 'white') associated with this plant. Such low diversity is unusual for an ectomycorrhizal plant. The yellow morphotype was the most widespread and prevalent and was identified by morphological and molecular methods to have been formed with *Scleroderma sinnamariense*. Propagules of this fungus were present in soil collected from farm lands, cocoa plantations, *Chromolaena* and bush fallows, as well as in a relatively undisturbed forest harbouring ectomycorrhizal legumes. The fungus responsible for the white morphotype was identified as also belonging to the genus *Scleroderma* by ITS sequence similarity. Arbuscular mycorrhizal structures were absent in cleared and stained portions of the roots.

Keywords *Gnetum* · Ectomycorrhiza · *Scleroderma sinnamariense* · DNA · Molecular phylogeny

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Introduction

Gnetum spp. are found in Asia, South-America and Africa. They are characterized as evergreen under storey lianas, with a few species being small trees. There are about 40 species (Mialoundama and Paulet 1986) with some reports proposing up to 72 species (Anonymous 1999) of this lone genus of the family Gnetaceae. *Gnetum africanum* Welw. and *Gnetum buchholzianum* Engl. are found in Africa and distributed in the humid tropical forests in Nigeria through Southern Cameroon, to the Central African Republic, Gabon, Democratic Republic of Congo and Angola (Mialoundama 1993). In these countries, *Gnetum* leaves which are mainly collected from the wild resource, are edible and are used in the preparation of local dishes. For this reason, there is an indiscriminate and unsustainable harvesting, thus, imparting a negative influence on the resource. *Gnetum* plants have been listed as endangered species and there is a need for the designation of proper management and conservation strategies.

Previous studies have reported the formation of ectomycorrhiza (EM) by *Gnetum* spp. (Fassi 1957; St. John 1980; Mialoundama and Mbou 1992; Ingleby 1999; Cadiz and Florido 2001) but only the work of Ingleby (1999) gave a detailed description of mycorrhiza formed between *G. africanum* and *Scleroderma sinnamariense*. Onguene (2000) observed hyphal coils (arbuscular mycorrhiza structure) in portions of *Gnetum* roots in the absence of EM. However, all of these studies reported only the existence of one distinctive yellow EM morphotype with *Gnetum*, with the exception of Onguene (2000), which documented the existence of four EM morphotypes. It was therefore necessary to know the diversity of mycorrhizas of this plant and to identify the fungal associates. Such information would improve knowledge on diversity and

ecology of tropical mycorrhizas as well as serve in the development of appropriate management strategies for the plant.

The main objective of the study reported in this paper was therefore;

1. To determine the extent and diversity of ectomycorrhizal colonisation of *Gnetum* in Cameroon. The key questions were:
   a. Are *Gnetum* always ectomycorrhizal?
   b. How many different EM fungi are involved?
   c. Are the EM fungi in *Gnetum* farms the same as those in natural situations?
   d. What is the extent of host specificity of EM fungi of *Gnetum?*
   e. Does *Gnetum* share EM fungi with other EM hosts (e.g. members of Caesalpiniaceae)?

Materials and methods

Survey sites

Gnetum spp. are found principally in the lowland humid forest region of the country. Five of the ten administrative regions, which are characterized by this type of eco-climatic zone; the Centre, East, Littoral, South and South West regions were surveyed in this study. Within these regions, sites were chosen to cover a variety of different locations and disturbance regimes. Table 1 shows the region, location and site description of the different areas surveyed in this study.

Mycorrhizal investigations

Roots were traced from the base of a total of 475 selected *Gnetum* stems until they led to ultimate order fine roots.

Table 1 Frequency of occurrence of ectomycorrhiza morphotypes on *Gnetum* at a number of sites in Cameroon

Region	Location	Distance and direction from Yaoundé (km)	Site description	No. of plants sampled	Yellow (%)	White (%)	Non-EM (%)	Yellow + white (%)
Centre	Ovoa Abang	150 S	Banana farm	15	80	0	20	0
			Chromolaena fallow	20	70	10	20	0
			Secondary forest	15	90	10	0	0
	Nkol Owondo	150 S	*Chromolaena* fallow	20	70	10	20	0
			Secondary forest	25	90	5	5	0
	Nkong Meyos	100 N	*Chromolaena* fallow	20	60	20	20	0
			Secondary forest	20	70	20	10	0
	Mode Mallam	200 S	Secondary forest	10	60	10	30	0
	Yaoundé	0	Nursery ICRAF	20	90	8	0	2
East	Kobi	600 E	Palm/cocoyam/cassava farm	20	60	10	30	0
Littoral	Pouma	150 SW	Palm/maize farm	20	100	0	0	0
South	Ebimimbang	450 S	*Chromolaena* fallow	20	80	20	0	0
			Cocoa plantation	30	90	0	10	0
			Secondary forest	15	70	20	10	0
			Undisturbed forest	15	60	0	40	0
South West	Mbanga Pongo	600 SW	Farmland	15	60	10	30	0
			Chromolaena fallow	20	70	10	20	0
			Secondary forest	20	60	40	0	0
	Kumba	450 SW	Cassava farm	15	0	90	0	10
			Gnetum plantation	20	100	0	0	0
	Limbe Bot. Garden	250 SW	Nursery	10	95	0	10	0
			Demonstration farm	15	70	20	10	0
			Secondary forest quadrat 1	40	100	0	0	0
			Secondary forest quadrat 2	20	0	100	0	0
	NGO Limbe	250 SW	Trial farm	15	60	0	40	0
Total (per column (mean ± standard error))				19±1.2	70.2±5	16.5±5	13±2.6	0.48±0.4

ICRAF International Centre for Agroforestry Research

Field based examinations were done with the aid of a10× hand lens (Sight Savers and Bausch and Lomb, Rochester, NY). Colour of mantle in daylight was noted. Samples bearing from 20 or more root tips were cut and fixed in 50% ethanol and stored at 4°C. Two tips from each morphotype and each collection was stored in 500 µl of 2× cetyltrimethylammonium bromide (CTAB) at −20°C. Potential EM root tips were identified under the dissecting microscope. Representative sample of between four to six tips were excised and observed by use of a compound microscope for the presence of a mantle and Hartig net. Representative portions of non-EM roots as well as those collected from portions of EM roots which had no EM tips were cleared and stained (Kormanik et al. 1980) and examined microscopically for the presence of AM structures.

A detailed description of the white ectomycorrhiza morphotype found on the *Gnetum* roots was done following the method described in Ingleby (1999). The yellow morphotype had already been described in detail by Ingleby (1999).

Molecular identification of fungi involved

Dried basidiomata of nine collections of *S. sinnamariense* associated with dipterocarps, and a single collection of an undetermined yellow species of *Scleroderma* associated with *Gnetum gnemon*, were obtained from the herbarium of the Forest Research Institute of Malaysia (FRIM). A living culture of *S. sinnamariense* isolated from a basidiome collected beneath *Hopea odorata* (from Malaysia) was also obtained. DNA from this material was extracted and compared with DNA extracted from yellow mycorrhizas collected in Cameroon, and with DNA extracted from basidiomata formed in association with ectomycorrhizal *Gnetum* plants obtained from Cameroon and growing in the greenhouse in Aberdeen. This approach also allowed the taxonomic position of the fungus forming the white ectomycorrhizal morphotype to be established, since this fungus had been tentatively placed in the genus *Scleroderma* on the basis of ectomycorrhizal morphology. The origin of all fungal material is shown in Table 2.

DNA extraction, amplification and analysis

Tissue samples for DNA extraction consisted of about one to two fresh clean mycorrhiza tips which had been collected from a number of locations in Cameroon, areas of mycelium of approximately 1×0.5 cm in diameter which had been scraped from a culture grown on modified Melin–Nokrans agar (Marx 1969), small pieces of about 1×3 mm in diameter of fresh young basidiomes or dried herbarium material (materials indicated in Table 2). DNA extraction was based on the method of Gardes and Bruns (1993) with slight modifications. In the extraction process, tissue samples were individually transferred to sterile Eppendorf tubes containing 300 µl of 2× CTAB extraction buffer (100 mM Tris, 1.4 M NaCl, 20 mM EDTA, 2% CTAB, 1% PVP, 0.2% ß-mercaptoethanol). Samples were then subjected to alternating freezing and thawing (three times) between liquid nitrogen and a 65°C water bath. After the final thaw, material was finely crushed in a mortar and pestle.

DNA was extracted by placing the sample in a Bio-101 red multimix tube (Anachem Ltd) along with 0.5 ml of CTAB extraction buffer and 0.5 ml of phenol/chloroform/isoamyl alcohol (25:24:1; pH 8.0). The sample was then lysed for 2× 15 s at a speed of 5.5 m s^{-1} in a FastPrep bead beating system (Bio-101, Vista, California, USA) and the aqueous phase was separated by centrifugation at 13, 500 rpm for 7 min at 4°C. The sample was extracted further with 600 µl of chloroform before precipitating the nucleic acids with Na acetate (3 M) and isopropanol by centrifugation at 13,500 rpm for 30 min. Pelleted nucleic acids were then washed with cold 70% (*v*/*v*) ethanol and air-dried overnight prior to resuspension in 100 µl of TE buffer (pH 7.4).

The universal primers ITS1 and ITS4 developed by White et al. (1990) were used for PCR amplification. For all the vouchers, approximately 50 ng of template DNA was used for each reaction. The DNA was PCR-amplified as previously described (White et al. 1990). Fifty-microlitre reaction volumes each containing: approximately 50 ng of template DNA, 20 pmol of each primer, 2 mM $MgCl_2$, 250 µM of each dATP, dCTP, dGTP and dTTP, 10× buffer (20 mM Tris–HCl (pH 7.5), 100 mM KCl, 1 mM dithiothreitol (DTT), 0.1 mM EDTA, 0.5% Tween 20 (*v*/*v*), 0.5% Nonidet P40 (*v*/*v*), and 50% glycerol (*v*/*v*)) and 2.5 U *Taq* DNA polymerase (Bioline, UK) were used. The samples were run in a PTC-200 Thermal Cycler (MJ Research) with the following cycling parameters: initial denaturation for 85 s at 93°C, followed by 30 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 55°C and extension for 30 s at 72°C. The thermal cycling was ended by a final extension at 72°C for 10 min. Reactions were performed in duplicate and negative controls were run to check for DNA contamination of reagents.

The amplified PCR products were separated by electrophoresis in 1.5% agarose gels, stained with ethidium bromide and visualised under UV light. PCR products were purified using the QIAquick PCR purification Kit (Qiagen). Sequencing reactions were set up with primers ITS1 and ITS4 using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit with an automated DNA sequencer (ABI model 377, Applied Biosystems, UK). Each reaction mix included 2 µl BigDye (with *AmpliTaq* polymerase), 8 pmol primer and 3.5 µl PCR product. The PCR programme was: 96°C denaturation for 2 min, 47°C annealing for 15 s and 60°C extension for 4 min in 35 cycles. Cycled sequencing products were run

Table 2 Origin of material included in the phylogenetic analysis of *Scleroderma* basidiomes and mycorrhizas

ID number	Material	Origin
LW1	Yellow ectomycorrhiza	Limbe Cameroon
PW1	Yellow ectomycorrhiza	Pouma Cameroon
LW2	White ectomycorrhiza	Limbe Cameroon
KW2	White ectomycorrhiza	Kumba Cameroon
IY1	White ectomycorrhiza	Yaoundé Cameroon
FRIM161	Fungal culture	From basidiome collected beneath *Hopea odorata*, Malaysia
IA-S1	Basidiome: *Scleroderma* sp.	Collected beneath *Gnetum* sp. Aberdeen
IA-S2	Basidiome: *Scleroderma citrinum*	Collected beneath *Tilia europea* in Aberdeen
FP3058	Basidiome: *Scleroderma* sp.	Collected beneath *Gnetum gnemon*, Malaysia
AB099901	Basidiome: *Scleroderma bovista*	Thailand
AB099900	Basidiome: *Scleroderma* sp.	Thailand
AF270782	Basidiome: *Pisolithus* sp.	Australia
AF270777	Basidiome: *Pisolithus tinctorius*	Australia

on an ABI 377 automated DNA sequencer with a 5.25% polyacrylamide gel (PAGE PLUS, 7 M Urea, Amresco). Forward and reverse sequences were edited, and a consensus sequence obtained, using the Sequencher™ software (version 3.0; Gene Codes Corporation, MI, USA).

The sequences obtained were supplemented with two *Scleroderma* ITS sequences downloaded from GenBank. Details of these, and of the two *Pisolithus* sequences used as an outgroup, are given in Table 2. All sequences were preliminarily aligned using ClustalW (version 1.8.2 (Thompson et al. 1994)) and manual adjustments were made to the alignment where necessary in the editor of PAUP* 4.0b10 (Swofford 2002) and submitted to TREE-PUZZLE (version 5.0). The transition/transversion ratio and the gamma distribution parameter were estimated using TREE-PUZZLE (version 5.0) before conducting a neighbour-joining analysis using the F84 model in PAUP* (version 4.0b10 (Swofford 2002)) with 1,000 bootstrap replicates.

Inoculation trial

This experiment was carried out to evaluate the distribution of propagules of fungi forming mycorrhiza with *Gnetum*. It enabled us determine the different possible EM fungi associated with this plant in natural conditions. Soil was collected at Ebimimbang from *Chromolaena* fallows, cocoa plantations, a secondary forest and a relatively undisturbed forest containing clumps of EM Caesalpiniaceae-like *Monopetalanthus* spp.; Leguminosae such as *Berlinia* spp. as well as Euphorbiaceae-like *Uapaca* spp. The soil collections were stored in plastic bags and transported to Yaoundé. The planting was done within 72 h of collection time. During collection, at each of the four randomly selected points within each collection site, unconsolidated litter was moved aside and a sample of 20 cm depth taken. These replicates were bulked, mixed and subsampled to *c*. 3 kg. To serve as a negative control, soil was collected from the courtyard of a residential area, enclosed by a cement wall. This area has been used solely for habitation for over 30 years. Propagules of EM fungi associated with *Gnetum* were not expected to be found here.

Five hundred grammes of each subsample was used to fill a black polythene bag. Six months old rooted *Gnetum* cuttings with bare roots which had earlier been obtained from a farmers group in Limbe were transplanted into the polythene bag with one plantlet per bag. There were three replicates per test soil. Three other rooted *Gnetum* cuttings were transplanted into a pot containing a *Monopetalanthus* sp. which was colonised by an unidentified white EM fungus. Two rooted cuttings were also transplanted into a pot containing *Gnetum* which was mycorrhizal with the yellow fungus. The transplanted plantlets were kept on bench tops in the shade house and watered daily with tap water. The temperature in the shade house varied from 25°C to 35°C on a very hot day.

Random root samples were collected every 3 months and scored for EM formation following microscopy. Final harvest was done at 9 months with the separation of bulk soil from the roots. This was followed by visual and microscopic examination of roots.

Results

Field survey

Of the 475 *Gnetum* plants sampled from 25 different sites, 87% were ectomycorrhizal (Table 1). However, only two

morphotypes, denoted as 'yellow' and 'white' (Fig. 1), were found. The yellow morphotype was the most common and widespread and was found on 70% of all the plants sampled. It was present in all vegetation types and different disturbance regimes. Where it occurred, it was the most frequent type. An exception was the cassava farm in Kumba and the quadrat 2 in the Limbe Botanic Garden (LBG) secondary forest, where the white morphotype was the most frequent (Table 1). Although the white type was not the major ectomycorrhiza morphotype of *Gnetum* spp., it also occurred in all regions sampled, but not in all plots, except in the Littoral region where only one site was sampled (Table 1). Longitudinal sections of the EM root tips showed well developed Hartig net, a structure which was absent from the uncolonised root tips. It was observed at the secondary forest in the LBG that all the plants were ectomycorrhizal exclusively with the yellow morphotype in one of the quadrats, while in the adjacent quadrat all the plants had the white morphotype. This spatial separation of morphotypes was unique to this site. However, elsewhere both the white and yellow morphotypes were found co-existing on the same roots of the plant as observed at Kumba cassava farm (10% of plants) and International Centre for Agroforestry Research (2% of plants).

Fig. 1 The different ectomycorrhiza morphotypes observed on *Gnetum* species in Cameroon. **a** Yellow morphotype and **b** the white morphotype. *Bar* is 3 mm

Description of white morphotype

Unidentified fungus + *G. africanum* Welw.

Short description Ectomycorrhizae were white, silvery from trapped air between hyphae, simple to infrequently branched, covered with a dense cottony to woolly network of frequently ramifying, emanating hyphae containing few visible clamp connections and occasionally binding soil particles. Mycorrhizae had abundant highly differentiated, partially hairy and compact rhizomorphs which originated from the base and ramified around the root and mycorrhizal surfaces. Sometimes the central hyphae of rhizomorph was vessel like with a wide core and partially dissolved septa. The mantle was plectenchymatous. Hyphae emanating from the mantle and rhizomorph surfaces had few visible clamp connections.

Morphological characters Mycorrhizal systems: simple to infrequently branched with abundant rhizomorphs; emanating hyphae occurred frequently on mycorrhiza, forming a dense covering. (1) *Main axes*: up to 10–12 mm long and 0.3–0.5 mm diam. (2) *Unramified ends* 2.5–4 mm long and 0.3 mm in diameter, straight to bent, silvery-white in colour, with narrowing tips. (3) *Rhizomorphs*: originated from base of mycorrhiza and sometimes grew along the mantle surface, silvery-white in colour; emanating hyphae from rhizomorphs had few clamp connections. *Sclerotia*: not observed.

Anatomical characters of mantle in plan views Mantle: a thin loosely plectenchymatous network of interwoven hyphal branches with few visible clamp connections (Fig. 2), which covered almost all of the mycorrhiza.(1) *Outer mantle layer*: below emanating hyphae was densely plectenchymatous with more compact interwoven hyphae that had no clamp connections; hyphal cells of 10–40 μm long and 2–3 μm wide. (2) *Middle mantle layers*: plectenchymatous with cells 1.4–2.3 μm in diameter. (3) *Inner mantle layer*: densely plectenchymatous with hyphae interwoven with cells slightly larger than previous layers; cell diameter of 2.3–3.2 μm and 3–10 μm in length.

Anatomical characters of emanating elements Rhizomorphs: compact and highly differentiated (Agerer 1991, type F) with very few emanating hyphae (Fig. 2). Hyphae arranged in parallel in young rhizomorphs with no visible clamps. Cells are 8.3–16.7 μm long and 1.7–2.5 μm wide. Older rhizomorphs, cells of peripheral hyphae are shorter (length, 7.7–11.4 μm) and inflated (4.1–5.9 μm diam.). Central hyphae cells diameter of 2.7–5.9 μm, length is more than 50 μm, and wall thickness is 2 μm. (1) *Emanating hyphae*: few with few clamp connections. Cells are 10–40 μm long and 2–3 μm in diameter. (2) *Cystidia*: not observed.

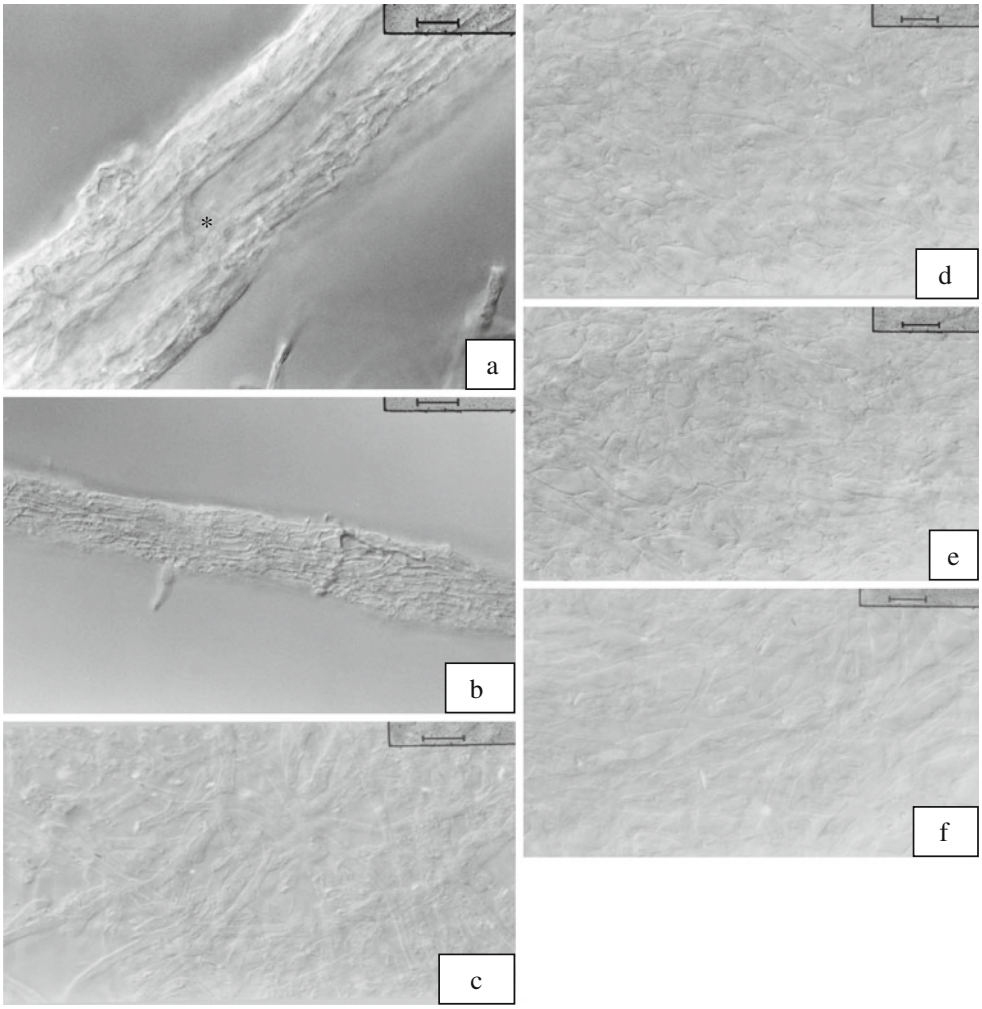

Fig. 2 **a** Older rhizomorph showing thick-walled, vessel-like hyphae with partially dissolved septum (*asterisk*), **b** younger rhizomorph with shorter hyphal cells, **c** hyphal network on outer mantle layer. **d** view of outer middle mantle layer, **e** view of inner middle mantle layer, **f** view of inner mantle layer (all showing plectenchymatous cellular arrangement). *Bar* is 10 μm

Anatomical characters of longitudinal section Mantle: 30–40 (45) μm thick.

Colour reaction with different reagents Whole EM tip: toluidine blue O mycorrhiza strongly blue; Melzer's reagent and iodine potassium iodide: amyloid, mycorrhiza blue-grey; sulfovanillin: mycorrhiza reddish-black; 70% ethanol: mycorrhiza light brown; 15% KOH: mycorrhiza light brown; 70% lactic acid: no reaction; iron (III) chloride: no reaction; distilled water: no reaction. Samples of the EM are kept at the Department of Plant and Soil Science, Cruickshank Building, University of Aberdeen.

Molecular identification of fungi

From the dendogram, the *Scleroderma* material fell within a clade (A in Fig. 3) which was quite different from the *Pisolithus* material (B in Fig. 3). Within the *Scleroderma* clade, all the tropical material fell within a well-supported sub-clade (A1 Fig. 3), separate from the reference material obtained from temperate basidiomes or from GenBank (A2 Fig. 3). Within this sub-clade (A1) there are four groups. The first of these (a Fig. 3) contains the three almost identical sequences from white mycorrhizas, confirming that the mycobiont of this morphotype is indeed a species of *Scleroderma* and that it is probably a separate species from that forming the yellow mycorrhizas. The second group (b Fig. 3) contains a sequence from a yellow mycorrhiza from Cameroon and a very similar sequence from the *Scleroderma* basidiome collected beneath *G. gnemon* in Malaysia. A third group (c in Fig. 3) contains two nearly identical sequences, one from a basidiome growing with *Gnetum* in the greenhouse in Aberdeen and the other from a culture of *S. sinnamariense* isolated from a basidiome growing with *H. odorata*. The fourth branch (d in Fig. 3) contains a single sequence from a yellow mycorrhiza.

Unfortunately only two sequences were obtained from the material sent from Malaysia. One of these was from the living culture obtained from a basidiome of *S. sinnamariense* growing with *H. odorata*, and the other was from the dried basidiome collected beneath *G. gnemon*.

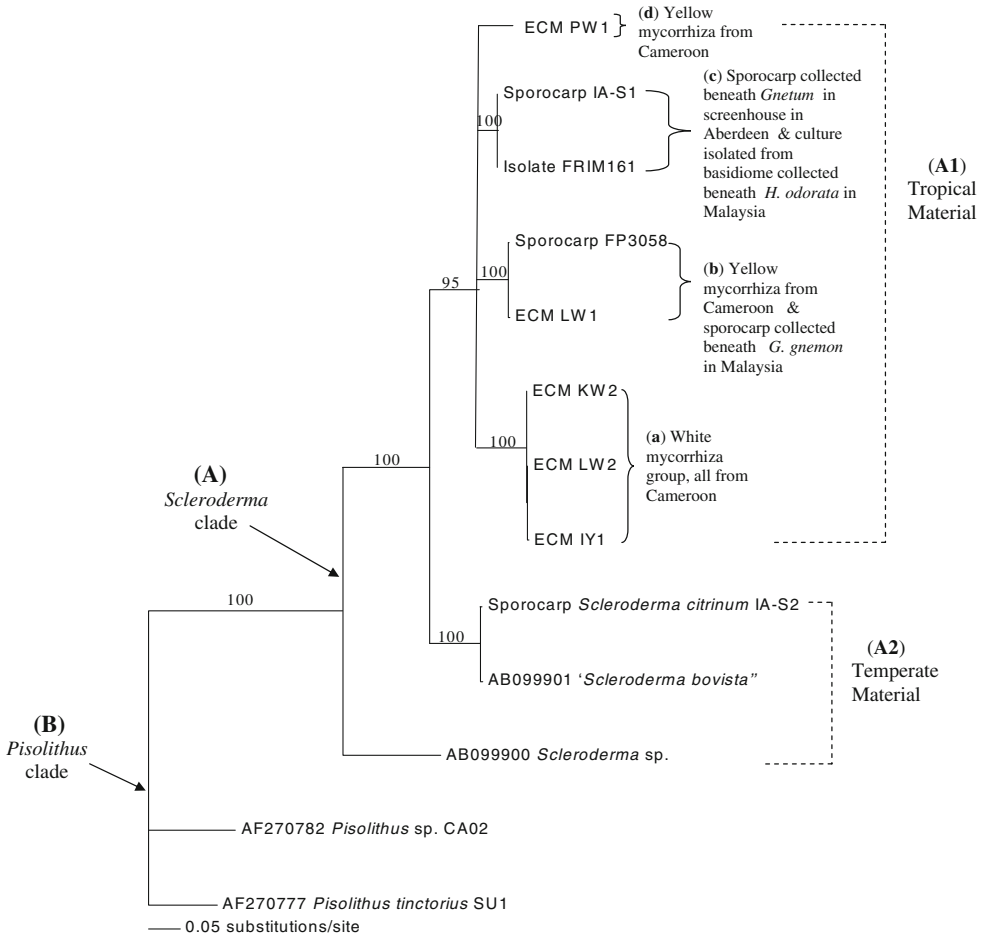

Fig. 3 Molecular phylogenetic tree-diagram of single linkage analysis of RFLP patterns from digestion of the ITS region of rDNA amplified from EM root tips from *Gnetum*, mycelium and EM fruiting bodies. *Numbers* at nodes indicate bootstrap indices over 50% obtained after 1,000 replicates

Pot inoculation

At the end of the 9 months experimental period, all cuttings planted had formed the yellow ectomycorrhizas, except those planted in soil collected from a residential area and in the pot containing *Monopetalanthus* (Table 3). Cuttings potted with soil from the undisturbed forest were the first to form EM, just like those planted in the pot containing a mature *Gnetum* plant.

Table 3 Morphotypes of EM formed following growth of *Gnetum* rooted cuttings in soil from different sources

Source	3 months	6 months	9 months
Chromolaena fallow	None	Yellow	Yellow
Cocoa plantation	None	Yellow	Yellow
Secondary forest	None	Yellow	Yellow
Leguminous forest	Yellow	Yellow	Yellow
Residential area	None	None	None
Monopetalanthus pot	None	None	None
Gnetum pot	Yellow	Yellow	Yellow

There were three replicate plants in each source

Discussion

Field survey

Earlier studies had indicated that an important minority of tropical plants are ectomycorrhizal (Alexander 1989a, b; Brundrett 2009). In this study, plants of *G. africanum* were almost always ectomycorrhizal, suggesting that colonisation might be a prerequisite for normal growth, development and survival of the plantlets. Nevertheless, there were a few uncolonised plants in all the regions but not on all plots, except in the Littoral and colonisation seemed to vary with location: suggesting that the inoculum potential may vary with the sites. Just two ectomycorrhiza morphotypes: the yellow and white morphotypes, were observed on *Gnetum* plants in Cameroon. The yellow morphotype was the most frequently observed and such a low diversity of EM morphotypes is not common for an ectomycorrhizal plant species (Chen et al. 2007; Malvárez and Oliveira 2003; Smith and Read 2008).

In a previous study by Bechem and Alexander (2009), *Gnetum* formed EM with mycelia of *S. sinnamariense* isolated from *Gnetum* root tips from Cameroon but not with

isolates of *Pisolithus tinctorius* from *Pinus* sp. or *Acacia mangium*. Mycorrhization of this plant was not possible with the use of *Paxillus involutus* isolated from *Larix* sp. Inoculation with *Glomus mosseae* (BEG 12) and *Gigaspora margarita* (BEG 34) also failed to produce positive results (Bechem and Alexander 2009). Mycelia of our isolate of *S. sinnamariense* failed to colonise the roots of *H. odorata* from Malaysia although this species forms EM with *H. odorata* in Malaysia (Sims et al. 1999). The inability of *Gnetum* to form associations with broad host spectrum mycorrhizal fungi is unusual and merits further investigation (but see Tedersoo et al. 2009).

Gnetum spp. are tropical plants, thriving in environments where there is a seasonal fluctuation of nutrients, therefore mycorrhization would help the plant to stand such nutrient fluctuations and the competition which thus arises. However, such specificity to one or a few fungi places the plant at risk, in that, where this particular inoculum is absent, environmental factors would greatly affect the plants' establishment and survival. It would seem ecologically more advantageous if several ectomycorrhizal fungi species were present on these plants.

The extreme specificity between *Gnetum* and the yellow fungus was a rarity and the basis of such specificity if determined may help in answering some questions as to whether the distribution of plant species is determined by their fungal partners or vice versa. The molecular basis of specificity if determined would also provide important information needed to answer some questions in ecology especially pertaining to host/fungus interaction and functioning.

From the research findings, it can be concluded that *Gnetum* spp. are ectomycorrhizal in the nursery, in *Chromolaena* fallows, bush fallows, secondary forest and even in a relatively undisturbed forest, the most widespread and abundant fungal partner being the yellow fungus assumed to be *S. sinnamariense*. Any further research on *Gnetum* mycorrhiza and possible improvement of plant growth should therefore be concentrated on the EM formed with the yellow fungus.

Description of white morphotype

Species of the genus *Scleroderma* are widespread and common in both established forests and disturbed sites of Europe, Asia, North-America (Sims et al. 1995) and Africa. Several *Scleroderma* isolates have been used as ectomycorrhizal inoculum especially in the tropics (Marx et al. 1991). One species of *Scleroderma, S. sinnamariense* which is tropical and associated with *G. africanum* has been comprehensively described (Ingleby 1999) so far. This species was described as chrome yellow making it quite different from the white species in the current description. Species of *Scleroderma* ectomycorrhizae in this study, described as silvery-white is quite similar to several species of *Scleroderma* ectomycorrhizae which have been described in literature: *Scleroderma areolatum* Ehrenb. (Godbout and Fortin 1985), *Scleroderma bovista* Fr. (Jakucs 1999), *Scleroderma citrinum* Pers. (Mohan et al. 1994), *Scleroderma dictyosporum* Pat. (Ducousso 1991), *Scleroderma hypogaeum* Zeller (Molina and Trappe 1982), *Scleroderma geaster* Fr. (Rose et al. 1981) and *Scleroderma verrucosum* Pers. (Thoen and Ducousso 1989).

Most of these *Scleroderma* species are described as having a plectenchymatous outer mantle layer just like the white type in this study. Other similarities between this *Scleroderma* sp. ectomycorrhizae and those of other *Scleroderma* species were seen in the compact, frequently branched and highly differentiated rhizomorphs composed of hyphae arranged in parallel and having a central core with wide vessel-like hyphae. The presence of clamp connections, an important feature of all basidiomycetes, in all these *Scleroderma* species was another important similarity. Although the yellow and white ectomycorrhiza both had plectenchymatous inner mantle layers, their outer inner mantle layers were different. The yellow EM had a pseudoparenchymatous outer inner mantle layer with numerous visible clamp connections in the emanating elements while the white EM had a plectenchymatous outer inner mantle layer with fewer visible clamp connections on the emanating elements

Molecular identification of fungi

DNA analysis showed that, although all EM fungal associates of *Gnetum* in this study were *Scleroderma*, they belonged to different species. The yellow fungus from *Gnetum* in the current study was shown to be similar to the yellow fungus from *G. gnemon* and *H. odorata* from Malaysia.

The conclusion from this molecular phylogenetic analysis must be that, based on the limited material available, there is no evidence to support the notion that the Cameroon material is distinct from *S. sinnamariense* or that there is a distinctive taxon of yellow *Scleroderma* associated only with *Gnetum* as opposed to dipterocarps. However, this conclusion is based on a single 'non-*Gnetum*' sequence (FRIM 161). In addition, there is some variation (Fig. 3) within ITS sequences obtained from yellow mycorrhizas and yellow basidiomes, and even between different yellow mycorrhizas from Cameroon. Clearly, a more detailed molecular phylogeny, including a larger number of sequences, supplemented by morphological analysis of basidiomes and cross-inoculation experiments will be required to fully resolve this issue. We would therefore continue to refer to the yellow fungus as *S. sinnamariense*.

Pot inoculation

The inability of *Gnetum* plants to form EM with the fungus present on the roots of the *Monopetalanthus* plant was probably due to incompatibility between the fungus and the *Gnetum* plant. Such an observation, coupled with previous field observations and bioassays, emphasizes the likelihood of fungus-plant specificity between *Gnetum* and *S. sinnamariense* in Cameroon. From the bioassay, *Gnetum* only formed EM with the yellow fungus following growth in soil collected from stands where EM trees occurred in clumps. This observation further confirms the possibility of host–fungus specificity. St. John (1980) reported the occurrence of EM on *Gnetum* sp. in the Amazonian forest of Brazil. These mycorrhizal tips were also bright yellow in colour (personal communication). This observation goes to reiterate the possible specificity between this fungus and plant not only in Cameroon but probably in other tropical regions where they occur. This strict specificity between fungus and plant merits further investigation.

Implications of results for conservation and growth of *Gnetum*

From the results obtained in this study it is conclusive that ectomycorrhizas are an integral part of the plant's physiology. Any useful and effective conservation strategy therefore would have to incorporate the diversity and biology of the fungal partner. There is a possibility therefore that the survival of plantlets following outplanting would depend on their mycorrhizal status and inoculation may occur naturally only if the inoculum potential of the planting site is good. Because the potential planting sites in Cameroon however have been devoid of the plant due to excessive collection, coupled with the extreme specificity observed between this plant and the fungal partner, it is possible that the inoculum potential of such sites would be quite low. Inoculation in nursery prior to outplanting would therefore be imperative. It would be necessary to select candidate isolates for inoculation purposes by screening fungal isolates from different provenances for their ability to improve plant growth, survive competition from other soil microbes and withstand other soil physiological properties in the planting site. The selected isolate should also have the ability to withstand the physical manipulations involved in inoculum production.

The results from this study serve as baseline information for the biology of the fungi and also the physiology of the plant. The information obtained from the field survey serves as a qualitative inventory of the plants distribution and ecology in Cameroon. However before any definite and effective conservation strategy can be drawn, more of such studies would be needed.

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