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Application of PCR-DGGE in determining food origin: Cases studies of fish and fruits

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Summary

The determination of geographical origin is a demand of the traceability system of import-export food products. For this purpose, molecular techniques employing 16S, 26S rDNA profiles generated by PCR-DGGE (polymerase chain reaction - denaturing gradient gel electrophoresis) were used to detect the variation in microbial community (bacteria, yeast) structures of Pangasius fish from Viet Nam harvested in different aquaculture farms and during different seasons and two fruits Physalis from Egypt and mandarins from Spain and Morocco. In Physalis case, ecology of yeasts was also studied for the same purpose. Bacterial band profiles of fish and mandarins, and yeast band profiles of Physalis were specific for each location and could be used as a bar code to certify the origin of fish and fruits. This method is a new traceability tool which provides food with a unique biological bar code and makes it possible to trace back the food to their original location.

Key words: Traceability, PCR-DGGE, Pangasius fish, Physalis, mandarins, microbial communities, origin

Introduction

The issues surrounding food safety continue to be hot topics throughout the supply chain. BSE (Bovine Spongiform Encephalopathy), Salmonella and avian influenza remain embedded in the memories of European consumers. Regulations across Europe continue to be tightened in order to provide a greater degree of insurance in quality and safety. Meanwhile, the traceability and labeling of imported products in European countries remains a compulsory issue (EU regulation 178/2002). The need for vigilance and strict monitoring is necessary. One of the great concerns of the customers is the traceability of the products. Traceability is the capacity to find the history, use or origin of a food by registered methods (ISO 8402, 1994). For a long time the food industry has used simple traceability systems. In view of the difficulties of installing these 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 during international trade. Currently, there are no existing

analytical methods which permit the determination the origin of food or to follow them during international trade.

In the case of fish, the predominant bacterial flora would permit the determination of the capture area, production process or hygienic conditions during post harvest operations (Leesing *et al*., 2005; Le Nguyen *et al*., 2008). Aquatic microorganisms are known to be closely associated with the physiological status of fish (Al Harbi & Uddin, 2003; Grisez *et al*., 1997; Leesing, 2005; Spanggaard *et al*., 2000). The water composition, temperature and weather conditions can influence the bacterial communities (De Sousa & Silva-Sousa, 2001; Wong *et al*., 1999). In case of fruits, Physalis and mandarins are considered as fruits which are preferred by European people. The mature berry of Physalis has a golden yellow skin, with many minute seeds in a juicy pulp which is sweet and tangy, and resembles a Chinese lantern. Nowadays, Physalis is included in the priority list of many governments' horticulture and fruit export plan. It is relatively unknown in importing markets and remains an exotic fruit. In Egypt, Physalis has been known for a long time. Recently, the economic importance of Physalis has risen, due to its high acceptance for local consumption, achieving a great success in Arabic and European markets (El Sheikha, 2004). Mandarins are an oblate, medium-sized fruit, with an exterior which is a deep orange color with a smooth, glossy appearance. The two biggest exporters of mandarins are Spain with 249,965 Mt (million tonnes) and Morocco with 22,938 Mt (Saint-Charles International, 2006).

For economic reasons and for profitability, several batches of fruits of various pieces or various cultures could be mixed. It is thus very difficult to check their exact geographical origin. Traceability is only assured by rigorous labeling and administrative documentation without any analytical control. In case of doubt or fraud, it is necessary to find a precise and fast analytical technique in order to determine their geographical origin. In addition, certain species such as Corsica mandarins obtained a Protected Geographical Indication (PGI) from Europe and could be sensitive to the development of analytical methods. Among the most popular analytical methods which allow us to ensure the determination of origin (2D code, spectrophotometers, stable isotope of strontium, etc.) (Peres *et al*., 2007), no molecular biology method in general or PCR-DGGE in particular, were described and used. This tool will permit to give reliable results with very short times in adequacy with the speed of trade concerning these products. The multitude of Physalis and mandarin varieties are not specific of a particular geographical area. Moreover, the classification of these varieties is very complex and little information exists on their genetic specificities (El Sheikha *et al*., 2008; Bretó *et al*., 2001). It thus seems difficult to use fruit genomic markers to ensure the traceability of Physalis and mandarins. However, 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 DNA of microorganisms present on the products. This method is based on the assumption that the bacterial communities of the fruits are specific for a geographical area.

The purpose of our study is to apply the PCR-DGGE method to analyse the microorganisms in food in order to create an analytical technique to link microbial communities to the geographical origin and avoid the individual analysis of each microbial strain. The acquired band patterns for the microbial species of different fish (bacteria) or fruits (bacteria and yeasts) were compared and analysed statistically to determine their geographical origin.

Materials and Methods

Fish sampling

The Pangasius fish samples *Pangasius hypophthalmus* were collected in a unique pond in five aquaculture farms of five different districts from the South Viet Nam namely Chau Phu, An Phu,

Phu Tan, Chau Doc, and Tan Chau of An Giang province. This province supplies about 2/3 (about 80,000 MT in 2005) of Pangasius fish for export (Ministry of Aquaculture, Viet Nam, 2005). The samples were collected in two seasons in Viet Nam: the rainy season (October 2005) and the dry season (February 2006). The samples were taken from the same pond and aseptically transferred to storage bags, then maintained on ice and transported to the laboratory. Then the skin, gills and intestines were aseptically removed from each fish specimen and put in separate sealed plastic bags, then kept frozen at -20°C until analysis.

Fruits sampling

Samples of Physalis (*Physalis ixocarpa* Brat, *Physalis pubescens* L., *Physalis pruinosa* L.) came from four Egyptian governments (Qalyoubia, Minufiya, Beheira, Alexandria). The fruits were gathered to preserve their initial flora. They were collected directly on the tree using gloves and put in sterile bags in May 2008. These bags were kept into a refrigerator then transferred by plane to CIRAD Montpellier (France) and the yeast DNA was extracted immediately on the fresh fruits. The origin of the samples was defined by country, site and date of harvest.

Mandarins (*Citrus reticulata blanco* var. clementine) were provided by the Marché Gare Saint-Charles of Perpignan (France) and came from two countries, Spain and Morocco. The samples arrived ar CIRAD in January 2007 and the bacterial DNA was extracted immediately on the fresh products. Three varieties (Clemenvilla, Clemenule, and Hernandine) came from Valencia region in Spain, provided by three different companies. Two varieties (Nour and Nour tardive) were supplied by a company from the Berkane region in Morocco. Three mandarins from each variety were randomly taken for analysis from the various packages.

DNA extraction from bacteria

DNA extraction from bacteria was based on the methods of Ampe *et al*. (1999) and Leesing (2005) but modified and optimized. For fish samples, around 2 g each of gills, skin and intestine were homogenized by vortex for 3 min after addition of 6 mL sterile peptone water (pH 7.0, Dickinson, France). But for mandarins, fruits were peeled, with all the skin put in a sterile Stomacher bag in 20 mL of peptone water. The mixture was crushed during 30 s in a Stomacher® (Seward, UK) as described by Le Nguyen *et al.* (2008). Four 1.5 mL tubes containing each samples (fish or mandarins) were then centrifuged at 10,000×***g*** for 10 min. 100 µL of lysis buffer TE (10 mM Tris; 1 mM EDTA; pH 8.0, Promega, France) and 100 µL of lysozyme solution (25 µg L^{-1}, Eurobio, France) and 50 µL of proteinase K solution (10 µg L^{-1}, Eurobio) were added to each pellet. Samples were vortexed for 5 min and incubated at 42°C for 20 min. Then 50 µL of 20% SDS (Sigma) were added to each tube and were incubated at 42°C for 10 min. A volume of 300 µL of MATAB (Sigma) was added and the tubes were incubated at 65°C for 10 min. The lysates were then purified by repeated extraction with 700 µL of phenol/chloroform/isoamyl alcohol (25/24/1, Carlo Erba), and the residual phenol was removed by extraction with an equal volume of chloroform/ isoamyl alcohol (24/1). The DNA was precipitated with isopropanol, washed with 70% ethanol and then air dried at room temperature for 2 h. Finally, the DNA was resuspended in 50 µL of ultra pure water and stored at -20°C until analysis.

DNA extraction from yeast

For yeast DNA extraction, we improved the protocol of Ros Chumillas *et al*. (2007), which mentioned the fast detection of DNA by PCR starting from orange juice. Two fruits of Physalis with or without husks were put in sterile Stomacher bag containing 6 mL peptone water then crushed by hand. 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]. Aliquots of 100 µL TE (10 mM Tris-HCl; 1 mM EDTA; pH 8.0), 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. Following this 50 µL of 20% SDS were added to each tube, then incubated at 42°C for 10 min. A volume of 400 µL of MATAB was 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 an 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.

PCR-Denaturing Gradient Gel Electrophoresis (DGGE) analysis

For bacteria : The V3 variable region of bacterial 16S rDNA from fish was amplified using primers gc-338f (5`-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG-3`, Sigma) and 518r (5`-ATT ACC GCG GCT GCT GG-3`, Sigma) (Ampe *et al*., 1999; Leesing, 2005; Øvreas *et al*., 1997). A 40-bp GC-clamp (Sigma) was added to the forward primer 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). Each mixture (final volume 50 µL) contained about 100 ng of template DNA, all the primers at 0.2 µM, all the deoxyribonucleotide triphosphate (dNTPs) at 200 µM, 1.5 mM $MgCl_2$, 5 µL of 10× of reaction Taq buffer $MgCl_2$ free and 5 U of Taq polymerase (Promega). In order to increase the specificity of amplification and to reduce the formation of spurious by-products, a ‘‘touchdown’’ PCR was performed according to the protocol of Díez *et al*. (2001). An initial denaturation at 94°C for 1 min and 10 touchdown cycles of denaturation at 94°C for 1 min, then annealing at 65°C (with the temperature decreasing 1°C per cycle) for 1 min, and extension at 72°C for 3 min, followed by 20 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 3 min. During the last cycle, the extension step was increased to 10 min.

For yeasts: A fragment of 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`) and the a reverse primer LS2 (5`-ATT CCC AAA CAA CTC GAC TC-3`, Sigma) amplifying an approximately 250 bp fragment (Kurtzman & Robnett, 1998; Cocolin *et al*., 2000). 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 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, 1.25 U of Taq DNA polymerase, and 2 µL of the extracted DNA (≈ 30 ng). The amplification was carried out as follows: An initial denaturation at 95°C for 5 min, 30 cycles of 95°C for 60 s, 52°C for 2 min and 72°C for 2 min, and a final extension at 72°C for 7 min.

For all DNA, 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 0.5 µg mL^{-1} in TAE 1× and quantified by using a standard (DNA mass ladder 100 bp).

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 Leesing (2005). 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 standardised with two reference patterns included in all gels, *Escherichia coli* DNA and *Lactobacillus plantarum* DNA for bacteria and *H. anomala* DNA, *C. parapsilosis* DNA and *Pichia pastoris* DNA for yeast. This software permitted identifiation of 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 bacteria or 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 sequences. 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) : $S_D = 2\ N_c / N_a + N_b$ 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 indeces were expressed within a range of 0 (completely dissimilar) to 100 (perfect similarity). Dendograms were constructed using the Statistica version 6 software (StatSoft, France). Similarities in community structure were determined using the cluster analysis by the single linkage method with the Euclidean distance measure. Significant differences of bacterial communities of fish between seasons were determined by factorial correspondence analysis using the first two variances which described most of the variation in the data set.

Results

DGGE pattern of bacterial DNA from fish within the same sampling period

The PCR-DGGE patterns of five replicates for each location revealed the presence of 8–12 bands of bacteria in the fish (Fig. 1). Some of the bands are common to all the different regions. The bacterial community for five replicates of the same pond of one farm in each district were totally similar among the same season. High similarities were also observed on bacteria patterns for the samples from the same districts, as well as the neighboring districts where the water is supplied by the same branch of the Mekong River. The statistical analysis of the DGGE gel patterns for the five replicates of fish samples from five different districts of An Giang province harvested in the rainy season (25 samples), showed a 30% dissimilarity among the different geographical locations where the fish samples were collected (Fig. 2).

DGGE pattern of bacterial DNA from mandarins among different locations and countries

The PCR-DGGE patterns of 3 replicates for each location was totally similar and revealed the presence of three to six bands for mandarins (Fig. 3). High similarities were observed on bacteria patterns for the samples in the same region. Cluster analysis by Statistica of the DGGE

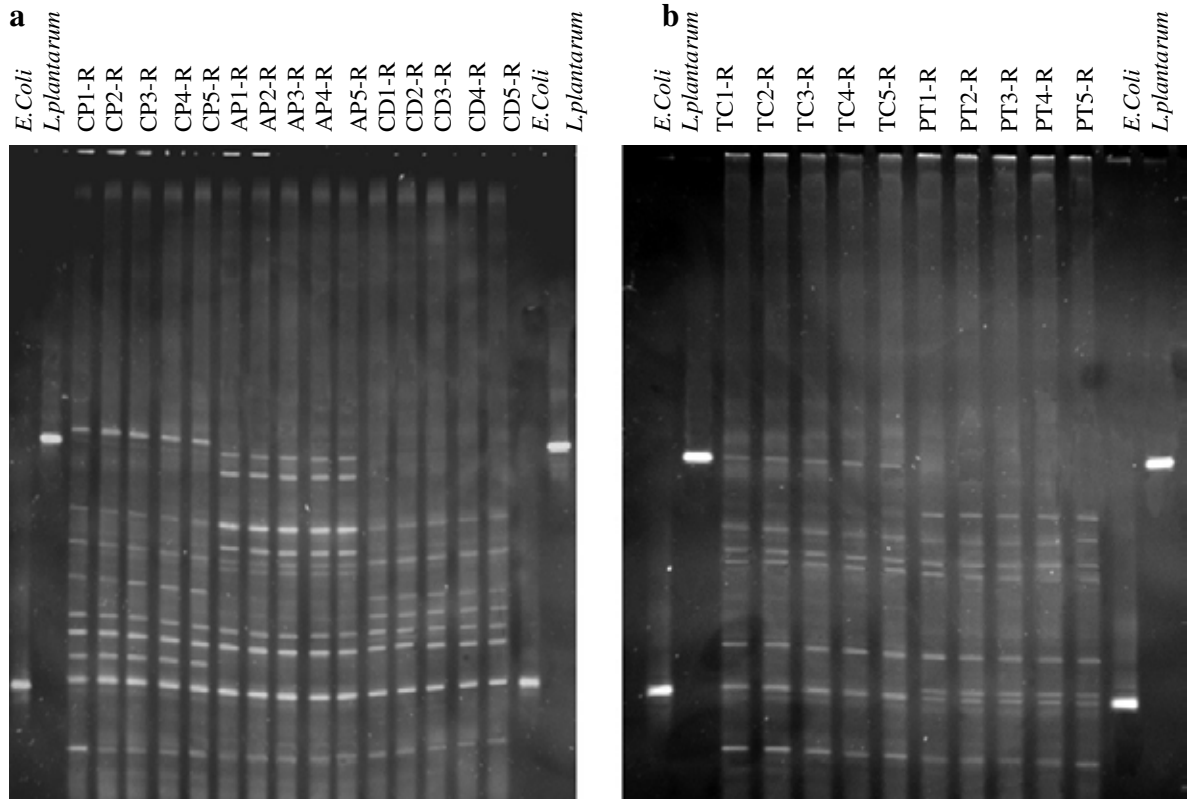

Fig. 1. PCR-DGGE 16S rDNA banding profiles of fish bacteria from five districts of An Giang province (five fish from the same pond in the same farm in each district), Viet Nam in rainy season (R) 2006. (a) CP: Chau Phu district; AP: An Phu district; CD: Chau Doc district; (b) TC: Tan Chau district, PT: Phu Tan district. 1–5: replicate of fish.

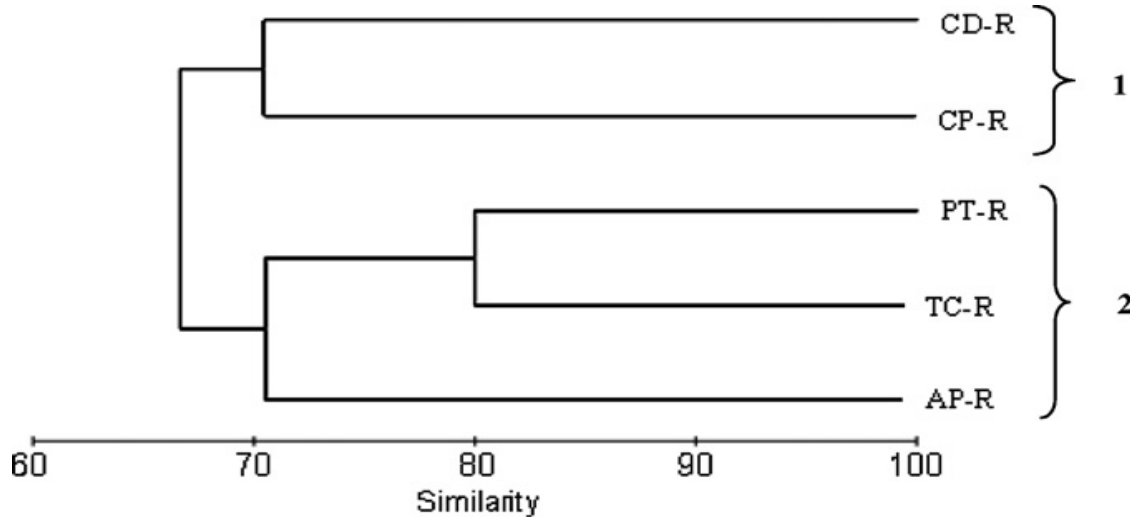

Fig. 2. Cluster analysis of 16S rDNA banding profiles for fish bacterial communities from five districts of An Giang province, Viet Nam in rainy season (R) 2006. CP: Chau Phu district; AP: An Phu district; CD: Chau Doc district; TC: Tan Chau district; PT: Phu Tan district.

gel patterns for the three replicates of mandarins from 2 different countries and various varieties showed 30% dissimilarity among the geographical locations where the fruits were collected (Fig. 4), the first cluster included the samples from Spain and the second cluster comprised the samples from Morocco. The bacterial communities of mandarins from Spain were closely related at 94% similarity as well as for Morocco mandarins, but had 30% dissimilarity in between the two countries (Fig. 4).

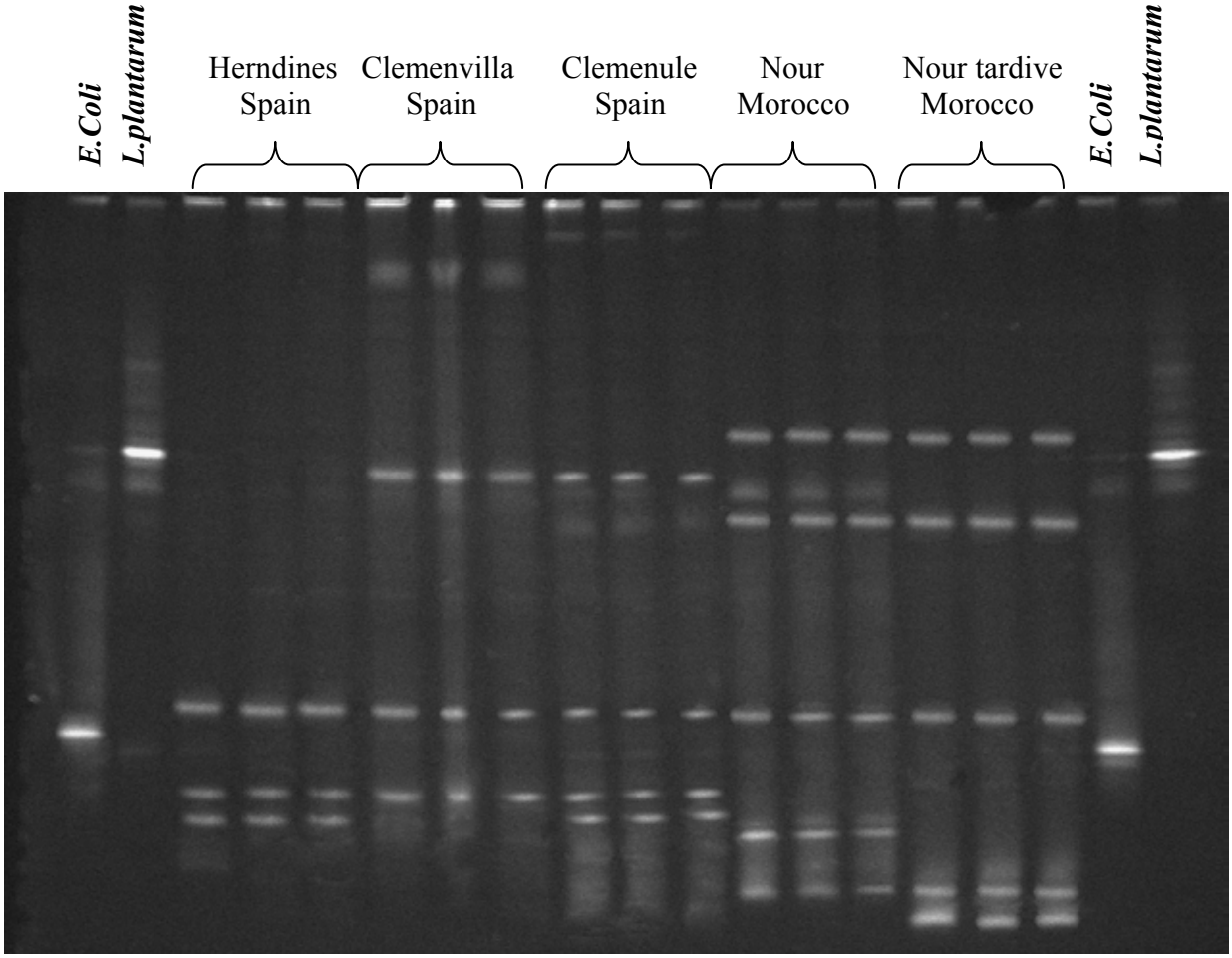

Fig. 3. PCR-DGGE 16S rDNA band profiles of different mandarin varieties from Spain and Morocco.

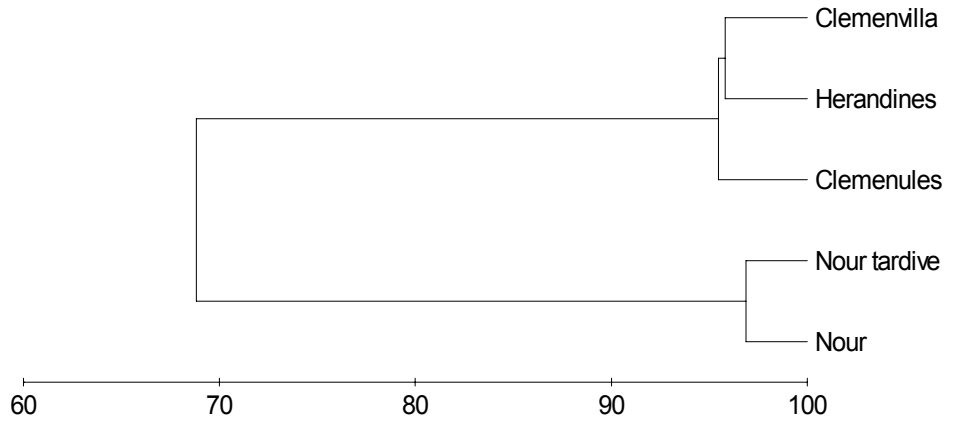

Fig. 4. Cluster analysis of 16S rDNA band profiles of different mandarin varieties from Spain and Morocco.

DGGE pattern of yeast DNA from Physalis among different locations

The DGGE gel patterns for Physalis samples with husk and without husk harvested in the 4 regions showed a community similarity among the geographical locations. This similarity confirms that the 4 areas are rather close geographically one to the other (Fig. 5), 16% of dissimilarity was observed between the geographical locations where the fruits without husk were collected (Fig. 6).

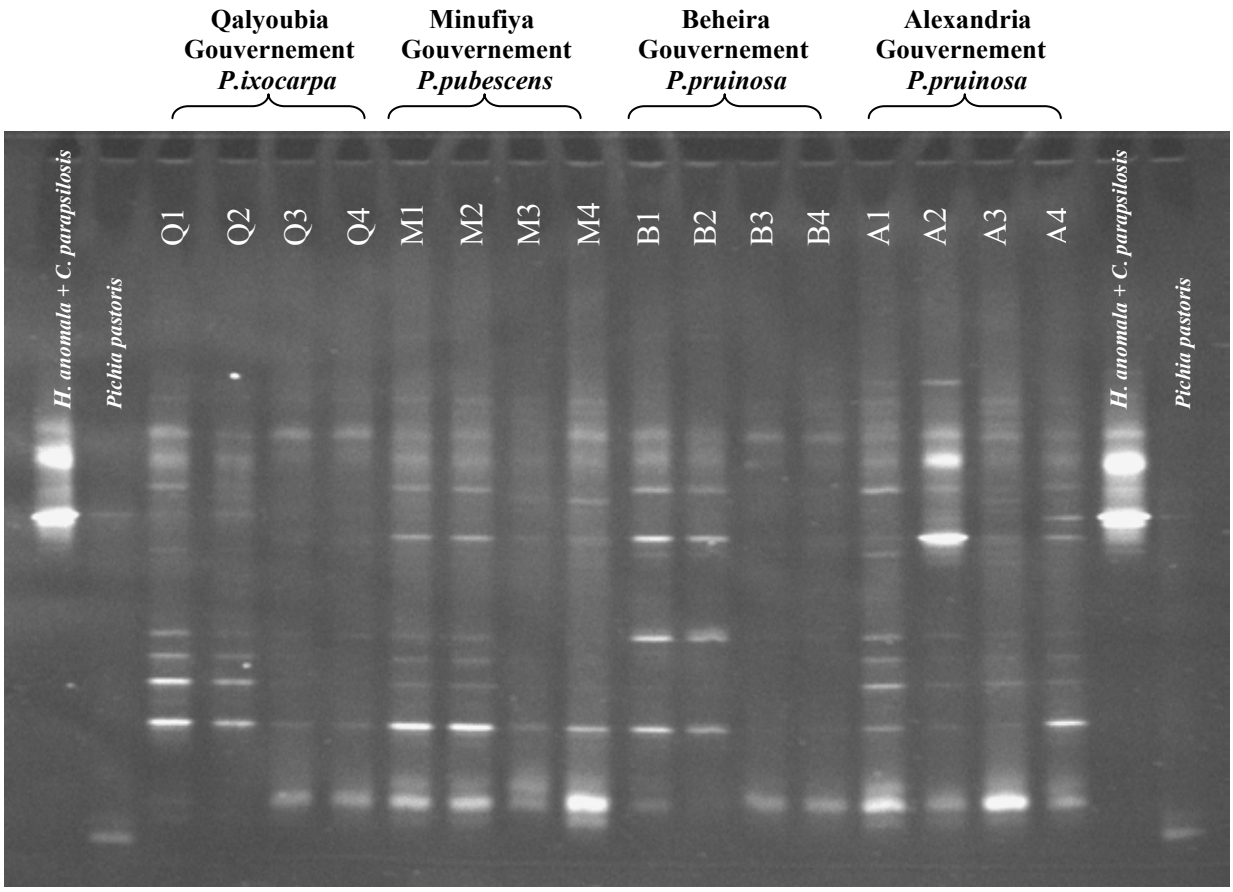

Fig. 5. PCR-DGGE 26S rDNA band profiles of different Physalis from four regions of Egypt. Q: Qalyoubia Gouvernement; M: Minufiya Gouvernement; B: Beheira Gouvernement; A: Alexandria Gouvernement. (1,2) Fruits with husks; (3,4) Fruits without husks.

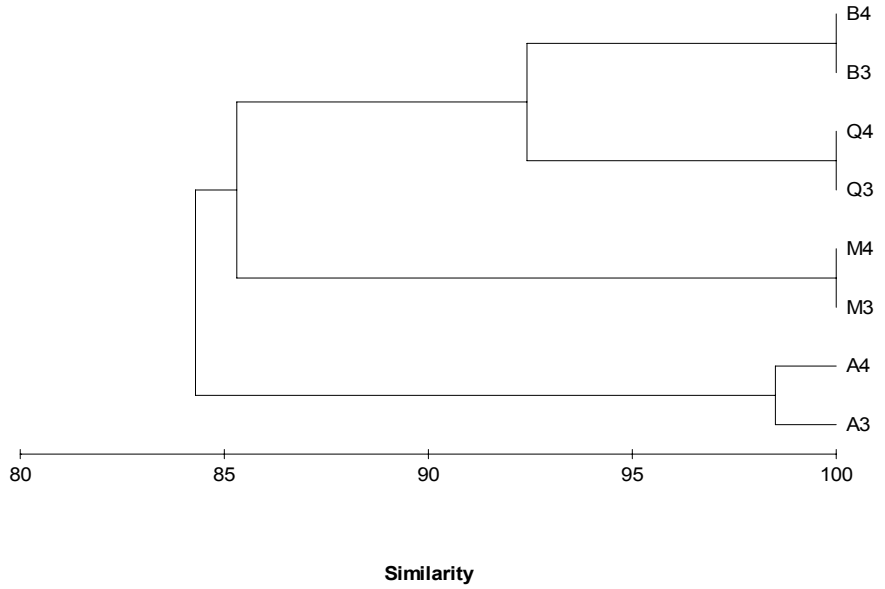

Fig. 6. Cluster analysis of 26S rDNA banding profiles for Physalis yeast communities without husks from four regions of Egypt 2008.

Discussion

Analysis of bacterial communities in fish samples has been often investigated using culture dependent methods and culture-independent methods by random amplified polymorphic DNA (RAPD) (Spanggaard *et al*., 2000). There are only a few published works that analysed the bacterial communities in fish samples by PCR-DGGE (Huber *et al*., 2004; Spanggaard *et al*., 2000). Analysis of bacterial communities in mandarins has been often investigated using culture dependent methods and culture independent methods by PCR. Most of the work was done on fruit pathogen identification by PCR (Jagoueix *et al*., 1996; Hocquellet *et al*., 1999; Do Carmo Teixeira *et al*., 2005) or real-time PCR (Li *et al*., 2006; Lacava *et al*., 2006; Li *et al*., 2006; Picchi *et al*., 2006) but no references have been found whch deal with the application of DGGE for fruit. This is the first publication which introduces the analysis of the yeast communities in Physalis samples by PCR/DGGE. Previous work on Physalis has focused on general proximate composition and physico-chemical properties of the fruit (Watt & Merrill, 1963; El Sheikha, 2004; El Sheikha *et al.,* 2008).

We found that the band patterns of the bacterial and yeast communities isolated from fish, mandarins and Physalis obtained by PCR-DGGE were strongly linked to the microbial environment of the fish and fruits.

The fish skin and gills are in direct and constant contact with the water. The analysis of fish samples from different locations within the same period (rainy season) showed some significant differences in the migration patterns on DGGE. The five replicates for each sampling location had statistically similar DGGE patterns throughout the study. The differences in the band profiles can be attributed to the differences of the feeding methods in between farms and the type of aquaculture system applied. The variations may also be due to the water supply which can be affected by the pollution from urban life. Furthermore, the antibiotics needed to cure diseases together with stress factors could also affect the microbial communities of the fish (Sarter *et al*., 2007). However, some common bands obtained by DGGE have been found in all the profiles within the same sampling periods and origin. We could conclude that there were enough differences in the water quality and the environment of the fish to result in a major effect on the bacterial ecology.

The analysis of mandarins from different locations showed some significant differences in the migration patterns on the DGGE gel. However, the three replicates for each sampling location had statistically similar DGGE patterns throughout the study. The differences in the band profiles can be attributed to the differences of environment in between farms and the type of processing system applied. Furthermore, the disease treatment of fruits could also affect the microbial communities of mandarins. In the gel, one band appeared in all the samples, independently of the origin and the variety. This band can be a common bacterium for all the mandarins. A specific band for each variety can also be found. Many common bands have been found in all the profiles within the same sampling origin.

For Physalis, the DGGE gel showed some significant differences in the migration patterns. However, the duplicates for each sampling location with and without husk gave statistically similar DGGE patterns throughout the study. We demonstrated that there was a link between the yeast populations and the geographical area.

In conclusion, the PCR-DGGE analysis of bacterial and yeast communities suggests that this technique could be applied to differentiate geographical location. We showed that the biological markers for the specific locations were sufficient statistically to discriminate regions. This global technique is quicker (less than 24 h) than all of the classical microbial techniques and avoids the precise analysis of bacteria and yeast by biochemistry or molecular biology (sequencing). This method can thus be proposed as a rapid analytical traceability tool for fish and fruits.

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