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NOVEL MOLECULAR FINGERPRINTING FOR DETERMINING THE GEOGRAPHICAL ORIGIN OF FRUITS: AN APPLICATION TO SHEA TREE FRUITS FROM MALI

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ABSTRACT

The consumer is more and more demanding and sensitive to the quality and the origin of the foodstuffs. There are difficulties of installing these documentary systems in developing country, in particular the countries of sub-Saharan Africa. Therefore become an urgent need for the new strategies of traceability. The new molecular tool of tracing the products of vegetable origin based on the analysis of the DNA of micro-organisms present on the fruits is an interesting tool. Regarding Shea tree fruits, only seven countries have statistic. Nigeria accounts for more than 60% of the production of Shea butter in 2005. It is followed by Mali, Ghana and Burkina Faso, which 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.

Purpose: A molecular biology technique employing 26S rDNA profiles generated by PCR-DGGE was used to detect the variation in yeast community structures of Shea tree fruits (*Vitellaria paradoxa*) from two regions of Mali.
Results: The 26S rDNA profiles were analyzed by image analysis and multivariate analysis, distinct microbial communities were detected on Shea tree fruits. The band profiles of yeasts from different regions were specific for each location and could be used as a bar code to discriminate the origin of the fruits.

Significance of paper: This novel molecular traceability tool which provides fruit products with a unique fingerprint and makes it possible to trace back the fruits to their original location.

Keywords: molecular fingerprinting; traceability; Shea tree fruits; yeast communities; Mali

INTRODUCTION

Traceability is one the great concerns of the customers of the foodstuffs. 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 the molecular fingerprinting which comes from the environment of the food to assure their traceability. Currently, there are no existing analytical methods which permit the efficient determination 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 origin.

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". The tree can be between ten and fifteen meters in height and has a short barrel (three meters) with a diameter of up to one meter. Its life time is estimated at two or three hundred years. Its root system is very tortuous. Mungo Park (1771-1806) was the first to give the botanical characteristics of this tree. The fruits were collected between June and September. In Egypt, where they also found traces of Shea fruits, we think that the tree was used to make statues, about three centuries ago. 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 **[El Sheikha, (2009)]**.

The most popular analytical methods used to ensure the determination of origin are bar code and stable isotopes **[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)]**. 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 main objective of this study is to apply PCR-DGGE method to analyze in a unique step all the yeasts present on the fruit in order to create an analytical technique that will permit the linkage of yeast communities to the geographical origin and avoid the individual analysis of each yeast strain. The acquired band patterns for the yeast communities of different species of Shea tree fruits from different harvesting locations were compared and analyzed statistically to determine the Shea tree fruits geographical origin. To the best of our knowledge, this is the first paper describing a molecular

method PCR-DGGE which permit to create a unique "biological fingerprinting" for Shea tree fruits from different locations.

MATERIALS AND METHODS

Fruits sampling

Mature fruits of Shea tree (*Vitellaria paradoxa*) were collected in different districts from two different regions in Mali. The first two districts were from Daelan village in Ségou region and the second two districts were from Nafégué village in Sikasso region. Table (1) and Fig.(1) give the geographical coordinates of the sampling sites. In addition, Sikasso region follows the tropical Sudanian climate which divided into two sets: the Sudanian zone and Guinean zone wet is the wettest region of Mali and the wettest (700 to 1500 mm / year). The average annual temperature is 27°C. Ségou region, it is mainly located in the Sahelian zone where it enjoys a semi-arid climate (average annual rainfall: 513 mm). The presence of several rivers allows irrigated crops. It is indeed crossing the Niger River of 292 km and the Bani River. The fruits were gathered to preserve their initial flora. The fruits 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.

Table (1). Description of sampling sites.

Country	Region	Sites	GPS		Altitude (m)
			Longitude	Latitude	
Mali	Sikasso	Naféué	10° 29' 31.68" N	5° 58' 17.16" W	343
	Ségou	Daelan	13° 15' 06.96" N	4° 59' 16.74" W	282

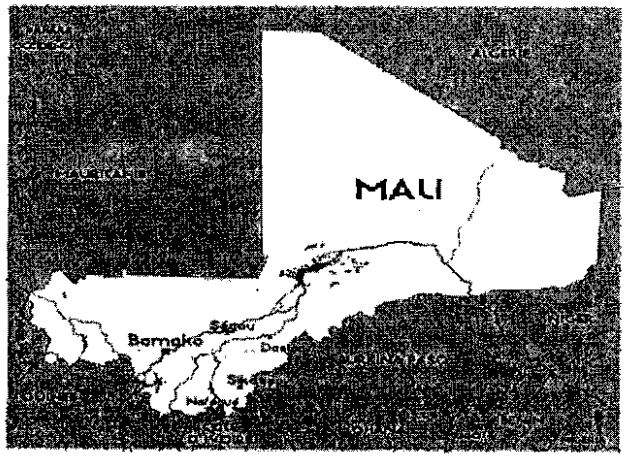

Fig. (1) : Localization of sampling sites of Shea tree fruits in Mali. (http://www.geoatlas.fr/fr/detailFR_cdrom.php?cdrom=7)

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 fruits were put in sterile Stomacher bag containing 6 mL peptone water then crushed by hands. The two Eppendorff 2 mL vials containing the resulting suspension were 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 (Sigma)/100 mM NaCl/ 10 mM Tris pH 8.0/ 1 mM EDTA pH 8.0]. 100 µL TE (10 mM Tris-HCl; 1 mM EDTA; pH 8.0) and 100 µL of lysozyme solution (25 $mg.mL^{-1}$) and 100 µL of proteinase K solution (20 $mg.mL^{-1}$) 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 were added to each tube, then incubated at 65°C for 10 min. The tubes were vortexed vigorously for 5 min. The lysates were then purified twice by repeated extraction with 700 µL of phenol/chloroform/isoamyl alcohol (25/24/1) and the tubes were vortexed for 5 min and then centrifuged 15 min at 12,000 ×*g*. 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 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 500µL 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.

PCR-Denaturing Gradient Gel Electrophoresis (DGGE) analysis :

The 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 **[Kurtzman and Robnett 1998; 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 $MgCl_2$, 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 (≈ 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 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 Na_2-EDTA), stained with ethidium bromide 50 $\mu g.mL^{-1}$ 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 **[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 Na_2-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, France).

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 three 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 **[Van Hannen *et al.* 1999; 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).

RESULTS

Efficiency of yeast DNA extraction of 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. On the gel, the bands with a molecular weight greater than 16 kb corresponding to genomic yeast DNA were clearly observed.

Verification of the PCR amplification of the extracted DNA :

The yeast DNA obtained after extraction was amplified by classic PCR according to [**Kurtzman and Robnett (1998) and 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 analyze these amplicons by the DGGE method.

DGGE pattern of yeast DNA from Shea tree fruits among different regions :

On DGGE gel, the observed bands had sufficient intensities to analyze samples of yeast DNA extracted from Shea tree fruits from two different regions of Mali **Fig. (2)**, 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 fruits 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 and revealed the presence of 6 to 11 bands for each Shea tree fruit.

Clusters analysis by Statistica software of the DGGE gel patterns for the duplicate Shea tree samples from two different regions showed a community similarity among the geographical locations where the fruit samples were collected. At 84% similarity level, two main clusters were observed: the first cluster contains the samples from Sikasso region and the second cluster contains the samples from Ségou region **Fig.(3)**.

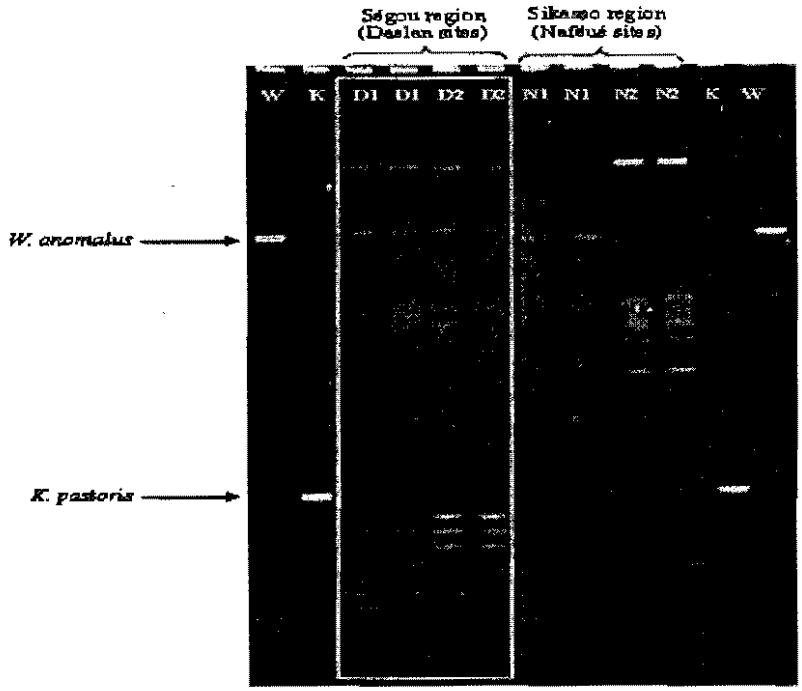

Fig. (2): DGGE Profiles of 26S rDNA of yeast strains isolated from Shea tree fruits from two different regions of Mali: Ségou region (D1, D2: Daelan sites) and Sikasso region (N1, N2: Nafégué sites).

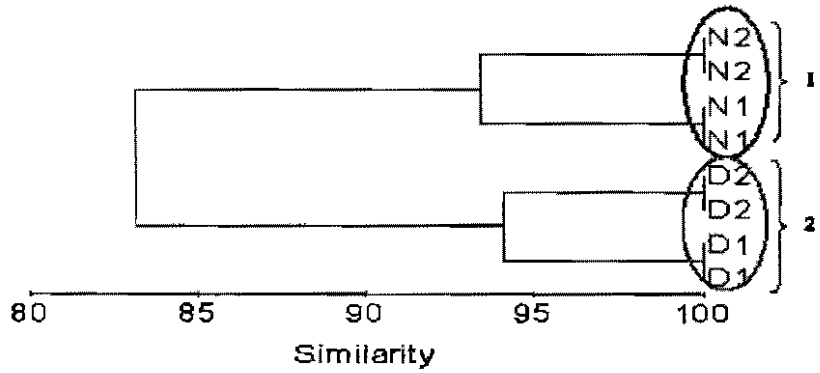

Fig.(3) :Cluster analysis of 26S rDNA profiles of yeast strains isolated from Shea tree fruits two different regions of Mali: Ségou region (D1, D2: Daelan sites) and Sikasso region (N1, N2: Nafégué sites).

DISCUSSION

Some teams already proposed this method to analyze the yeast communities in fruits and fruit products [**Tournasa *et al.* 2006; Fleet 2007; Prakitchaiwattanaa *et al.* (2007)**] and just one publication published by our French team described the linkage between the yeast communities and the geographical origin of Physalis fruits from Egypt [**El Sheikha *et al.*, (2009)**]. But we think that this is the second publication that introduces a unique "biological fingerprinting" using 26S rDNA fingerprinting of yeasts for Shea tree fruits.

In our study, we proved that the DGGE pattern of the DNA of yeast communities of Shea tree fruits was strongly linked to the microbial environment of the fruit. The analysis of Shea tree fruit samples from two regions 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 regions. 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 regions of fruit sampling. We could conclude that there were enough environmental differences between the two regions 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.

In 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 region 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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الملخص العربي

بصمة جزيئية جديدة لتحديد الأصل الجغرافي للفواكه : تطبيق على ثمار شجرة الشيا من مالى

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مقدمة : إن حساسية المستهلك تزداد بشكل مستمر نحو جودة ومنشأ المواد الغذائية . توجد العديد من الصعوبات لتثبيت الأنظمة الوثائقية كأنظمة للتتبع فى البلدان النامية ، ولا سيما بلدان أفريقيا جنوب الصحراء . لذا أصبحت الحاجة ملحة لوضع استراتيجيات جديدة لعملية التتبع . وسيلة جزئية لتتبع منشأ المنتجات النباتية حيث تعتمد هذه الوسيلة على تحليل الحمض النووي للحمولة الميكروبية لتلك المنتجات لذا تعد هذه الوسيلة كأداة جديدة للتتبع مثيرة للإهتمام . بالنظر إلي ثمار شجرة الشيا ، فقط سبع دول تمتلك إحصائيات إنتاجية . حيث يمثل إنتاجية نيجريا من زبدة الشيا ٦٠% من الإنتاج العالمى لعام ٢٠٠٥ . يليها فى ذلك دول مالى وغانا وبوركينا فاسو حيث تحتل انتاجية هذه الدول مجتمعة المرتبة الثالثة على مستوى العالم لعام ٢٠٠٥ . فى أوروبا ، يعد ٩٥% من الاستخدام الرئيسي لزبدة الشيا هو الاستخدام فى صناعة الشيكولاته . أما عن كميات زبدة الشيا الموردة إلي اليابان والولايات المتحدة الأمريكية وسويسرا فيتركز الاستخدام الرئيسي لها فى مستحضرات التجميل والمنتجات الدوائية .

الفرض : تم توظيف التقنية الجزيئية 26S للنوع الريبوسومى من الحمض النووى rDNA وذلك باستخدام تقنية PCR-DGGE للكشف عن الاختلاف فى تجمعات الخمائر لثمار شجرة الشيا التي تم جمعها من منطقتين مختلفتين فى مالى .

النتائج : تشكيلات المادة النووية المتحصل عليها من الحمض النووي للخمائر تم تحليلها كصورة بواسطة برنامج تحليل الصور وكذلك تم تحليلها احصائياً بواسطة التحليل ذات المتغيرات المتعددة ؛ وقد تم الكشف عن التجمعات الميكروبية على ثمار الشيا بشكل واضح . وكانت تشكيلات المادة النووية للخمائر لثمار الشيا التى جمعها من مناطق مختلفة محددة لكل موقع من مواقع الانتاج لذا يمكن اعتبار تلك التشكيلات للحمض النووى للخمائر بمثابة باركود مميز لمنشأ الفاكهة .

أهمية الدراسة : هذه الأداة الجزيئية الجديدة للتتبع توفر لمنتجات الفاكهة بصمة فريدة من نوعها كما تجعل من الممكن تتبع المواقع الأصلية للفواكه .

كلمات البحث : البصمة الجزيئية ؛ التتبع ؛ ثمار شجرة الشيا ؛ تجمعات الخميرة ؛ مالى