Proceeding of 2nd AFSSA Conference on Food Safety and Food Security
August 15 – 17, 2014
Asian Food Safety And Security Association
ISSN: 2306-2150

Novel microbial fingerprinting of fruits by PCR-DGGE: An application to determine the geographical origin of Shea tree fruits from Senegal

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Abstract:

Food traceability is essential to preserve the identity of unique quality traits against fraud or commercials disputes. Therefore, there is growing demand of new traceability systems for the collection of information related to units/batches of food ingredients and products. A rapid method based on a molecular technique employing 28S rDNA profiles generated by PCR-DGGE was used to detect the variation in fungal community structures of Shea tree fruit (*Vitellaria paradoxa*) from Senegal. When the 28S rDNA profiles were analyzed by multivariate analysis, distinct microbial communities were detected. The 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 the fruits. This method is a new traceability tool, which provides fruit products with a unique biological bar code and makes it possible to trace back the fruits to their original location.

Keywords: Molecular markers, Shea tree fruits, Fungal communities, Origin

1. Introduction

In the past years, the development of biological identification technologies has greatly contributed to support and check traceability systems. In parallel, computer technology provided many new and innovative tools for tracing products [1]. Biological, analytical and informatics tools have been synergistically proposed and utilized for traceability in the wine industry [2]. Currently, there are not molecular biology techniques for determining the geographical origin of food.

The Shea tree has been exploited by African communities for about 3000 years and offers an opportunity for sustainable development in Sudanian countries [3]. Shea trees produce fruits, which form an important part of the diet of Africans. It is a perennial tree which starts producing fruits at the age of 10 - 15 years. It takes 20 - 30 years for the complete growth of Shea trees. The botanical name of Shea is *Vitellaria paradoxa*. The region where Shea trees grow extends from eastern Senegal to northern Uganda. Shea tree seeds or nuts are used in the preparation of Shea butter products, which have a great commercial value. Shea tree is very important from the point of sustainable development of the African rural economy [4]. The estimated yield of dry kernel is about 600,000 tons per year, and exports have increased over the past decade, reaching 350,000 tons today, mainly to USA and Europe. Total production potential has been estimated at over 2.5 million MT kernels [5].

Shea fruit is often consumed as fresh fruit for its sweet edible pulp. The fruit pulp is a particularly rich source of ascorbic acid, i.e. 196.1 mg/100g compared with 50 mg/100 g for oranges. The iron content compares favorably with raspberries: 1.93 mg/100g compared with 0.92mg/100 g. The solidified Shea oil is called 'butter'. It is constituted primarily of heavier carbon molecules and is more or less solid at room temperature. Each kernel contains a fat for about half its weight. Shea butter is completely natural and very rich in vitamins (A and E) and essential fatty acids [6].

In Africa, Shea tree fruit is also nicknamed "The Gold of Women", because of the 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 [4]. Demand for Shea butter produced in West Africa has increased by over 1200% over the last 10 years. The increased demand has led to new businesses. There are now more companies operating at every level of the Shea value chain. 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 [5].

In Senegal, Shea butter (kare in Pulaar and karité in French) is an extremely popular product, people use it as a lip balm and a body moisturizer and they also cook with it. Making Shea butter is astoundingly hard work. The Shea nuts have to be gathered, dried, shelled, pounded, ground, soaked, strained, boiled, re-strained, melted down, and poured into containers. The women's groups often get together to make and sell Shea butter. Usually they just pour it into old water bottles or whatever containers happen to be on hand, and a half-liter goes for around 1,000 CFA, or about $2.00 USD [6].

Certification of Shea kernel and butter has become increasingly important for a number of reasons – The EU started demanding that all agricultural products are traceable from source from 1st January 2005. A number of cosmetic companies are asking for organically certified Shea butter for formulation of organically labelled 'botanical' products and the demand for consistent 'Quality @ Quantity' from rural producers is increasing the need for quality assurance [5]. The idea was to create a 'biological bar code' [7] 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 [6, 8, 9, 10, 11, 12].

The main objective of this study is to apply polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) method to analyze 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, there are few papers published by our team 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.

2. Materials and Methods

2.1. Fruit samples

Mature fruits of Shea tree (*V. paradoxa*) were collected in three different locations from Senegal (two different districts for each location). These locations were: Kenioto, Saraya and Samecouta, Tambacounda region in Senegal. Table 1 gives the geographical coordinates of the sampling sites. Additionally, 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).

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.

2.2. Fungal DNA extraction from Shea tree fruit samples

We applied a new protocol created by El Sheikha [6] and takes into account the methods of Karakousis et al. [13] developed for fungal DNA extraction and El Sheikha et al. [11] 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 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 re-suspended in 300 mL 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, Charbonnie`res-les-Bains, France)]. Then, 100 mL TE (10 mM Tris-HCl; 1 mM EDTA; pH 8.0, Promega) and 100 mL of lysozyme solution (25 $mg.mL^{-1}$, Eurobio, Les Ulis, France) and 100 mL of proteinase K solution (20 $mg.mL^{-1}$, Eurobio) were added and incubated at 42 °C for 20 min. Then 50 mL 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 mL 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 mL 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 mL 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 mL 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 re-suspended in 50 mL 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 (40mM Tris-HCl pH 7.4, 20 mM sodium acetate, 1.0 mM Na2–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 $mg.mL^{-1}$, 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).

2.3. PCR-Denaturing gradient gel electrophoresis (DGGE) analysis

For fungi, a fragment of region of the 28S rDNA 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 [6, 12, 14, 15, 16, 17, 18, 19, 20, 21]. A 30-bp GC-clamp (Sigma) was added to the forward primer (the GC-clamp is underlined) to ensure that the fragment of DNA will remain partially double-stranded and that the region screened is in the lowest melting domain. PCR was performed in a final volume of 50 µL containing 2.5 µL DMSO, 0.4 µM each primers, all the deoxyribonucleotide triphosphate (dNTPs) 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 sec, 50 °C for 50 sec and 72 °C for 90 sec, and a final extension at 72 °C for 5 min. Aliquots (5 µL) of PCR products were analyzed 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 analyzed by DGGE by using a Bio-Rad DcodeTM universal mutation detection system (Bio-Rad Laboratories, USA), using the procedure first described by El Sheikha [6]. 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 30–40 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, Les Ulis, France).

2.4. Image and statistical analysis

Individual lanes of the gel images were straightened and aligned using Image Quant TL 7.0 (GE Healthcare, USA). This software permitted to identify the bands and their relative position. The DGGE fingerprints were manually scored by the presence and absence of co-migrating bands, independent of intensity. Analysis of data was done using the Dice similarity coefficient (SD), which is calculated according to the formula $SD = 2\ N_c/N_a + N_b$ (1) [22], 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). Genogram was constructed by using software of Primer version 6 (PRIMER-E Ltd, United Kingdom).

2.5. 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 mL 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 analyzed 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 [23].

3. Results

3.1. Performance of fungal DNA extraction methods

Eight µL of genomic DNA extraction of the fungal communities were verified on a 0.8% (w/v) agarose gel and achieved an admirable success. On the gel, the bands with a molecular weight greater than 16 kb corresponding to genomic fungal DNA were clearly observed.

3.2. Verification of the PCR amplification of the extracted DNA

The fungal genomic DNA obtained after extraction were amplified by PCR using the protocols first described by El Sheikha [6]. In order to verify the efficiency of this fragment, five µL of 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. Successful amplification permits one to continue to analyze these amplicons by the DGGE method.

3.3. DGGE pattern of fungal DNA from Shea tree fruits among different districts of Senegal

On DGGE gel, the observed bands had sufficient intensities to analyse samples of fungal DNA extracted from Shea tree fruits from various geographical locations in Senegal (Fig. 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 to ensure the determination of Shea tree fruits origin.

The reference DNA of *Mucor racemosus* and *Trichoderma harzianum* indicates that DGGE was done successfully. Each vertical line represents a fruit and each spot represents a fungus. The PCR-DGGE patterns of duplicate Shea tree fruits for each location were similar and revealed the presence of four to twelve bands for each Shea tree fruit (Fig. 1). Some spots appeared double or smear because of the presence of single-strand DNA [24].

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 districts showed a community similarity among the geographical locations where the fruit samples were collected (Fig. 2). At 89% similarity level, two main clusters were observed: the first cluster included the samples from Saraya and Kenioto and the second cluster comprised the samples from Samecouta.

3.4. Identification by sequencing of dominant fungi on Shea tree fruits

To our knowledge, there is no information on the populations of fungi from Senegal 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 (Results not shown).

4. Discussion

There are only a few publications published by our team that analyzed the fungal communities in fruits and described the linkage between the fungal communities and the geographical origin of fruits [12, 19, 20, 21]. Flórez and Mayo [25] used 26S rDNA to detect the fu ngal species in Cabrales cheese during the manufacture and ripening. Durand et al. [26] used 28S rDNA to improve the knowledge about fungi dynamics and biodiversity of potentially OTA producing fungi during coffee processing.

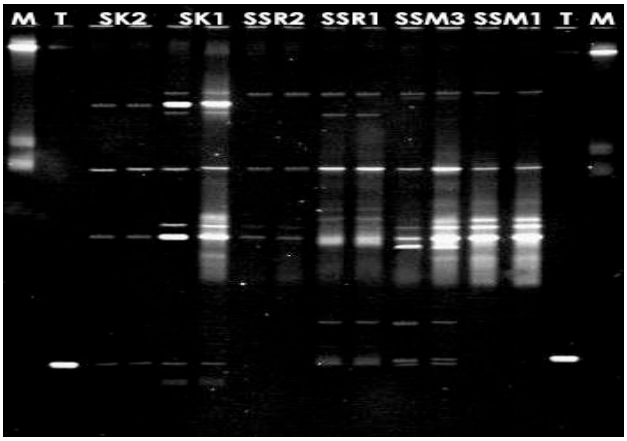

Fig 1. *PCR-DGGE band profiles of 28S rDNA of Shea tree fruits from three different locations of Senegal. Kenioto (SK2, SK1); Saraya (SSR2, SSR1); Samecouta (SSM3, SSM1); M: Mucor racemosus; T: Trichoderma harzianum.*

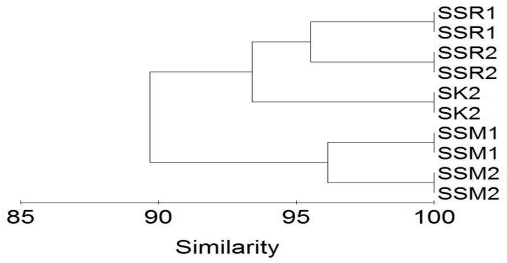

Fig 2. *Cluster analysis of 28S rDNA banding profiles of Shea tree fruits from three different locations of Senegal (two different districts for each location). Kenioto (SK2, SK1); Saraya (SSR2, SSR1); Samecouta (SSM3, SSM1).*

Previous work on Shea tree has focused on morphological, genetic diversity and technological studies [3, 27, 28, 29]. In our study, we proved that the DGGE pattern that the band pattern of the fungal communities isolated from Shea tree fruits obtained by PCR-DGGE was strongly linked to the microbial environment of the fruits. For Shea tree fruits, the DGGE gel showed some significant differences in the migration patterns. 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 [30] (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.

Table 1. *Geographic description of sampling sites*

Country	Region	Site	GPS		Altitude (m)
			Longitude	Latitude	
Senegal	Tambacounda	Saraya	12° 49' 53.62" N	11° 45' 21.10" W	189
		Samecouta	12° 36' 10.00" N	12° 07' 80.13" W	120
		Kenioto	12° 34' 20.11" N	12° 09' 60.16" W	120

5. Conclusion

We could conclude that 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. These meanings also as 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. 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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