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Biological bar code for determining the geographical origin of fruits using 28S rDNA fingerprinting of fungal communities by PCR-DGGE: an application to Shea tree fruits

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ABSTRACT

Objectives Shea tree is a multi-purpose tree daily used by rural African communities. Economic importance of Shea tree fruits has been rising and achieving a great success in African, American and European markets. Shea butter is used mainly in chocolate industry, cosmetic or pharmacological products. Traceability is now one of the great concerns of the customers and the lawyers. In view of the difficulties of installing these documentary systems in developing country particularly the countries of sub-Saharan Africa, the new strategies of traceability emerge. *Methods* Molecular technique using 28S rDNA profiles generated by polymerase chain reaction denaturing gradient gel electrophoresis was used to detect the variation in fungal community structures of Shea tree fruit from Senegal, Mali and Cameroon. *Results* 28S rDNA profiles were analysed by multivariate analysis, distinct microbial communities were detected. Band profiles of Shea tree fruit fungi from different countries were specific for each location and could be used as a bar code to discriminate the origin of fruits. *Conclusion* We propose the polymerase chain reaction denaturing gradient gel electrophoresis method as the fingerprinting of Shea tree fruits using 28S rDNA of fungi that provides the fruits with a unique bar code and make it possible to trace back the Shea tree fruit to their original locations.

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Introduction

Traceability is now one of the great concerns of the customers and the lawyers. For long time the food industry has used simple traceability systems. The UK Food Standards Agency (FSA) displays an interesting report on labelling research, showing that consumers strongly support Country of Origin Labelling and that consumers think it is important that labelling of food always clearly identifies the country of origin of the ingredients (FSA, 2007). Traceability is defined as the ability for the retrieval of the history and use or origin of an article or its relevant components or an activity through a registered method [International Organization for Standardization (ISO), 2007]. In view of the difficulties of installing these documentary systems in developing country, in particular the countries of sub-Saharan Africa, the new strategies of traceability emerge. To follow the geographical origins of Shea tree fruits product during processing, we proposed to identify and validate some pertinent biological markers, which come from the environment of the fruits to assure their traceability. Stable isotopes are the only ones which are referenced as an European regulation for wine origin determination (Ghidini *et al*., 2006).

The Shea tree (*Vitellaria paradoxa*) is a multi-purpose tree daily used by rural African communities. The species belongs to *Ebenales* order which contains 600 species distributed in 50 genera (Leroy, 1982; Guignard, 1986). The family *Sapotaceae* to which Shea belongs contains 25 genera representing 50 species (Diarrassouba *et al*., 2007). The Shea tree was scientifically known in the past as *Butyrospermum paradoxum*, which grows wild in West Africa within a geographical area stretching from Mali to Sudan in the north Togo and Uganda in the south (nearly 5000 km). It is called by traders 'Shea belt'. Mungo Park (1771–1806) was the first to give the botanical characteristics of Shea tree. The tree can be between 10 and 15 m in height and has a short barrel (3 m) with a diameter of up to 1 m. Its lifetime is estimated at 200 or 300 years. Its root system is very tortuous. The fruits are ovoid with a size of 4–8 cm long, green to dark brown. Shea fruit contains one or two hard kernels, a whitish hue surrounded a thin shell and pulp (55%). The fruit-harvesting season extends from June to September. In Egypt, where they also found traces of Shea fruits, we think that the tree used to make statues, about three centuries before our century. In Africa, Shea tree fruit is also nicknamed 'The Gold of Women', because Shea butter is not only used as a cosmetic by women, but also used as a fat for cooking, mainly in rural areas which account for 80% of total consumption [United Nations Conference on Trade and Development (UNCTAD), 2006]. The economic importance of Shea tree fruits has been rising and achieving a great success in African, American, European markets. The African continent with 16 countries is now a unique supplier of this product in the entire world. Shea tree butter is traditionally used as cooking fat, soap or a source of energy and provides export opportunities by it use in chocolate and cosmetics/pharmacological industries. Mali, Ghana and Burkina Faso, together account for just under a third of world production in 2005. In Europe, Shea butter is used mainly (95%) by the chocolate industry. The quantities exported to Japan, the United States or Switzerland would be mainly used for cosmetic or pharmacological [Food and Agriculture Organization of the United Nations Statistics (FAOSTAT), 2007]. Currently, there are only a few existing analytical methods which permit the efficient determination of the origin of food or to follow them during international trade. In case of doubt or fraud, it is necessary to find a precise and fast analytical technique in order to determine their geographical origins. The most popular analytical methods, which allow us to ensure the determination of origin are using bar code, spectroscopy, stable isotope, etc. (Peres *et al*., 2007). It thus seems difficult to use fruit genomic markers to ensure the traceability of Shea tree fruits. However, the skin of fresh fruits is not sterile and can carry microorganisms or their fragments. The presence of various microorganisms must depend on the external environment of the fruit (soil ecology, spoilage, insects, diseases), but also microorganisms brought by human activity (Sodeko *et al*., 1987).

The idea was to create a 'biological bar code' (Montet *et al*., 2004) based on the analysis of the DNA of microorganisms present on the products. This method is based on the assumption that the microbial communities of the fruits are specific for a geographical area (Le Nguyen *et al*., 2008; Montet *et al*., 2008, 2010; El Sheikha *et al*., 2009, 2010; El Sheikha, 2010).

The main objective of this study is to apply polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) method to analyse in a unique step all the fungi present on the fruit in order to create an analytical technique that will permit the linkage of fungi communities to the geographical origin and avoid the individual analysis of each fungus strain. To the best of our knowledge, this is the first paper describing a molecular method of fungi ecology, the PCR-DGGE that will permit the certification of Shea tree fruit using 28S rDNA fingerprinting of fungi.

Materials and methods

Fruits sampling

Mature fruits of Shea tree (*V. paradoxa*) were collected in two different districts from three countries: Mali and Senegal and one district from Cameroon. These districts were Tori and Sassamburu, Mopti region in Mali; Kenioto and Saraya, Tambacounda region in Senegal and Adamawa in Cameroon. Table 1 gives the geographical coordinates of the sampling sites. Additionally, Tori and Sassamburu, Mopti region in Mali follow the central fringe of the African Sahel, called 'The Sahel nomads'. Thus it has a semiarid climate with a total annual rainfall of 467 mm and a rainy season extending from late May to early October. August is the wettest with a total rainfall of 156 mm. Temperatures are also experiencing significant seasonal variations. The warmest temperatures are recorded in May ($T_{\max} = 40.6\,^{\circ}\mathrm{C}$) and coldest in January ($T_{\min} = 15.0\,^{\circ}\mathrm{C}$). These conditions are associated with sparsely vegetated steppe and roaming livestock farming is the main resource. In Senegal,

Table 1 Description of sampling sites

			GPS		
Country	Region	Sites	Longitude	Latitude	Altitude (m)
Mali	Mopti	Tori	13°37′00.00″N	3°43′00.00″W	295
		Sassambourou	14°18′00.00″N	3°28′48.00″W	535
Senegal	Tambacounda	Saraya	12°49′53.62″N	11°45′21.10″W	189
		Kenioto	12°34′20.11″N	12°09′60.16″W	120
Cameroon	Adamaoua	Mbe	7°53′12.05″N	13°35′04.56″E	607

Tambacounda region follows the Sudano-Guinean area climate; these forests are very large and dense savannah. The trees are varied: baobab, kapok, palm, casuarina and Roni. August is the wettest with a total rainfall of 308 mm. Temperatures are also experiencing significant seasonal variations. It records the hottest temperatures in April ($T_{\max} = 40$ °C) and coldest in January ($T_{\min} = 18$ °C). In Cameroon, the high altitude of Adamawa region gives a relatively cool climate of between 22 and 25 °C. In the southern region, an equatorial climate type of Guinea with four seasons: a long dry season from December to May, a small wet season from May to June, a short dry season from July to October, and a long rainy season from October to November. On the Adamawa plateau, the climate is tropical Sudanian. There are only two seasons: the dry season goes from November to April and then comes the wet season. The average annual rainfall of 900–1500 mm and decrease further north. The third type of climate in southwestern Adamawa (department of Mayo-Banyo) is a Cameroonian-type equatorial climate. Rainfall ranges from 1500 to 2000 mm with a long dry season followed by a long rainy season.

The fruits were gathered to preserve their initial flora. They were collected directly on the tree using gloves and put in sterile bags in July 2008. These bags were kept into a refrigerator then transferred by plane to CIRAD Montpellier (France) where the fungal DNA was extracted immediately from the fresh fruits. The origin of the samples was defined by country, site and date of harvest.

Fungal DNA extraction from Shea tree fruit samples

For fungal DNA extraction, we created a new protocol which takes into account the methods of Karakousis *et al.* (2006) developed for fungal DNA extraction and El Sheikha *et al.* (2009) developed for yeast DNA extraction. Two fruits of Shea tree were randomly taken and put in sterile Stomacher bag containing 6 mL peptone. The two Eppendorff 2 mL vials contained the resulting suspension with 0.3 g of 0.5 mm diameter acid washed glass beads 425–600 μm (Sigma-Aldrich Chimie S.a.r.l., Lyon, France). The mixture was vortexed vigorously for 30 min in a bead beater instrument (Vortex Genie 2 SI–A256, Bohemia, NY, USA) then centrifuged at 12 000 × *g* for 15 min and the supernatant discarded. The cell pellet was resuspended in 300 μL of breaking buffer [2% Triton X-100 (Prolabo, Fontenay-sous-Bois, France); 1% sodium dodecyl sulphate (SDS, Sigma); 100 mM NaCl (Sigma); 10 mM Tris-HCl pH 8.0; 1 mM EDTA pH 8.0 (Promega, Charbonnières-les-Bains, France)]. Then, 100 μL TE (10 mM Tris-HCl; 1 mM EDTA; pH 8.0, Promega) and 100 μL of lysozyme solution (25 mg mL^{-1}, Eurobio, Les Ulis, France) and 100 μL of proteinase K solution (20 mg mL^{-1}, Eurobio) were added and incubated at 42 °C for 20 min. Then 50 μL of 20% SDS were added to each tube, and the tubes were incubated at 42 °C for 10 min. Four hundred microlitres of mixed alkyltrimethyl ammonium bromide (Sigma) were added to each tube, and the tubes were incubated at 65 °C for 10 min. The tubes were vortexed vigorously for 5 min after each addition. The lysates were then purified by twice repeated extraction with 700 μL of phenol–chloroform–isoamyl alcohol (25:24:1, Carlo Erba, Val De Reuil, France) and the tubes were vortexed for 5 min and then centrifuged at 12 000 × *g* for 15 min. The aqueous layer was transferred to an Eppendorff vial and the residual phenol was removed by extraction with 600 μL of chloroform–isoamyl alcohol (24:1) and centrifuged at 12 000 × *g* for 15 min. The aqueous phase was collected and the DNA was stabilized with 30 μL of sodium acetate (3 M, pH 5), followed by precipitation by adding equal volume of ice-cold isopropanol and stored at −20 °C for 12 h (overnight). After centrifugation at 12 000 × *g* for 15 min, the supernatant was eliminated, DNA pellets were washed with 500 μL 70% ethanol, and tubes were centrifuged at 12 000 × *g* for 15 min. The ethanol was then discarded and the pellets were air dried at room temperature for 45–60 min. Finally, the DNA was resuspended in 50 μL of ultra pure water and stored at −20 °C until analysis. In order to check DNA extraction, an electrophoresis on agarose gel was done. The entire DNA was loaded into 0.8% agarose gel in 1 × TAE buffer (40 mM Tris-HCl pH 7.4, 20 mM sodium acetate, 1.0 mM Na_2–EDTA, Eppendorff, Hamburg, Germany) with molecular weight ladder 16.21 kb as reference (Supercoiled DNA ladder, Invitrogen, Carlsbad, CA, USA). After running at 100 V for 30 min, the gels were stained for 30 min with ethidium bromide solution ($50\,\mu g\,mL^{-1}$, Promega), rinsed for 20 min in distilled water, then observed and photographed on a UV transilluminator using black and white camera (Scion Company, Bethesda, MD, USA) and Gel Smart 7.3 system software (Clara Vision, Les Ulis, France).

PCR-DGGE analysis

A fragment of region of the 28S rDNA gene was amplified using eukaryotic universal primers U1 (5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GTG AAA TTG TTG AAA GGG AA-3′, Sigma) and the reverse primer U2 (5′-GAC TCC TTG GTC CGT GTT-3′, Sigma) amplifying an approximately 260 bp fragment (Wu *et al*., 2002; Li *et al*., 2008). A 30-bp GC-clamp (Sigma) was added to the forward primer (the GC-clamp is underlined) in order to insure that the fragment of DNA will remain partially double-stranded and that the region screened is in the lowest melting domain (Sheffield *et al*., 1989). PCR was performed in a final volume of 50 μL containing 2.5 μL DMSO, 0.4 μM each primers, all the deoxyribonucleotide triphosphate at 200 μM, 3 mM $MgCl_2$, 5 μL of 10 × of reaction *Taq* buffer $MgCl_2$ free (Promega), 1.25 U of *Taq* DNA polymerase (Promega) and 2 μL of the extracted DNA. The amplification was carried out as follows: an initial denaturation at 94 °C for 3 min, 30 cycles of 94 °C for 45 s, 50 °C for 50 s and 72 °C for 90 s, and a final extension at 72 °C for 5 min. Aliquots (5 μL) of PCR products were analysed first by conventional electrophoresis in 2% (w/v) agarose gel with TAE 1 × buffer (40 mM Tris-HCl pH 7.4, 20 mM sodium acetate, 1.0 mM Na_2–EDTA), stained with ethidium bromide $50\,\mu g\,mL^{-1}$ in TAE 1 × and quantified using a standard (DNA mass ladder 100 bp, Promega).

The PCR products were analysed by DGGE using a Bio-Rad DcodeTM universal mutation detection system (Bio-Rad Laboratories, Benicia, CA, USA). Samples containing approximately equal amounts of PCR amplicons were loaded into 8% (w/v) polyacrylamide gels (acrylamide/*N*,*N*′-methylene bisacrylamide, 37.5/1, Promega) in 1 × TAE buffer (40 mM Tris-HCl pH 7.4, 20 mM sodium acetate, 1.0 mM Na_2–EDTA).

All electrophoresis experiments were performed at 60 °C using a denaturing gradient ranging from 30–40% to 60–70%, and were finally standardized at 40–70% [100% corresponded to 7 M urea and 40% (v/v) formamide, Promega]. The gels were electrophoresed at 20 V for 10 min and then at 80 V for 16 h. After electrophoresis, the gels were stained for 30 min with ethidium bromide and rinsed for 20 min in distilled water and then photographed on a UV transilluminator with the Gel Smart 7.3 system (Clara Vision).

Image and statistical analysis

Individual lanes of the gel images were straightened and aligned using ImageQuant TL software v.2003 (Amesham Biosciences, Arlington Heights, IL, USA). Banding patterns were standardized with three reference patterns included in all gels, *Mucor racemosus* DNA and *Trichoderma harzianum* DNA. This software permitted to identify the bands relative positions compared with the standard patterns.

In DGGE analysis, the generated banding pattern is considered as an image of all of the major fungi in the populations. An individual discrete band refers to a unique ‘sequence type’ or phylotype (Muyzer *et al*., 1995; Van Hannen *et al*., 1999). This was confirmed by Kowalchuk *et al*. (1997) who showed that co-migrating bands generally corresponded to identical sequence. The DGGE fingerprints were manually scored by the presence and absence of co-migrating bands, independent of intensity. Pair wise community similarities were quantified using the Dice similarity coefficient (S_D) (Heyndrickx *et al*., 1996).

$$S_D = 2N_c/(N_a + N_b) \tag{1}$$

where N_a represented the number of bands detected in the sample A, N_b represented the number of bands in the sample B and N_c represented the numbers of bands common to both sample. Similarity index were expressed within a range of 0 (completely dissimilar) to 100 (perfect similarity). Agglomerative Hierarchical Clustering (group average) was carried out on the table of proximity values. Agglomerative Hierarchical Clustering does not evaluate the accuracy of the created clusters (Suzuki & Shimodaira, 2004). A simple resampling method has been used to evaluate it. Each of the five samples was doubled. In the five new hierarchical trees, we noted that the clusters were similar to the original ones. Significant differences of fungal communities of Shea tree fruits were determined by factorial correspondence analysis (FCA) using the first two variances, which described most of the variation in the data set.

Sequence analysis of DNA bands and fungi identification

DNA bands from DGGE gel were carefully selected and excised from the gel using sterile razor blades. The pieces of gel were soaked in 100 µL of TE buffer overnight at 4 °C. Eluted DNA for each band was purified by kit Wizard PCR Preps DNA Purification system (Promega), and then the purified DNA was re-amplified by the same PCR conditions as described above using the primers without GC-clamp. The amplicons were sequenced by GATC Biotech (Konstanz, Germany). DNA base sequences were analysed by comparison with the GenBank databases of the National Centre for Biotechnology Information. Searches in GenBank with BLAST program were performed to determine the closet known relative of partial 28S rDNA sequences (Altschul *et al*., 1997).

Results

Efficiency of the new protocol for the extraction of fungal DNA from Shea tree fruits

DNA extraction of the fungal community was done on the Shea tree fruits using our new protocol, which achieved admirable success; we verified the extraction efficiency with a 0.8% (w/v) agarose gel. On the gel, the bands with a molecular weight greater than 16 kb corresponding to genomic fungal DNA were clearly observed.

Verification of the PCR amplification of the extracted DNA

The fungal DNA obtained after extraction was amplified by classic PCR using a protocol improved by us. In order to verify the efficiency of this fraction, the PCR amplicon were electrophoresed on 2% (w/v) agarose gel at 100 V for 30 min in the TEA buffer as described above. All of the bands were clearly observed and had a molecular weight of 260 bp, the expected size of the amplicon. The intensity of the bands representing the PCR amplicons was important. After successful amplification which permits to continue to analyze these amplicons by the DGGE method.

DGGE pattern of fungal DNA from Shea tree fruits among different countries

On DGGE gel, the observed bands had sufficient intensities to analyze samples of fungal DNA extracted from Shea tree fruits from three various geographical areas (Figure 1), so the total quantity of DNA deposited in the wells of DGGE gel was sufficient to consider that fungal DNA could be used as potential markers. The reference DNA of *M. racemosus* and *T. harzianum* indicates that DGGE was perfectly done. Each vertical line represents a fruit and each spot represents a species of fungi. Some spots appeared double or smear because of the presence of single-strand DNA (Osborn & Smith, 2005).

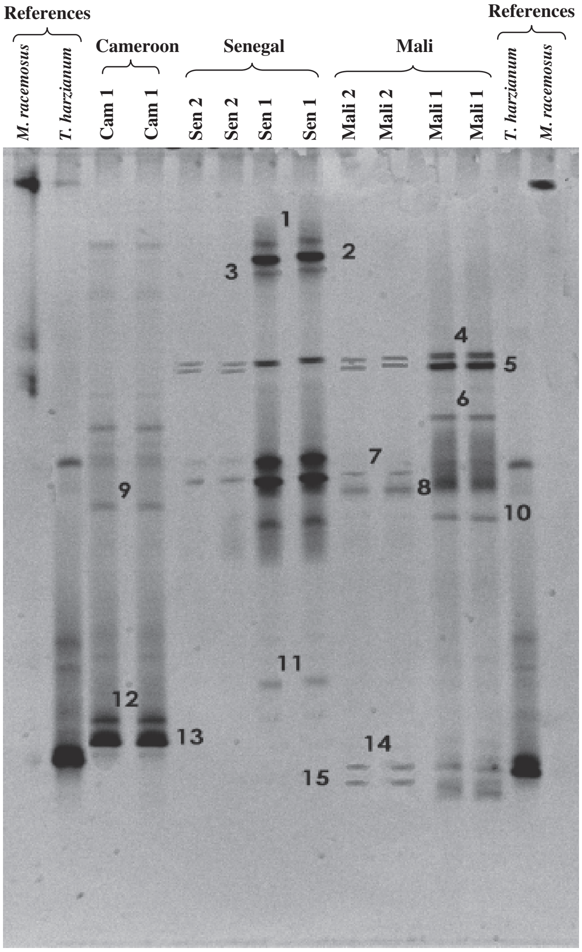

Figure 1 Polymerase chain reaction denaturing gradient gel electrophoresis 28S rDNA band profiles of Shea tree fruit from three countries Mali, Senegal and Cameroon. Mali, Mali; Sen, Senegal; Cam, Cameroon. (1, 2) Two different districts. Numbers 1–15 are the sequenced bands.

The duplicate of PCR-DGGE patterns of Shea tree fruits for each location were similar for each country and revealed the presence of 6–10 bands for each Shea tree fruit (Figure 1).

Clusters analysis by Statistica version 6 software (StatSoft, Maisons-Alfort, France) of the DGGE gel patterns for the duplicate Shea tree fruit samples from three different countries showed a community similarity among the geographical locations where the fruit samples were collected (Figure 2). At 45% similarity level, two main clusters were observed: the first cluster included the samples from Senegal and Mali the second cluster comprised the samples from

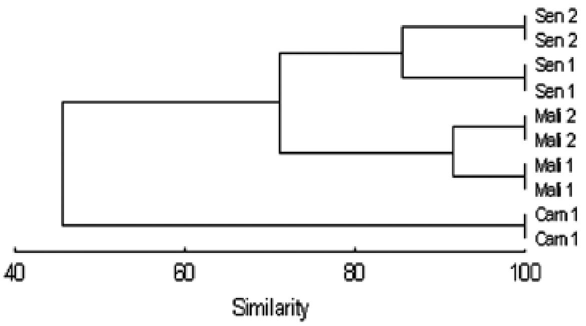

Figure 2 Cluster analysis of 28S rDNA band profiles of Shea tree fruit from three countries Mali, Senegal and Cameroon. Mali, Mali; Sen, Senegal; Cam, Cameroon. (1, 2) Two different districts.

Cameroon. Doubling one sample never involved changes in the constitution of the two established classes (100% stability). This organization in two classes thus represents a strong structuring of the data. The cluster analysis also showed different similarities levels between the different countries. The similarities among districts (i.e. 87–93%) are more closed than the similarities among countries (45–72%). For example, there was 70% similarity between Senegal and Mali.

FCA proved to be a useful statistical tool to compare the similarity of the fungal communities of Shea tree fruit samples from the three different countries in harvested season. The first two variances, which described most of the variation (86%) in the data set (Figure 3).

Identification by sequencing of dominant fungi on Shea tree fruits

To our knowledge, there is no information on the populations of fungi from Mali, Senegal and Cameroon by culture dependent methods. The bands cut from DGGE gel profiles of extracted fungi DNA from Shea tree fruits were sequenced in order to know the identity of strains of fungi present in the fruit samples. Each sequenced band corresponds to a unique sequence which has a sufficiently long to allow an identification by comparison between the sequences and those listed in GenBank (Table 2).

Discussion

The PCR-DGGE approach has been also profitably applied to study fungal communities that very often play an important role in food fermentations. Ben Omar and Ampe (2000) and Ampe *et al.* (2001) looked for fungi in pozol and cassava samples, respectively, during fermentation. The authors performed analyses using PCR-DGGE amplicons of 18S rDNA. This 18S rDNA PCR-DGGE was also used by Röling *et al.* (2001) to identify fungi in vanilla beans during curing. Flórez and Mayo (2006) used 26S rDNA to detect the fungal species in Cabrales cheese during the manufacture and ripening. Three papers were published by our team that described the linkage between bacterial, yeast and fungi communities and the geographical origin of fruits (Le Nguyen *et al.*, 2008; Montet *et al.*, 2008; El Sheikha *et al.*, 2009, 2010; El Sheikha, 2010). But we think that our paper is the first publication, which introduces a unique 'biological bar code' of the Shea tree fruit using 28S rDNA fingerprinting of fungi. In our study, we proved that the DGGE pattern

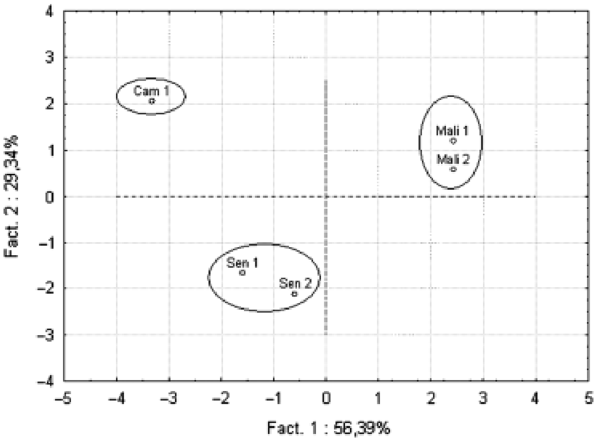

Figure 3 Factorial variance analysis of 28S rDNA band profiles of Shea tree fruit from three countries Mali, Senegal and Cameroon. Mali, Mali; Sen, Senegal; Cam, Cameroon. (1, 2) Two different districts.

Table 2 Sequencing results of the bands cut from the DGGE gel

Band (s)	Closest relative	Identity (%)*	Source
1	*Mucor hiemalis*	93	GU566266
2	*Penicillium griseofulvum*	90	GU566224
3	*Penicillium oxalicum*	85	FJ810802
4	*Aspergillus versicolor*	98	HQ316565
5	*Cryptococcus neoformans*	97	AY217026
6	*Ceratocystis paradoxa*	96	HQ248205
7	*Paecilomyces variotii*	91	GU966517
8	*Penicillium sp*	87	GQ418173
9	*Fusarium oxysporum*	95	GU566301
10	*Aspergillus penicillioides*	96	GU017541
11	*Penicillium chrysogenum*	92	HQ026731
12	*Aspergillus sp*	89	HQ166611
13	*Trichoderma rossicum*	85	HQ342420
14	*Trichoderma harzianum*	98	GU060099
15	*Cladosporium sp*	97	HQ316573

*% Similarity with the reference strain.
DGGE, denaturing gradient gel electrophoresis.

of the DNA fungal communities from Shea tree fruit was strongly linked to the microbial environment of the fruit. The analysis of Shea tree fruit samples from different locations showed some significant differences in the migration patterns on the DGGE gel. However, the duplicates for each sampling location gave statistically similar DGGE patterns throughout the study. The differences in the band profiles can be attributed to the differences in environment between districts. In the gel some common bands appeared in all of the samples independently to the location. These bands could be common fungi for all of the Shea tree fruit samples. The fungi strains were identified from the environment, such as *Fusarium oxysporum* could be found on many plants (Anaissie *et al*., 2001). The presence of fungi on the fruit is the good reflection of the environment of the sampling areas thus they could serve as markers of the geographical origin of the fruit. These results can give an idea of the biodiversity of the fungi according to the geographical origin.

In fact, when comparing the different locations of fruits sampling with the statistical analysis of DGGE pattern throughout the study, we could noted that we obtained a complete statistical correspondence between the geographical areas and the fungal communities. We could conclude that there were enough environmental differences between the districts where the Shea tree fruits were harvested to obtain a major effect on the fungal ecology, whereupon we could create a statistical link between the fungi populations and the geographical area.

In conclusion, the analysis of Shea tree fruit fungi communities by PCR-DGGE could be applied to differentiate geographical locations. We showed that the biological markers for the specific locations were sufficient statistically to discriminate regions. This global technique is quicker (< 24 h) than all of the classical microbial techniques and avoids the precise analysis of fungi by biochemistry or molecular biology (sequencing). This method can thus be proposed as a rapid analytical traceability tool for fruits and could be considered as a provider of a unique biological bar code for each country. Furthermore, the ecological study of fungi in many other products in which they occur provide another area for future study.

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