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DIVERSITY OF FRUIT ORIGIN BY USING 26S rDNA FINGERPRINTING OF YEAST COMMUNITIES BY PCR-DGGE: AN APPLICATION TO SHEA TREE FRUITS

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ABSTRACT Aims: The economic importance of Shea tree fruits has been rising and achieving a great success in African, American, European markets. 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. The traceability of fruits is only documentary. In case of doubt or fraud, no standardized analysis makes it possible to trace back the origin of the fruit. The aim of this study is to use a new tool of tracing the products (PCR-DGGE) as a molecular technique to analyse in a unique step all the yeasts present on the fruit to create a linkage between yeast communities and the geographical origin. **Methods and Results**: A method of yeast ecology, the PCR-DGGE, was used to characterize the yeast flora of Shea tree fruit (*Vitellaria paradoxa*) from four countries (Ghana, Senegal, Mali, Cameroon). DGGE fingerprints analysed by multivariate analysis permitted to distinct different fruit origins by their microbial communities. **Conclusion:** the fingerprints of Shea tree fruit yeasts were specific for each country and could be used as a unique biological bar code to discriminate the country of origin of fruits. **Significance of Study:** Creation of a new traceability analytical tool by using 26S rDNA fingerprinting of yeasts that provides the fruits in general and Shea tree fruits in particular with a unique bar code for each country.

Keywords: traceability, PCR-DGGE, Shea tree fruits, 26S rDNA fingerprinting, geographical origin

INTRODUCTION Traceability is becoming a "buzzword" with regard to food, especially after a number of food safety incidents happened. The consumer is more and more demanding and sensitive to the quality and the origin of the foodstuffs that they buy. 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 (Food Standards Agency, 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 (ISO, 2007). In view of the difficulties of installing the documentary systems in developing countries, and to follow the product during processing, we proposed to identify and validate some pertinent biological markers which come from the environment of the food to assure their traceability. 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.

Shea tree (*Vitellaria paradoxa)* which was former named *Butyrospermum paradoxum* is a tree of the *Sapotaceae* family which grows wild in West Africa within a geographical area stretching from Mali to Sudan in the north Togo and Uganda in the south. It is called by traders "Shea belt". 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 (UNCTAD, 2001). The economic importance of Shea tree fruits has been rising and achieving a great success in African, American, European markets. The African continent with sixteen 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/Pharmacology 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 (FAOSTAT, 2007).

The most popular analytical methods which allow us to ensure the determination of origin are bar code, spectroscopy, stable isotope, etc. (Peres et al., 2007). Stable isotopes are the only ones which are referenced as an European regulation for wine origin determination (Ghidini et al., 2006).

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; El Sheikha et al., 2009).

MATERIALS AND METHODS

Fruits sampling Mature fruits of Shea tree (*Vitellaria paradoxa*) were collected in two different districts from three countries: Mali, Senegal and Ghana and one district from Cameroon. These districts were: Daelan and Nafégué in Mali; Kenioto and Saraya in Senegal; Sumbrungu and The piste between Mole Park and Nyawrupe in Ghana; Adamaoua in Cameroon. 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 yeast DNA was extracted immediately from the fresh fruits. The origin of the samples was defined by country, site and date of harvest.

DNA extraction from yeast For yeast DNA extraction, we applied the new protocol of El Sheikha et al. (2009). Briefly, two fruits of Shea tree were randomly taken and crushed in sterile Stomacher bag containing 6 mL peptone water. The two Eppendorff 2 mL vials containing the resulting suspension with 0.3 g of 0.5 mm diameter acid washed glass beads (425-600 µm, Sigma, France). The mixture was vortexed vigorously for 30 min in a bead beater instrument (Vortex Genie 2 SI-A256, 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, France); 1% SDS; 100 mM NaCl (Sigma); 10 mM Tris pH 8.0; 1 mM EDTA pH 8.0 (Promega, France)]. 100 µL TE (10 mM Tris-HCl; 1 mM EDTA (Promega); pH 8.0 and 100 µL of lysozyme solution (25 $mg.mL^{-1}$) and 100 µL of proteinase K solution (20 $mg.mL^{-1}$, Eurobio, France) were added and the mixture was incubated at 42°C for 20 min. Then 50 µL of 20% SDS were added to each tube, then incubated at 42°C for 10 min. 400 µL of MATAB (Sigma) were added to each tube, then incubated at 65°C for 10 min and vortexed vigorously for 5 min. Phenol/chloroform/isoamyl alcohol (25/24/1, 700 µL, Carlo Erba, France) was added twice 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 for 15 min at 12 000 ×*g*. 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. The extracted DNA (8 µL) was analysed in a 0.8% 1× TAE buffer (40 mM tris-HCl pH 7.4, 20 mM sodium acetate, 1.0 mM Na_2-EDTA, Eppendorf, Germany) agarose gel electrophoresis with molecular weight ladder (Supercoiled DNA ladder 16.21 kb, Invitrogen, USA). The gel was stained 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, USA) and Gel Smart 7.3 system software (Clara Vision, France).

PCR-Denaturing Gradient Gel Electrophoresis (DGGE) analysis D1/D2 region of the 26S rRNA gene was amplified using eukaryotic universal primers NL1GC (5`-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCC ATA TCA ATA AGC GGA GGA AAA G-3`, Sigma) and the reverse primer LS2 (5`-ATT CCC AAA CAA CTC GAC TC-3`, Sigma) amplifying a 250 bp fragment (Cocolin et al. 2000; El Sheikha et al. 2009). A 30-bp GC-clamp (Sigma) was added to the forward primer (the GC-clamp is underlined). PCR was performed in a final volume of 50 µL containing 0.2 µM of each primers, all the deoxyribonucleotide triphosphate (dNTPs) at 200 µM, 1.5 mM MgCl2, 5 µL of 10× of reaction Taq buffer MgCl2 free (Promega), 1.25 U of Taq DNA polymerase (Promega), and 2 µL of the extracted DNA (≈ 30 ng). PCR was run for 30 cycles with annealing at 52°C for 2, extension at 72°C for 2 min, and denaturation at 95°C for 60 sec (El Sheikha et al. 2009).

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 Na2-EDTA), stained with ethidium bromide 50 µg.mL-1 in TAE 1× and quantified by using a standard (DNA mass ladder 100 bp, Promega).

The PCR products were analysed by DGGE by using a DcodeTM universal mutation detection system (Bio-Rad Laboratories, USA) using the procedure first described by Muyzer et al. (1993) and improved by El Sheikha et al. (2009). 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 Na2-EDTA).

All electrophoresis experiments were performed at 60°C using a denaturing gradient ranging from 30% to 60% (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 12 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, Les Ulis).

Image and statistical analysis Individual lanes of the gel images were straightened and aligned using ImageQuant TL software v.2003 (Amesham Biosciences, USA). Banding patterns were standardized with two reference patterns included in all gels, DNA of *Wickerhamomyces anomalus* MTF 1103 and DNA of *Komagataella pastoris* ATCC 28484. 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 yeast in the populations. An individual discrete band refers to a unique ''sequence type'' or phylotype (Muyzer et al., 1995). 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).

$$S_D = 2\, N_c / N_a + N_b \qquad (1)$$

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). Significant differences of yeast communities of Shea tree fruits were determined by factorial correspondence analysis using the first 2 variances which described most of the variation in the data set.

RESULTS

Efficiency of the new protocol for the extraction of yeast DNA from Shea tree fruits DNA extraction of the yeast community present on Shea tree fruits was verified on a 0.8% (w/v) agarose gel and achieved admirable success.

Verification of the PCR amplification of the extracted DNA DNA was amplified by classic PCR according to Cocolin et al. (2000) improved by El Sheikha et al. (2009). In order to verify the efficiency of the amplification of the fraction, the PCR amplicon were electrophoresed on 2% (w/v) agarose gel at 100 V for 30 min in the TEA buffer. All of the bands were clearly observed and had a molecular weight of 250 bp, the expected size of the amplicon. The intensity of the bands representing the PCR amplicons was important and signifies that yeast DNA was amplified very well and thus it was possible to continue to analyse these amplicons by the DGGE method.

DGGE pattern of yeast DNA from Shea tree fruits among different countries On DGGE gel, the observed bands had sufficient intensities to analyse samples of yeast DNA extracted from Shea tree fruits from four various geographical areas (Fig. 1), so the total quantity of DNA deposited in the wells of DGGE gel was sufficient to consider that yeast DNA could be used as potential markers to ensure the determination of Shea tree fruit origin. The reference DNA of *Wickerhamomyces anomalus* and *Komagataella pastoris* indicates that DGGE was perfectly done. Each vertical line represents a fruit and each spot represents a species of yeast. The duplicate of PCR-DGGE patterns of Shea tree fruits for each location were similar for each country and revealed the presence of five to 12 bands for each Shea tree fruit.

Clusters analysis by Stastica software of the DGGE gel patterns for the duplicate Shea tree fruit samples from four different countries showed a community similarity among the geographical locations where the fruit samples were collected (Fig. 2). At 68% similarity level, two main clusters were observed: the first cluster included the samples from Senegal, Ghana and Mali; the second cluster comprised the samples from Cameroon. The cluster analysis also showed different similarities levels between the different countries. For example, there is 82% similarity between Senegal and Ghana.

Factorial Correspondence Analysis (FCA) proved to be a useful statistical tool for comparing the similarity of the yeast communities of Shea tree fruit samples from the four different countries in harvested season. For the fruits samples, the two variances described 97% in between the yeast communities (Fig. 3). We can observe clearly four different groups for the four different countries.

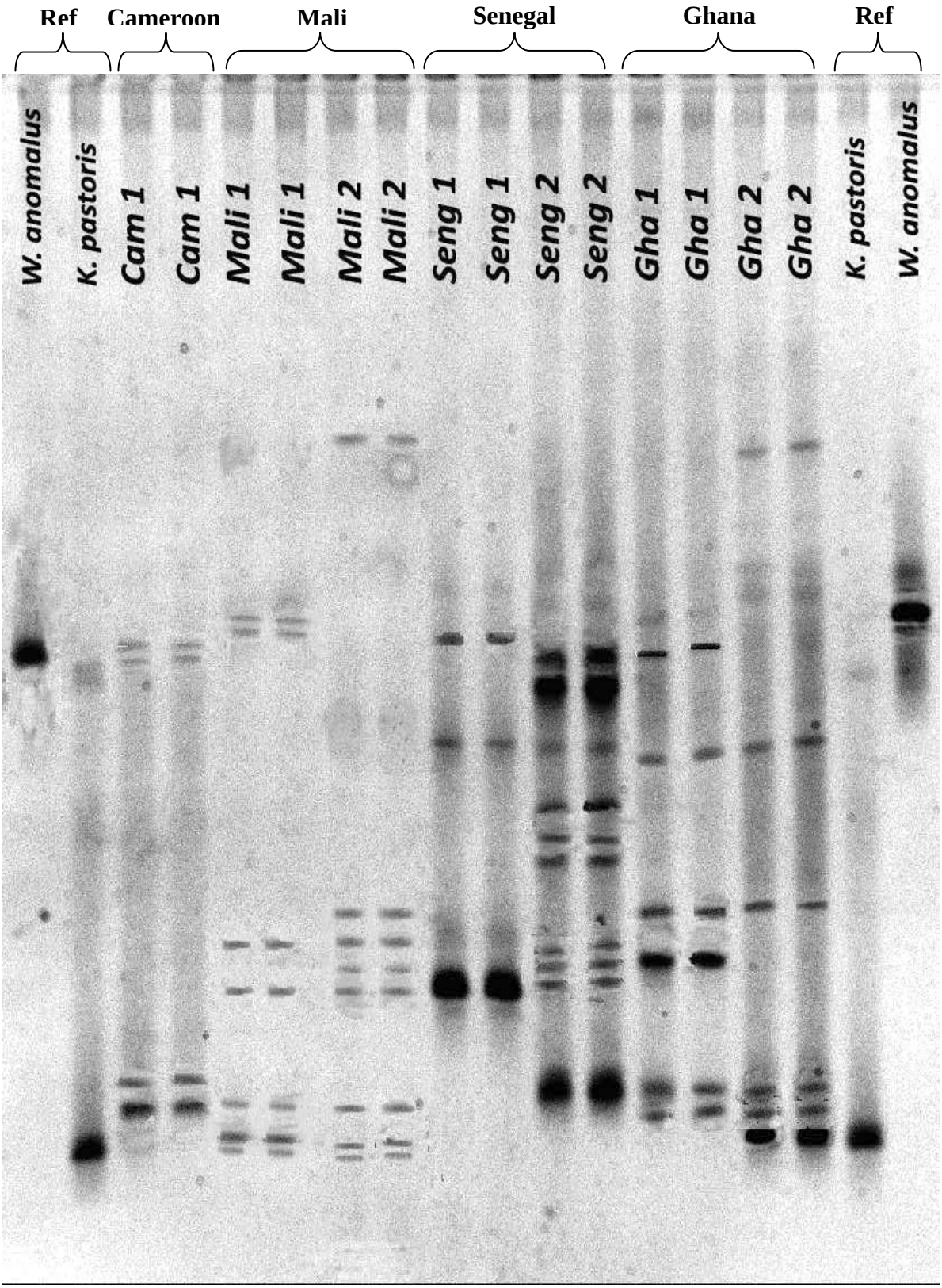

Figure 1. PCR-DGGE profiles of 26S rDNA of Shea tree fruits from four countries: Ghana, Senegal, Mali and Cameroon. Gha: Ghana; Seng: Senegal; Mali: Mali; Cam: Cameroon. (1,2) Two different districts.

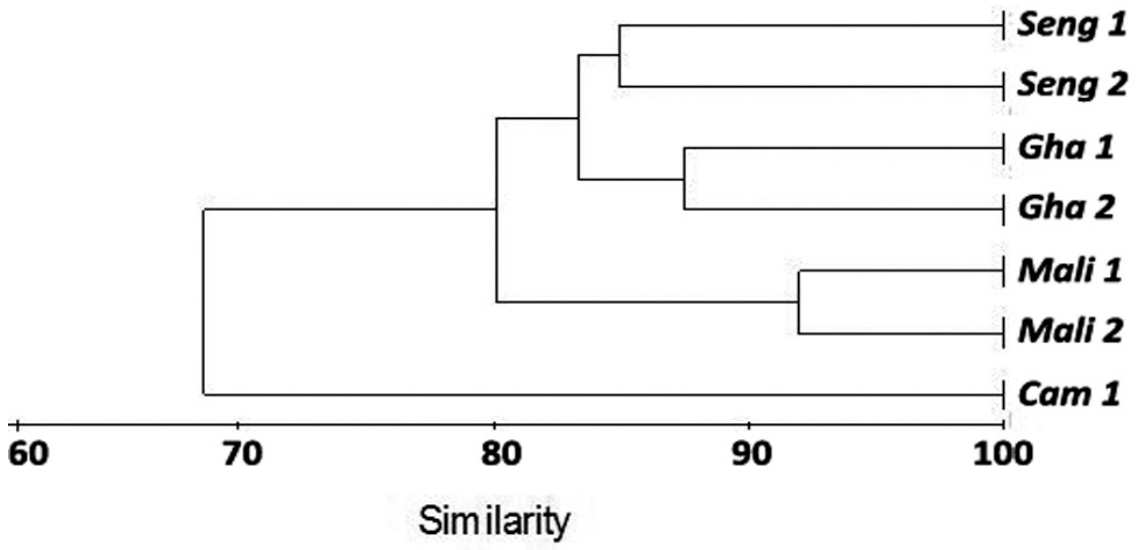

Figure 2. Cluster analysis of 26S rDNA profiles of Shea tree fruits from four countries: Ghana, Senegal, Mali and Cameroon. Gha: Ghana; Seng: Senegal; Mali: Mali; Cam: Cameroon. (1,2) Two different districts.

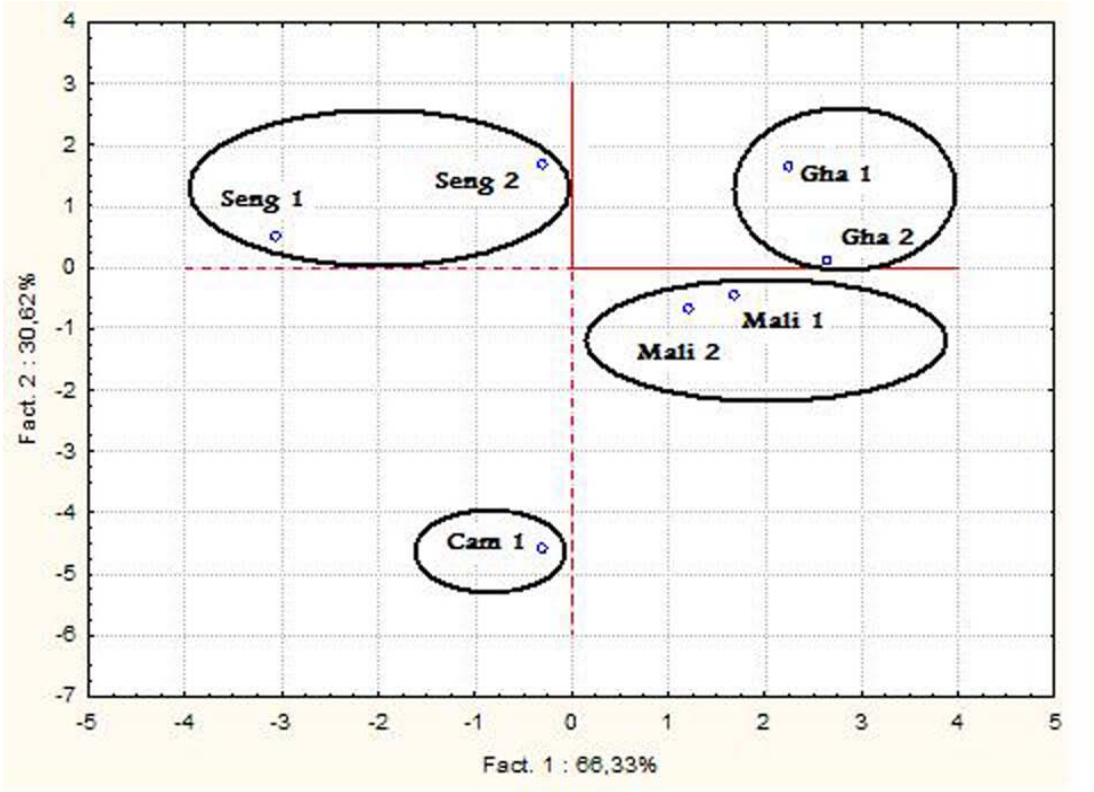

Figure 3. Factorial variance analysis of 26S rDNA profiles of Shea tree fruits from four countries: Ghana, Senegal, Mali and Cameroon. Gha: Ghana; Seng: Senegal; Mali: Mali; Cam: Cameroon. (1,2) Two different districts.

DISCUSSION Some teams already proposed this method to analyse the yeast communities in fruits and fruit products (Tournasa et al., 2006; Fleet 2007; Prakitchaiwattanaa et al., 2007); however, we believe that this is one of the first publication to introduce the analysis of yeast communities in Shea tree fruits by PCR-DGGE. In our study, we proved that the DGGE pattern of the DNA yeast communities of Shea tree fruits was strongly linked to the microbial environment of the fruit. The analysis of Shea tree fruit samples from four countries 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 countries. The types of processing system applied could also affect the microbial communities of Shea tree fruits. In the gel some common bands appeared in all of the samples independently to the district and country. These bands could be common yeasts for all of the Shea tree fruits.

In fact, we proved that there was a complete statistical correspondence between the geographical areas and the yeast communities when we compared by statistical analysis of DGGE pattern the different countries of fruits sampling. We could conclude that there were enough environmental differences between the four countries where the Shea tree fruits were harvested to obtain a major effect on the yeast ecology, whereupon we could create a statistical link between the yeast populations and the geographical area.

CONCLUSION The analysis of Shea tree fruits yeast communities by PCR-DGGE could be applied to differentiate the geographical areas. We showed that the biological markers for each country were sufficient statistically to discriminate the geographical origin. This global technique is quicker (less than 24 h) than all of the classical microbial techniques and avoids the precise analysis of yeast 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 diversity of Shea tree fruits and other fruits varieties and the ecological study of yeasts in many other products in which they occur provide another area for future study.

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