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MOLECULAR DIVERSITY OF ARBUSCULAR MYCORRHIZAL FUNGI ASSOCIATED WITH *PRUNUS AFRICANA* (HOOK F.) KALKMAN (ROSACEAE) IN HUMID FOREST ZONES OF CAMEROON

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ABSTRACT

Prunus africana is an endangered tree species, endemic to Africa and was included in Appendix II of the Convention on International Trade in Endangered Species (CITES). In this study, we described the composition of arbuscular mycorrhizal fungi (AMF) colonized roots and pool of spores of *P. africana* from three dry Afromountain forests of Cameroon. The large sub-unit (LSU) rRNA gene was amplified from these materials cloned and sequenced using the Glomeromycota specific primers FLR3 situated between the D1 and D2 domains of LSU rRNA and FLR4 in the D2 domain. Molecular phylogenetic analysis reveal that the sequences obtained belong to three families; *Gigasporaceae* (*Gigaspora margarita*), *Acaulosporaceae* (*Acaulospora tuberculate, A. longula* and *Entrophospora colombiana*) and Glomeraceae (*Glomus manihotis* and *G. etunicatum*). The composition of the AMF communities differed significantly with a slight tendency of specificity between sampled trees and host plants used. Isolation and utilization of the indigenous AMF taxa from the respective sites might be required for a successful enrichment of the plantations of *P. africana* species.

INTRODUCTION

Prunus africana (Hook. f.) Kalkman (Kalkman, 1965), is a geographically widespread pan-African mountain tree (Hall *et al.*, 2000; Kadu *et al.,* 2013). Bark extract from *P. africana* is used pharmaceutically world-wide, for the treatment of benign prostatic hyperplasia (BPH) and prostate gland hypertrophy (Stewart, 2003). BPH affects 50 percent of 60 year aged men and about 90 percent of over 80 year aged men. (Roehrborn *et al.,* 2002).

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Over-the-counter trade, the bark of trees is valued at over 220 million US$ per year (Cunningham and Mbenkum, 1993). The world-wide demand of *P. africana* bark is estimated at more than 4000 tons per year and Cameroon alone produces around 2000 tons per year (Ingram and Nsawir, 2007). Unfortunately, the increasing demand has led to irresponsible exploitation of *P. africana* with entire trees being girdled of their bark and left to die or, in other cases, felled to facilitate easier access to their bark (Cunningham *et al.,* 2002; Betti, 2008). This massive increase in the intensity of *P. africana* bark harvesting (Abanda and Nzino, 2014) over a relatively short period prompted the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) and the International Union for Conservation of Nature (UICN) to classify it respectively as an endangered and vulnerable species in Cameroon (Cunningham *et al.,* 1997).

Despite these protection measures, *P. africana* however, remains Cameroon's most intensively exported medicinal plant species by volume and, unfortunately decrease in its population over the time (Amougou *et al.,* 2010). Hence, putting in place an active program of multiplication and conservation of this species appears to be urgent pre-occupation to insure long-term sustainability of harvesting. Where the severity of the problem is more serious, incorporation into agroforestry systems has been suggested as a solution to decrease the pressure of local population in natural forests especially on *P. africana* (Cunningham and Mbenkum, 1993; Kebede *et al*., 2014). In Cameroon, several active plant protection techniques are applied to multiply plant material *ex situ* and to maintain natural populations of these species (Tchoundjeu *et al.,* 2002, Avana, 2006). However, problems associated with establishment after transplanting during vegetative propagation need to be solved. Success in domesticating *P. africana* involves its naturally associated soil microorganisms especially arbuscular mycorrhizal fungi (AMF). AMF are considered to be essential for plant performance and nutrition (Turnau and Haselwandter 2002; Sadhana, 2014) and are vital components of soil in natural and agricultural systems.

They colonize the roots of 90 percent plant families, where they facilitate mineral nutrient uptake from the soil in return for carbon assimilated by plants (Smith and Read, 1997). AMF are particularly important in tropical regions where soils usually present low fertility and extend plants root systems, facilitate plants uptake of soil nutrients of poor mobility, especially phosphorus (Smith and Read 2008). Identification of arbuscular mycorrhizal fungi (AMF), which have an undeniable feature on plant nutrition is essential to understand the relationship between plants and fungi in natural ecosystems (Helgason *et al.,* 2002; Wubet *et al.,* 2004). Therefore, the requirement to know the diversity of AMF that colonize the roots of plant species is capital to develop the strategies of domestication for endangered species. However, the differences can be observed in fungal diversity species, associated with plant families as well as the plant species itself (Fuchs and Haselwandter, 2008). For numerous medicinal plants, the mycorrhizal status has not so far been thoroughly investigated in Africa.

Nevertheless, there are some studies reporting on the colonization of medicinal plants by arbuscular mycorrhizal fungi (Wubet *et al.,* 2004; Fuchs and Haselwandter, 2008; Zeng *et al.,* 2013). Among those studies, Wubet *et al.,* (2004) have used molecular methods to determinate the diversity of AMF in *Prunus africana* root growing in dry Afromontane forests of Ethiopia, showing high diversity of AM Fungi with *Glomus* genus predominance. Wubet *et al.,* (2004) showed that the mycorrhizal community colonizing roots of *P. africana* is highly diverse. Isolation of the AMF species and screening for a combination of functionally complementary species that may be used in nursery propagation constitutes the future challenge. Thus, AMF may be useful in the development of effective methods for the maintenance and propagation of threatened plant species such as *P. africana* and may significantly improve the success of their conservation (Fisher and Jayachandran, 2002; Turnau and Haselwandter, 2002; Fuchs and Haselwandter, 2004).

This concept is far from being practically applied due to the lack of understanding of the functioning of AMF species (Scullion *et al.,* 1998). In Cameroon, no attention has been devoted in the study of AMF diversity of *P. africana* rhizosphere for its better improvement in agroforestry system. Thus, the prospects for exploiting them for production of mycorrhizal seedlings in Cameroon is still lacking. The aim of the present study was to assess the genetic variability of mycorrhizal fungi associated with *P. africana* and to determine their phylogenetic relationships in three agroecological zones of Cameroon.

MATERIALS AND METHODS

Study sites

Three sites were selected to collect the rhizosphere soil of *P. africana* tree. They were geographically separated and represent the three mostly harvested sites (Table 1; Fig. 1). The three sites were located in two different agro-ecological zones: (1) the mono-modal rainfall forest zone; and (2) the Western highlands agro-ecological zone (Fig. 1).

Sampling design and collection

According to the clustering of *P. africana* in each site, Adaptive Cluster Sampling (ACS) was used. ACS is a sample method use for classical forest inventory (Amougou *et al.,* 2010; Ojiambo and Scherm, 2010; Luke *et al.,* 2012). However, the effectiveness of ACS is dependent upon the density and clustering degree of *P. africana*. Using ACS method in each site, identifying cluster led to carry out a plot of 0.5 ha (100 m length X 50 m width). In each plot, fifty trees where randomly selected for soil and root sampling. Roots samples of randomly selected fifty trees of *P. africana* and soil closed to the roots were collected at a depth of 0-45 cm in 4 orientations (N, E, S, and W) in dry season (January 2011) in each of the plot. Roots were observed from the base of the stem to the terminal sections, to ascertain their origin. A portion of the terminal root system from each plant was collected, washed with water and stored in 50 percent alcohol.

Soil analysis

A mixture of soil samples from each plot were air dried, ground and sieved to 2 mm. Soil organic carbon (total C) was determined by colorimetric method after oxidation with H2SO4 and dichromate of potassium; total nitrogen (total N) by Kjeldahl digestion, total phosphorus (total P) by dosage in continuous flow and available phosphorus by the Olsen method (Olsen *et al.,* 1954). Soil pH was measured in 1/2.5 ratio of soil to distilled water. Soil analyses were performed at LASPEE (IRAD, Cameroon; ISO17025/200).

Trap culture

Trap culture was established for 6 mo under greenhouse conditions in 2 L pot filled with 1 kg of autoclaved loam/sand mixture (v/v). The mycorrhiza inoculum which consist of soil and root of each composite soil sample (MC, MM and MO) were placed on the surface of the substrate in the pots at position at which the trap plantlets subsequently were planted

Table 1. Characteristics of experimental sites where soil and root samples were obtained for culturing and molecular analyses of arbuscular mycorrhizal fungi

Sample places	Climatic zone	Coordinates	Altitude (m)	Predominant vegetation	Soils characteristics			
					pH	% N	P ($mg.kg^{-1}$)	% Org C
Mount Cameroon	Subequatorial humid	4º44'N, 9º 32'E	2231	Sterculiaceae, Ulmaceae, *Chromolaena ordorata*	5.87	1.32	19.51	8.95
Mount Oku	Tropical humid and warn	7°15'N, 11°15'E	2335	Caesalpiniaceae; *Chromolaena ordorata*	4.50	0.75	23.04	7.61
Mount Manenguba	pseudo-tropical humid	5°6'N, 10° 7'E	1935	*Agauris salicifolia, Nuxia congesta, Pittosporum manii, Schefflera abyssinica.*	6.11	1.24	66.40	7.95

P: available P, Org C: Organic Carbon

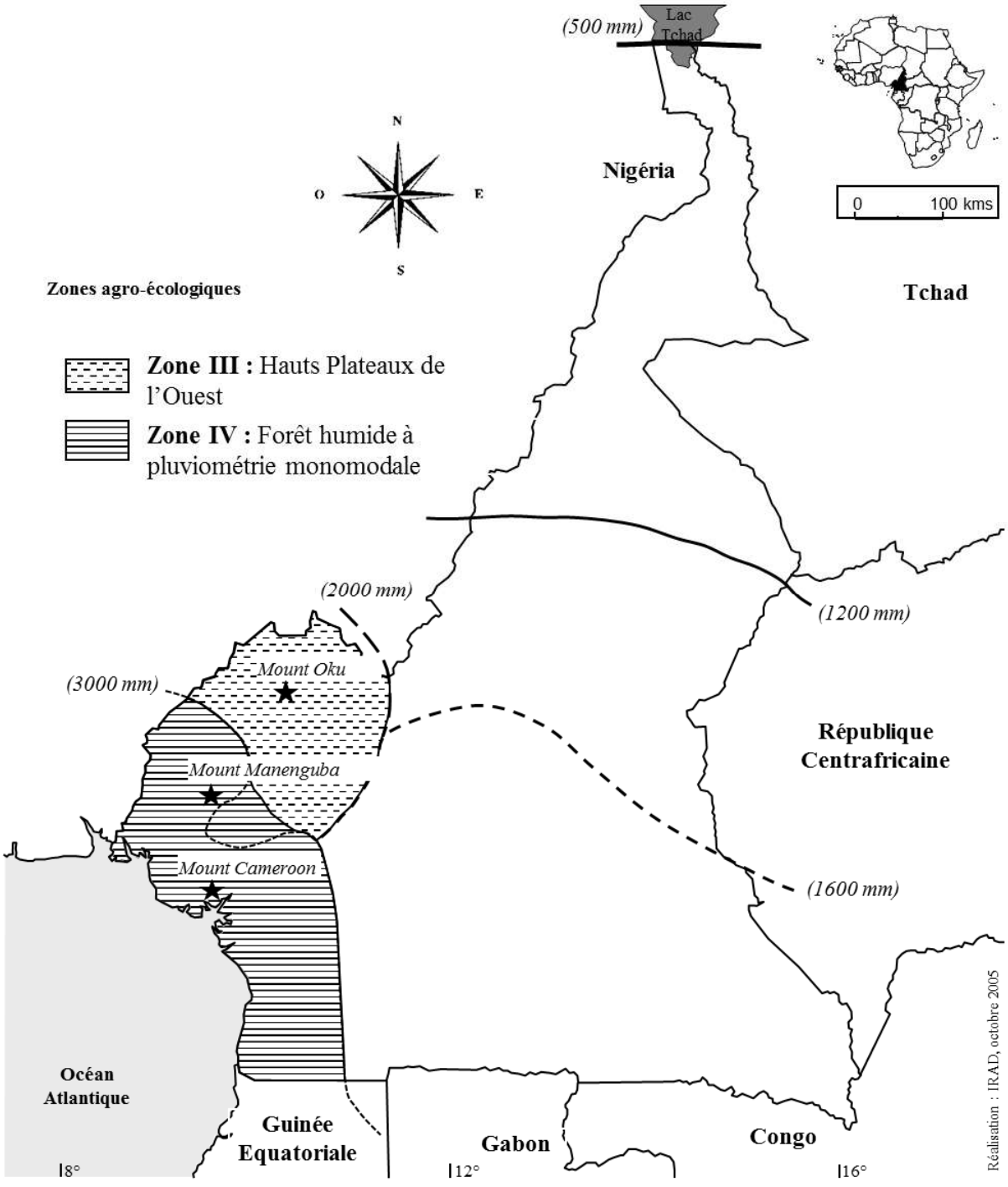

Fig. 1. Sampled Sites for arbuscular mycorrhizal fungi isolates (brown star) of *Prunus africana* in the mono modal rain forest and western highlands ecological zones of Cameroon

(each pot received 250 g of inoculum). Each of the inoculum was then covered with autoclaved sand. *Zea mays* seeds were sterilized in sodium hypochlorite 10 percent (w/v) for 15 min then rinsed three times with sterilized distilled water and sown as a trap plant in a density of three seeds per pot, with 3 replicates per soil origin. The trap plant species were chosen because they are well-known as AMF host plants and are frequently used for trap cultures (Ngonkeu, 2009). Pots were watered every two days and, twice a month, they were fertilized with 100 ml of Rorison's nutritive solution with low phosphorus 120.02 g/L MgSO4,7H2O, 238.04 g/L Ca(NO3)2,4H2O, 115.38 g/L KH2PO4,3H2O, 12.500 g/L [Fe EDTA, 1.121 g MnSO4,4H2O, 1.421 g H3BO3, 0.093 g (NH4)6Mo24,4H2O, 0.220 g ZnSO4,7H2O, 0.198 g CuSO4,5H2O]/L (Ngonkeu, 2009). The trap culture was kept in a greenhouse for 3 mo and this procedure was repeated 2 times. At the end of two growing cycle (three month/cycle), plants were harvested and roots of host plants collected. Part of the roots was dried at room temperature (25°C) for AMF molecular analysis and the remainder was used for mycorrhizal colonization rates analysis.

AMF colonization of *Prunus africana* and trapping plants

The percentage root length colonization of AMF of *Prunus africana* and trapping plants was calculated by the gridline intersect method (Ngonkeu 2009) after cleared in 10 percent KOH for 15 min at 90°C and stained with Fuchsin acid or Trypan blue (0.05%) for 30 min (Phillips and Hayman, 1970; Brundrett *et al.,* 1996). AM fungal spores were isolated from trapping soil by wet sieving and decanting technique (Ngonkeu, 2009). The spores were identified following current taxonomic criteria (Schenck and Perez, 1990) and also using information of INVAM (http://www.invam.caf.wdu.edu/).

Species diversity of AMF

The Shannon Weaver index (Legendre & Legendre 1984) was used to evaluate the specific diversity, and was calculated as $H = \Sigma\ Pi \log Pi$ (with $Pi = ni/n$ being the frequency of the species on the sites).

Molecular analysis

DNA extraction from roots

Dried composite root sample from each origin (±50 mg) was first ground in liquid nitrogen within a 1.5 mL microcentrifuge tube using a pellet pestle. DNA was extracted from the resulting powder using the Purelink Plant Total DNA Purification Kit (Invitrogen, France) following the manufacturer's instructions. DNA extracts were quantified by eye after electrophoresis in 1% agarose gel and either retained as neat extracts. Extracts were used as template DNA for amplification by polymerase chain reaction.

DNA extraction from pool of spores

AMF spores were cleaned by sterilization with chloramine T (4%) and streptomycin (0.004%) and by four rounds of sonication. 10 spores were crushed in 50 µL of 1X REDTaq PCR Reaction Buffer (10mM Tris-HCl pH 8.3, 50mM KCl, 1.1 mM MgCl2 and 0.01 percent gelatin) and incubated at 95°C for 15 min, centrifuged for 5 min at 10,000 rd/min, and the supernatant stored at -20°C (Lanfranco *et al.,* 2001).

Nested PCR amplification

At both two different DNA extractions process (from root and from spore), the large subunit (LSU) region of the nuclear rRNA was used as a target region for the PCR experiment. Reactions were carried out in a final volume of 25 µL containing 5 µL of 10X PCR reaction buffer, 2 µL dNTPs (2.5 mM), 0.625 µL each primer (20 µM), 0.5 µL BSA (10 mg/mL), 0.3 µL Go Taq (0.5 U) with 3 µL of DNA template. The first fungal DNA amplification was performed using the fungal primers LR1 (5′-GCA TAT CAA TAA GCG GAG GA-3′) (Van Tuinen *et al.,* 1998) and FLR2 (5′-GTC GTT TAA AGC CAT TAC GTC-3′) (Trouvelot *et al*., 1999). The PCR programme was as follows: 93°C for 5 min, followed by 35 cycles at 93°C for 1 min at 58°C for 1 min and at 72°C for 1 min and a final elongation at 72°C for 10 min. 1 µL aliquot of the first PCR product was directly used as template for the nested PCR using the Glomeromycota specific primers FLR3 (5′-TTG AAA GGG AAA CGATTG AAG T-3′) situated between the D1 and D2 domains of LSU rRNA and FLR4 (5′-TAC GTC AAC ATC CTT AAC GAA-3′) in the D2 domain (Gollotte *et al.,* 2004) under the same conditions described above. The nested PCR products were analyzed in electrophoresis using 1 % agarose gel (Sigma, France) in Tris-Acetate-EDTA buffer and visualized by ethidium bromide staining. In all PCR experiments, negative controls consisted of reaction mixture without template DNA. With a DNA size standard (Eurogentec Smartladder). The amplified fragments of about 400 bp were exceed under UV light and purified with the Purelink gel extraction kit (Invitrogen, France) according to the manufacturer's instructions (Ngonkeu, 2009).

Cloning and sequencing

The amplified products (400 bp) were purified on agarose gel using Purelink gel extraction kit (Invitrogen, France) according to the manufacturer's instructions and directly cloned into pGEM-T easy vector with a cloning kit (Promega/Catalys, Wallisellen, Switzerland) following the manufacturer's instructions. XL-2 Bleu ultracompetent cells (Stratagene) were transformed and plated on selective medium following the manufacturer's instructions. For each sample, twenty putative positive clones identified based on blue-white screening were chosen randomly and the plasmid DNA was extracted from each transformed Escherichia coli cells suspended in 30 µL of sterile water in a 1 ml tube in a thermocycler as followed: 2 min at 96°C followed by 5 cycles of 2 min at 96°C and 10s at 4°C. The plasmids containing supernatant were re-amplified by PCR using the Glomeromycota specific primers FLR3/FLR4 as described above and the products were revealed on agarose gels (Sigma, France) to confirm the presence of the inserts into the plasmids. The bands of AMF species obtained were exceed and purified using the Purelink gel extraction kit (Invitrogen, France) according to the manufacturer's instructions. The purified PCR products were sequenced using primers FLR3/FLR4. Sequencing reactions were analyzed on a 3730 XL (Applied Biosystems) 96 capillary sequencers using a

BigDye 3.1 sequencing kit (Genoscreen, France). The sequences obtaines were compared with sequences registered in the National Center for Biotechnology Information (NCBI) GenBank (http://www.ncbi.nlm.nih.gov) by BLAST. CLUSTAL W logarithm was use to performed multiple alignements and Neighbor-joining phylogenic trees were constructed using Mega 6.0.5 (Tamura *et al.,* 2013). Bootstrap values were estimated from 1,000 replicates. An out-group was *Mortierella chlamydospora* (accession no. AF157197).

Data analysis

The percentages of the root mycorrhizal colonization were transformed by arcsine (sqrt) before statistical analysis. Data regarding root colonization rates were then subjected to one-way analysis of variance (ANOVA). Means were compared using the Newman-Keuls test ($p<0.05$) in SPSS software, version 18.

RESULTS

Mycorrhizal colonization of *P. africana* root's and spore abundance

The natural occurrence of arbuscular mycorrhizal in *P. africana* roots was significantly different between the three sites of the two agroecological zones (monomodal rainfall forest represented by Mount Cameroon (MC) and Mount Manengouba (MM); and Western highlands by Mount Oku (MO). In MC and MM, percent root colonization were range between 80 – 91 % while in MO root colonization rate was estimate at 43 % (Fig. 2). But after six months trap culture with maize (Zea mays) as host plant, root colonization rates were nearly similar among the three soil origin when soils of *P. africana* rhizosphere were used as inoculum (Fig. 2).

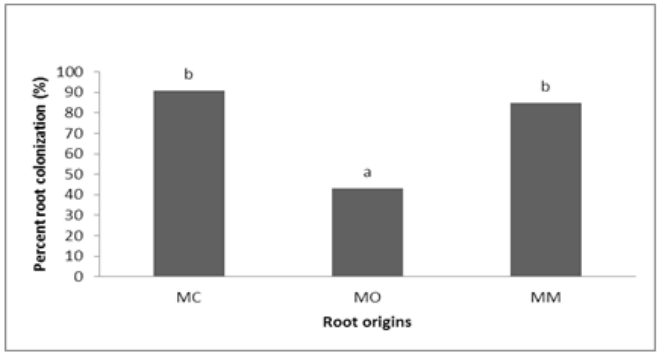

Fig. 2. Mycorrhizal root colonization of *P. africana* directly from native tree of three sites. For each site, bars with the same letter are not significantly different according to the Newman-Keuls test (P ≤ 0.05). MC: Mount Cameroon; MO: Mount Oku; MM: Mount Manenguba

Direct extraction performed on native *P. africana* soils resulted on empty spores (data not shown) while, in soils collected after trap culture, the density of AMF spores varied highly among the treatments with 5234 spores per 100 g soil in MC sample, 5564 in MM and 320 spores/100 g of soil in MO (Table 2). AMF spores abundance was significantly higher in soil samples from *P. africana* mono modal rainfall forest than those from Western highlands (Table 2).

Table 2. Arbuscular mycorrhizal fungi population in three sites of Cameroon according to different spores morphotypes

Soils origin	AMF spores Per 100g soil	H	E
MC	4828 a	0.40 b	0.83 a
MO	838 c	0.26 a	0.87 a
MM	1723 b	0.53 c	0.88 a

In each column, means with the same letter are not significantly different according to the Newman-Keuls test (P ≤ 0.05). MC: Mount Cameroon; MO: Mount Oku; MM: Mount Manenguba. H: Shannon – Weaver indices; E: Equitability.

AMF species richness, community composition and infection potential

Four AMF morphotypes belonging to four genus (*Gigaspora, Glomus*, *Acaulospora* and *Entrophospora*) were isolated and identified in rhizosphere of *P. africana* in three sites of the 2 agroecological zones (Table 2; Fig. 3). In mono modal rainfall, 3 and 4 morphotypes representing 4 genuses were found in *P. africana* of MC and MM sites respectively, compared to two morphotypes within one genus (*Acaulospora*) in MO. This low AMF species richness was confirmed by Shannon-Weaver indice where H were 0.40 and 0.53 in MC and MM sites respectively compared to 0.26 in MO site. Likewise, Pielou equitability index which measures the proportion of individual organism within each species was nearly similar between the three sites. Indeed, there was no significant difference between the indexes in the three sites. Indices was around 1 with 0.83, 0.87 and 0.88 respectively in MC, MO and MM sites (Table 2). Two spores' morphotypes (T4 *Acaulospora* sp. and T5 *Acaulospora* sp.) were detected only at MO site of Western highlands zone. All others spores morphotypes were only found in both MC and MM sites of mono modal rainfull zone except spore morphotype T2 *Glomus* sp.

Molecular characterization of AMF communities

Blast search results of large subunit (LSU) sequences obtained from these AMF spores isolated from root of natural *P. africana* confirmed that the species belonged to the Glomeromycota. Results of the phylogenetic analysis of the LSU sequences (Fig. 3) indicated the presence of three main families groups: The first group (*Acaulosporaceae*) included PR4MO and PR5MO species which represented 90 percent and 96 percent of similarity respectively with *Acaulospora tuberculata* and *A. longula*, and PR6MC and PR6MM species, both had 91 percent of similarity with *Entrophospora colombiana*; the second group (*Glomaceae*) formed by PR2MM species belonging to *Glomus* AB group has 99 percent of similarity with *Glomus manihotis* while, the species PR3MC and PR3MM belonged to the same group had 100 percent of homology with *Glomus etunicatum;* and the third group (*Gigasporaceae*), included PR1MC and PR1MM species which presented 76% of similarity with *Gigaspora margarita*. Phylogenetic analyses indicated a total of 6 species identified in the 3 study sites. The most diversified site was Mount Manenguba with 4 species of AMF, followed by Mount Cameroon with 3 species. The lowest diversified site was the Mount Oku with 2 species found (Fig. 4).

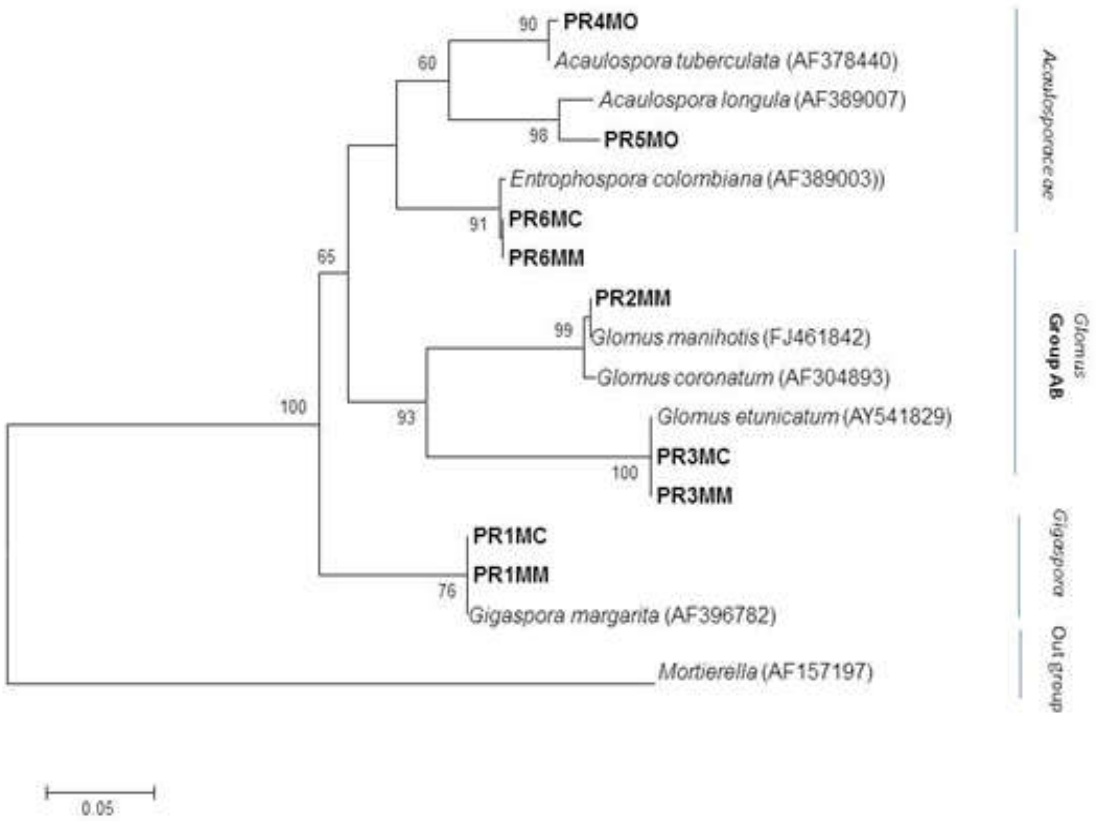

Fig. 3. Neighbour-joining phylogenetic tree showing the position of the 25S rRNA AMF sequences from the *Prunus africana* roots (in bold type) among representatives of Glomeromycota. Bootstrap values were estimated from 100 replicates and *Mortieriella chlamydospora* was used as an out group

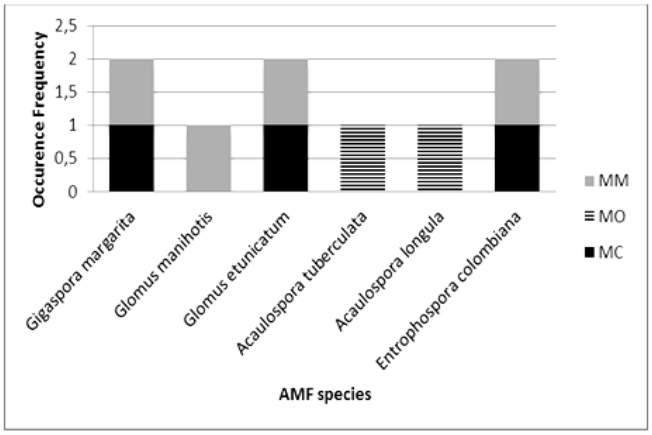

Fig. 4. Distribution of AMF species in *Prunus africana'* roots in tree study sites. MC: Mount Cameroon; MO: Mount Oku; MM: Mount Manenguba

DISCUSSION

AMF status of *Prunus Africana*

Prunus africana is pharmaceutically one of the most important medicinal tree, use for the treatment of BPH and prostate gland hypertrophy worldwide (Stewart, 2003). It is also one of the tree where establishment and growth are highly dependent of the presence of AMF in the soil (Wubet *et al.,* 2003; Wubet *et al.,* 2004). Our study confirm the presence of AMF in the root of *P. africana* as previous studies.

Species richness and community composition of AMF

The index of Shannon (H) for the study sites involving MC, MO and MM which are respectively 0.4, 0.26 and 0.53 show a very low diversity of AMF compared to the values found by Wubet *et al.* (2004). In our study, the determination of the diversity index was made on the basis of morphological characterization of spores obtained after trapping culture. This could explain the low diversity of AMF obtained compared with some previous studies (Husband *et al.,* 2002; Wubet *et al.,* 2004). Indeed, trapping culture reveals only partially diversity of AMF in soil (Bever *et al.,* 2001; Wubet *et al.,* 2004). In addition some AMF species could not be revealed because of selective association established between host plant and the fungus (Kardol *et al.,* 2006). However, a direct comparison could not be made between the diversity of AMF obtained on the basis of morphological characters of spores and that obtained by Wubet *et al.,* (2004), based on molecular analyzes (ITS rDNA) MFA contained in the roots of *Prunus africana*.

AMF specificity

The AMF communities in field differs with plant host (Wubet *et al.,* 2004). In the present study, the comparison of both phylogenetic trees of AMF associated with trapping plants and *Prunus africana*, reveals the specificity of AMF associated with *P. africana* in Cameroon (Fig. 5). This confirm that even endangered or common plant species inoculated with native AMF from different habitats show specificity with regard to

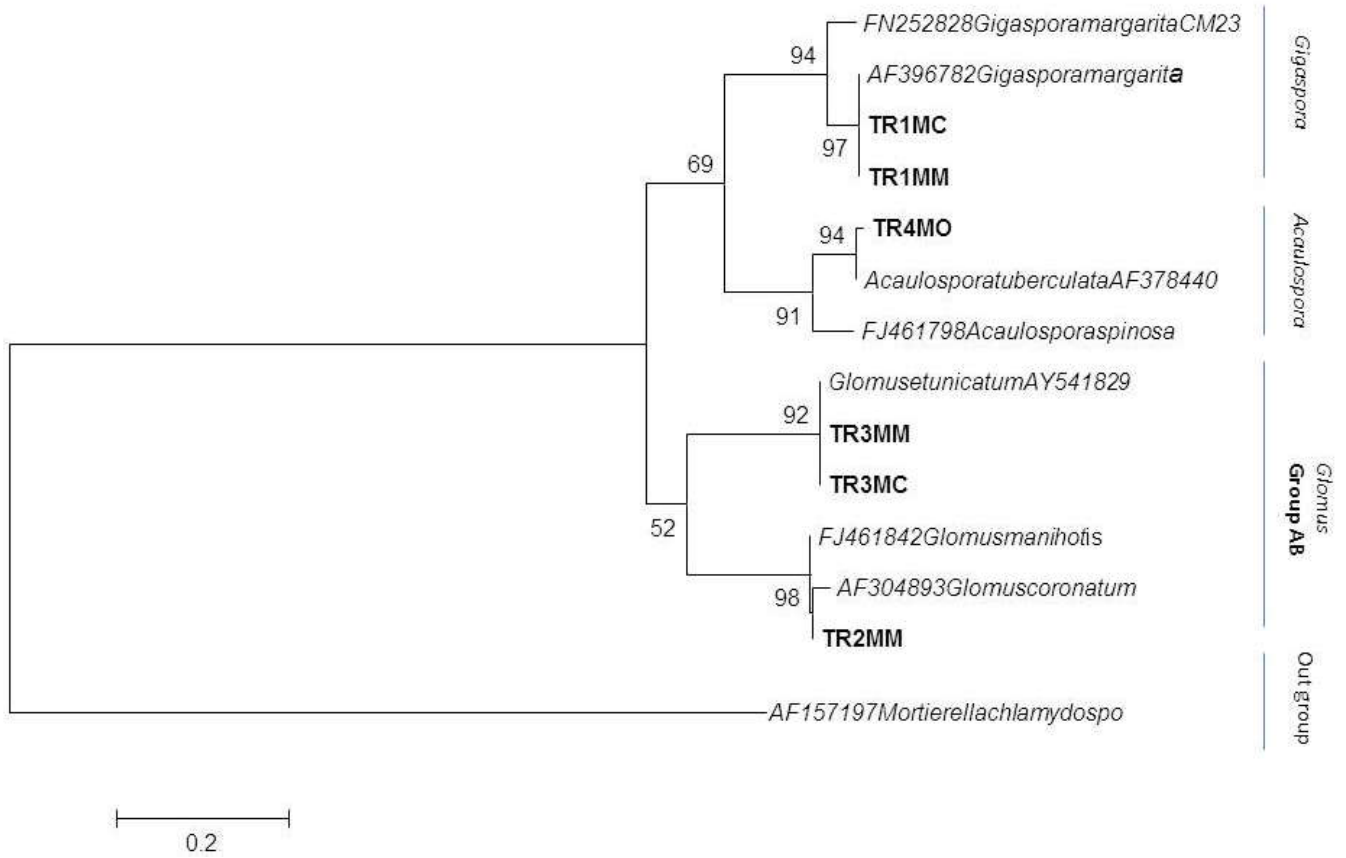

Fig. 5. Neighbour-joining phylogenetic tree showing the position of the 25S rRNA AMF sequences from the *Zea mays* roots (in bold type) among representatives of Glomeromycota. Bootstrap values were estimated from 100 replicates and *Mortieriella chlamydospora* was used as an out group

the inoculum they prefer (Moore *et al.,* 2004; Fuchs and Haselwandter 2008).

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