Original article

Determination of citrus fruit origin by using 16S rDNA fingerprinting of bacterial communities by PCR- DGGE: an application to clementine from Morocco and Spain

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Determination of citrus fruit origin by using 16S rDNA fingerprinting of bacterial communities by PCR- DGGE: an application to clementine from Morocco and Spain.

Abstract — Introduction. Consumption of citrus fruits and their juice has strongly grown in the last few decades. However, at the present time, the traceability of these fruits is only documentary. In case of doubt or fraud, no standardized analysis makes it possible to discriminate or determine the geographical origin of culture of the fruit. **Materials and methods**. A method of bacterial ecology, PCR-DGGE, was used to characterize the bacterial flora of clementines imported into France from Spain and Morocco in order to show that there is a relation between the bacterial communities of the fruits and their geographical origins. The principle rests on the determination of specific biological markers for a given region. Protocols of microbial extraction and DNA amplification were optimized. **Results**. DGGE profiles analyzed by multivariate analysis permitted us to distinguish microbial communities from different origins. **Conclusion**. We propose the PCR-DGGE method as a new tool for traceability that provides citrus products with a unique bar code and makes it possible to trace back the citrus fruit to their original location.

Morocco / Spain / *Citrus reticulata* / fruits / provenance / microbial proteins / DNA / PCR / DNA fingerprinting

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Détermination de l'origine de fruits d'agrumes par utilisation de l'empreinte de l'ADNr 16S des communautés bactériennes par PCR-DGGE : application à la clémentine du Maroc et de l'Espagne.

Résumé — introduction. La consommation des agrumes et de leur jus s'est fortement développée pendant ces dernières décennies. Cependant, à l'heure actuelle, la traçabilité de cette filière est uniquement documentaire. En cas de doute ou de fraude, aucune analyse normalisée ne permet de discriminer ou de déterminer l'origine géographique de la culture du fruit. **Matériel et méthodes**. Une méthode d'écologie bactérienne, la PCR-DGGE, a été utilisée pour caractériser la flore bactérienne des clémentines importées en France d'Espagne et du Maroc afin de montrer qu'il existe une relation entre les communautés bactériennes des fruits et leurs origines géographiques. Le principe repose sur la détermination de marqueurs biologiques spécifiques d'une localisation donnée. Les protocoles d'extraction et d'amplification des ADN microbiens ont été optimisés. **Résultats**. Le profil de DGGE étudié par une analyse multivariée a permis de déterminer les profils microbiens spécifiques des différentes régions étudiées. **Conclusion**. Nous proposons d'utiliser la méthode de la PCR-DGGE comme un nouvel outil de traçabilité qui fournirait un code barres unique aux produits issus d'agrumes et permettrait de relier les agrumes à leur zone de culture originale.

Maroc / Espagne / *Citrus reticulata* / fruits / provenance / protéine microbienne / ADN / PCR / empreinte ADN

1. Introduction

International trade has intensified, and extends to the entire planet. Foodstuffs are often consumed far from their zone of production. Consumers are more and more demanding and sensitive to the quality and the origin of the foodstuffs that they buy. The issues surrounding food safety and security continue to be a hot topic that concerns all the supply chain. Bovine Spongiform Encephalopathy (BSE), *Salmonella* and avian influenza remain embedded in the memories of European customers. Regulations across Europe continue to be tightened in order to provide a greater degree of assurance in quality and safety. Meanwhile, the traceability and labeling of imported products in European countries remains a compulsory issue (UE regulation 178/2002).

World citrus production and consumption has witnessed a period of strong growth since the mid-1980s. Production of oranges, tangerines, lemons and limes has all expanded rapidly. Larger production levels have enabled higher levels of total as well as per capita consumption of citrus. Even faster growth has been realized for processed citrus products, and improvements in transportation and packaging have lowered costs and improved quality [1].

Clementine is one of the varieties of citrus that is preferred by European people. Due to its high quality, it is one of the most important cultivated citrus mandarins. A clementine is an oblate, medium-sized fruit. The exterior is a deep orange color with a smooth, glossy appearance. This fruit is easy to peel, like a tangerine, but it lacks the tangerine's sourness and seeds. Clementine, usually grown in Morocco and Spain, has been available in Europe for many years. The consumption of clementines by Europeans is very high. For example, in 2006, France imported about 278 000 Mt of clementines. The two biggest exporters of clementine are Spain with 249 965 Mt and Morocco with 22 938 Mt[1].

[1]http://www.saintcharlesinternational.fr/public/

Clementines could be harvested by cooperatives or by big independent suppliers. 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 the exact geographical origin of various clementines. This is why traceability is only assured by rigorous labeling and administrative documentation without any analytical control means. However, in case of doubt or of fraud, it is necessary to find a precise and fast analytical technique in order to determine the geographical origin of the fruits. In addition, certain species, such as Corsica clementines, have obtained a Protected Geographical Indication (PGI) from Europe and could be sensitive to the development of analytical methods of traceability.

Among the most popular analytical methods which allow us to ensure the determination of origin (2D code, spectrophotometers, stable isotope of strontium, etc.) [2], no method of molecular biology in general or PCR-DGGE, in particular, has been described and used. However, the potential of this tool shows that it should be able to give reliable results in very short times in adequacy with the speed of trade concerning these products.

There is a multitude of clementine varieties. These varieties are not specific to a particular geographical area, since one variety can be cultivated in different countries located on different continents. Moreover, the classification of these varieties is very complex and little information exists on their genetic specificities [3]. It thus seems impossible to use fruit genomic markers to ensure the traceability of clementines.

However, fresh clementines are not sterile and can carry microorganisms or their fragments. More generally, the bacterial communities of the fruits do not resemble those of other foodstuffs. This is due partly to the ionic concentration and the pH of the fruits (from 2.2 to 4.9). This characteristic helps to protect the fruit from possible degradations caused by a majority of the microorganisms. Due to the acidity of the internal part of clementine, only the acidophilic microorganisms can multiply. The presence of various species of microorganisms must

depend on the external environment of the fruit (soil ecology, presence of spoilage fruits, insects and diseases of the fruit or the tree, etc.), but also microorganisms transferred by human activity during fruit harvesting [4].

Among the new means of tracing food and, in particular, citrus fruits, the idea of creating a "biological bar code" [5] based on the analysis of DNA of microorganisms present on the products is an interesting approach. This method is based on the assumption that the bacterial communities of the fruits are specific to a geographical area.

Our study relates to the determination of bacterial DNA present on clementines that come from different areas, by the method of PCR-DGGE adapted from the method developed by our team to determine the geographical origin of fish [6, 7].

PCR-DGGE should enable us to visualize microbial diversity in order to link it with the geographical origin of citrus fruits, like a genetic profile of the various species of microorganisms (biological markers) present in the fruit. Thus, we propose to build a "biological bar code" characterizing the bacterial communities of clementine. The identification of the origin will thus be carried out by visualizing the diversity of the microbial communities present in the product. It will be necessary to prove that there is a true relation between the profiles obtained and the various geographical areas of production (specific bacteria).

Denaturing gradient gel electrophoresis (DGGE) of 16S rDNA fragments, generated by the polymerase chain reaction (PCR), is now a popular method for assessing the ecology of microbial communities in environmental samples [8–13]. DGGE has the advantages that we can obtain information on the microorganisms present and the community changes by analyzing DNA bands that migrate separately on DGGE gel, according to the melting point of 16S rDNA fragments [14–15].

The purpose of our study was to establish that using the DGGE method to analyze the dominant bacteria in clementine is possible with the further objective of creating a technique to link the bacterial community to the geographical origin. In order to reach this objective, a DNA extraction method and DGGE running conditions were optimized and were evaluated for use in analysis of bacterial communities in clementines.

2. Materials and methods

2.1. Fruit sampling

The clementine samples (*Citrus reticulata blanco* var. clementine) were provided by the *Marché Gare Saint-Charles* of Perpignan (France) and came from two different countries: Spain and Morocco. They arrived at the CIRAD (Agricultural Research Center for International Development) laboratory in Montpellier (France) in January 2007 and the bacterial DNA was extracted immediately on the fresh products.

Three varieties came from the Valencia region in Spain. Among them, the Clemenvilla variety was supplied by a first company; the Clemenule variety was supplied by a second company; and the Hernandine variety was supplied by a third company.

Two varieties came from the Berkane region in Morocco where the Nour variety and the Nour tardive variety were harvested. Both of them were supplied by a fourth company.

Three clementines from each variety were randomly taken for analysis from the various packages; each package contained the different varieties of clementines.

2.2. DNA extraction

The clementines were peeled and all of the skin was put in a sterile Stomacher bag, then 20 mL of peptone water was supplemented. The mixture was crushed for 30 s in a Stomacher® (Seward, UK). Four 1.5-mL tubes containing the resulting suspension were then centrifuged at 10 000 *g* for 10 min. One hundred µL of lysis buffer TE (10 mM tris-HCl; 1 mM EDTA; pH 8.0, Promega, France), 100 µL of lysozyme solution (25 $\mu g \cdot \mu L^{-1}$, Eurobio, France) and 50 µL of proteinase K solution (10 $\mu g \cdot \mu L^{-1}$, Eurobio, France) were added to each pellet in each Eppendorf

tube. Samples were vortexed for 1 min and incubated at 42 °C for 30 min. Then 50 µL of 20% SDS (Sodium Dodecyl Sulfate, Sigma, France) were added to each tube, and the tubes were incubated at 42 °C for 10 min. Three hundred µL of MATAB (Mixed Alkyltrimethyl Ammonium Bromide, Sigma, France) were added to each tube, 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, France), and the residual phenol was removed by extraction with an equal volume of chloroform-isoamyl alcohol (24:1). The pellet of DNA was precipitated with isopropanol, washed with 70% ethanol, then air-dried at room temperature. Finally, the DNA was resuspended in 100 µL of ultrapure water and stored at –20 °C until analysis.

In order to check DNA extraction, electrophoresis on agarose gel was done. The entire DNA was loaded into 0.8% agarose gel in 1X TAE buffer (40 mM tris-HCl pH 7.4, 20 mM sodium acetate, 1.0 mM Na_2-EDTA, Eppendorf, Germany). After running at 100 V for 30 min, the gels were stained for 30 min with ethidium bromide solution (50 $\mu g \cdot mL^{-1}$, Promega, France), rinsed for 20 min in distilled water, then observed and photographed on a UV transilluminator using a black and white camera (Scion Company, USA) and Gel Smart 7.3 system software (Clara Vision, Les Ulis, France).

2.3. PCR-DGGE

The V3 variable region of bacterial 16S rDNA of the bacterial flora of the fruit was specifically amplified using the primers gc338f (5'CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGACTCCTACGGGAGGCAGCAG, Sigma, France) and 518r (5'-ATTACCGCGGCTGCTGG, Sigma, France) [6, 7, 11, 12] was added to the forward primers in order to insure that the fragment of DNA would remain partially double-stranded and that the region screened was in the lowest melting domain [18]. Each mixture (final volume 50 µL) contained about 100 ng of template DNA, 0.2 µM for all the primers, 200 µM for all the deoxyribonucleotide triphosphate (dNTPs), 1.5 mM $MgCl_2$, 5 µL of 10x of reaction *Tag* buffer ($MgCl_2$ free) (Promega, France) and 5U of Taq polymerase (Promega, France). In order to increase the specificity of amplification and to reduce the formation of spurious by-products, a "touchdown" PCR was performed [6, 7, 13]. 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 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. Aliquots (5 µL) of PCR products were analyzed first by conventional electrophoresis in 2% (w/v) agarose gel with TAE 1X buffer (40 mM tris-HCl pH 7.4, 20 mM sodium acetate, 1.0 mM Na_2-EDTA), stained with ethidium bromide (Sigma, France) 0.5 $\mu g \cdot mL^{-1}$ in TAE 1X and quantified by using a standard (DNA mass ladder 100 bp, Promega, France).

The PCR products were analyzed by Denaturing Gradient Gel Electrophoresis (DGGE) by using a Bio-Rad DCode TM universal mutation detection system (Bio-Rad Laboratories, Hercules, USA) and the procedure first described by Muyzer *et al.* [12] and improved by Leesing [6] and Le Nguyen *et al.* [7]. Samples containing approximately equal amounts of PCR amplicons were loaded into 8% (w/v) polyacrylamide gels (acrylamide/NN'-methylene bisacrylamide, 37.5:1, Promega, France) in 1X 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 7M urea and 40% [v/v] formamide, Promega, France). The gels were electrophoresed at 20 V for 10 min and then at 180 V for 12 h.

After electrophoresis, the DGGE gels were stained for 30 min with ethidium bromide solution (50 $\mu g \cdot mL^{-1}$, Promega, France), rinsed for 20 min in distilled water, then observed and photographed on a UV transilluminator using a black and white camera (Scion Company, USA) and Gel Smart 7.3 system software (Clara Vision, Les Ulis, France).

2.4. Image and statistical analysis

Individual lanes of the gel images were straightened and aligned using ImageQuant TL software version 2003 (Amesham Biosciences, USA). Banding patterns were standardized with the two reference patterns included in all gels, which are the patterns of *Escherichia coli* DNA and *Lactobacillus plantarum* DNA. This software permitted the identification of the bands and their relative position compared with the standard patterns.

In DGGE analysis, the generated banding pattern is considered as an "image" of all of the major bacterial species in the population. An individual discrete band refers to a unique "sequence type" or phylotype [19, 20], which is treated as a discrete bacterial population. It is expected that PCR fragments generated from a single population will display an identical electrophoretic mobility in the analysis. This was confirmed by Kowalchuk *et al.* [21], who showed that co-migrating bands generally corresponded to an identical sequence.

The DGGE fingerprints were manually scored by the presence and absence of co-migrating bands, independent of intensity. Pairwise community similarities were quantified using the Dice similarity coefficient (S_D) [22] : $S_D = [2\ N_c / (N_a + N_b)]$, where N_a represented the number of bands detected in the sample A, N_b represented the number of bands detected in the sample B and N_c represented the numbers of bands common to both samples. Similarity indexes were expressed within a range of 0 (completely dissimilar) to 1.0 (perfect similarity). Dendograms were constructed using the Statistica version 6 software (StatSoft, France). Similarities in community structure were determined using cluster analysis by the single linkage method with the Euclidean distance measure.

3. Results

3.1. Efficiency of the bacterial DNA extraction method for different clementines from Spain and Morocco

DNA extraction of the bacterial community was done on the skin of the clementines using the method of Leesing [6] improved by Le Nguyen *et al.* [7], which has been successfully applied to fish bacteria. We verified the extraction efficiency with a 2% (w/v) agarose gel. On the gel, the bands with a molecular weight greater than 10 kb corresponding to genomic bacterial DNA were clearly observed (*figure 1*).

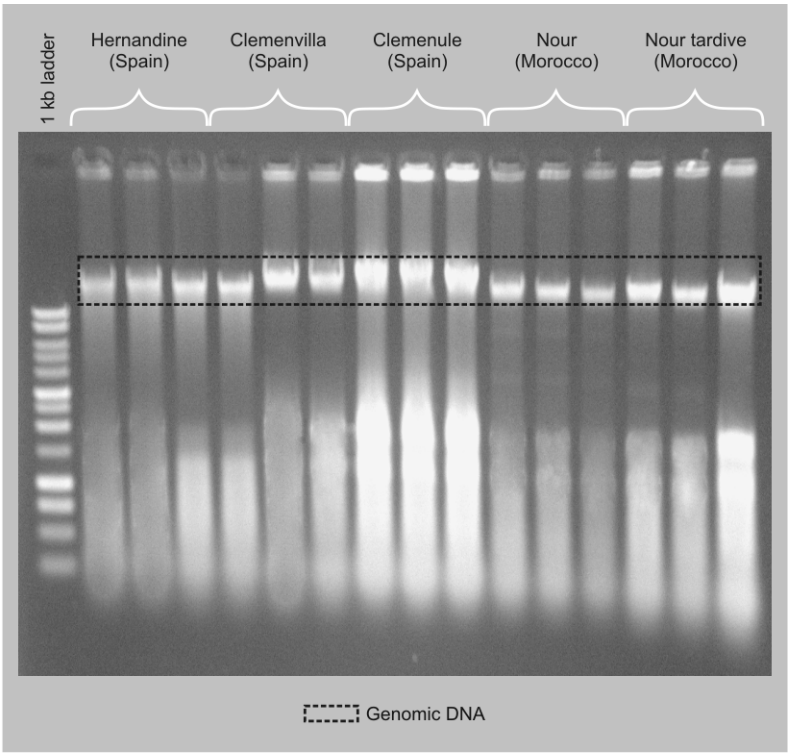

Figure 1.
Agarose gel for verification of bacterial DNA extraction from different varieties of clementine from Spain and Morocco.

3.2. Verification of the amplification of the extracted DNA by PCR

The bacterial DNA obtained after extraction was amplified by a "touchdown" PCR [6, 7]. In order to verify the efficiency of this amplification, the PCR amplicons were electrophoresed on 2% (w/v) agarose gel (*figure 2*) at 100 V for 30 min in the TAE buffer as described above. All of the bands were clearly observed and had a molecular weight of 236 bp, the expected size of the amplicon. PCR reaction was carried out well.

The intensity of the bands representing the PCR amplicons are important (*figure 2*). Bacterial DNA was amplified very well and

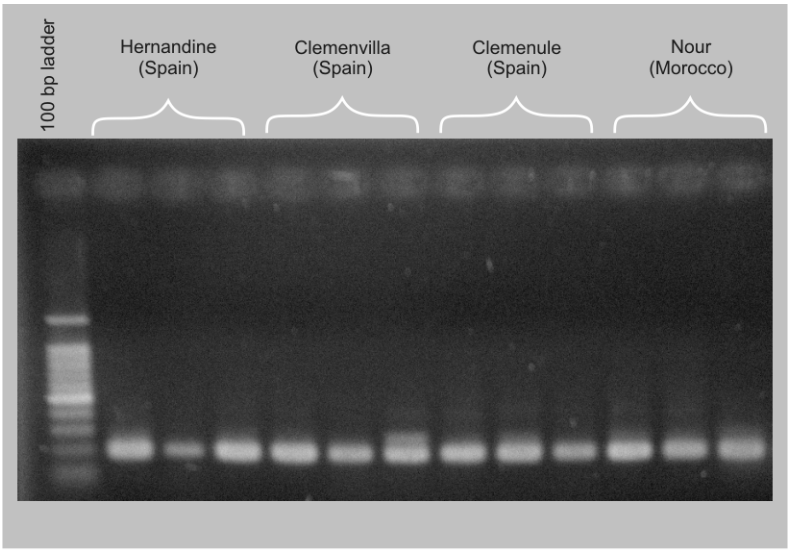

Figure 2.
Agarose gel for verification of PCR reaction of DNA extracted from different varieties of clementine from Spain and Morocco.

thus it was possible to continue to analyze these amplicons by the DGGE method.

3.3. Analyses of PCR amplicons of bacterial DNA from clementines by DGGE

On DGGE gel, the observed bands had sufficient intensities to analyze samples of bacterial DNA extracted from clementines of various origins (*figure 3*), so the total quantity of DNA deposited in the wells of DGGE was sufficient to consider that bacterial DNA was a potential marker to ensure the determination of clementine origin.

The PCR-DGGE patterns of three replicates for each location was totally similar and revealed the presence of three to six bands for clementines (*figure 3*). High similarities were observed in bacteria patterns for the samples in the same region.

Cluster analysis by Statistica software of the DGGE gel patterns for the three replicates of clementine samples from two different countries and various varieties show the community similarity among the geographical locations where the fruit samples were collected (*figure 4*). At the 70% similarity level, two main clusters were observed (*figure 4*): the first cluster included the samples from Spain; the second cluster comprised the samples from Morocco. The bacterial communities of Spain were closely related, at 94% similarity, with the bacterial communities from Morocco.

4. Discussion

Analysis of bacterial communities in clementine samples has often been investigated using culture-dependent methods and culture-independent methods by PCR. There are only a few publications that analyzed the bacterial communities in citrus samples by PCR methods. Most of the work was done on fruit pathogen identification on strains such as *Candidatus liberibacter* by PCR [23–25] or real-time PCR [26], *Methylobacterium mesophilicum* [27], *Xanthomonas axonopodis* pv. *citri* [28] and *Xylella fastidiosa* [29] but no references relate to the DGGE method.

In our study, we found that the DGGE pattern of the bacterial communities from clementines was strongly linked to the microbial environment of the fruit.

The analysis of clementine samples 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 in environment between farms and the types of processing system applied. Furthermore, the disease treatment of fruits could also affect the microbial communities of the clementines. In the gel, one band appears in all the samples, independently of the origin and the variety. This band could be a common bacterium for all the clementines. Some other bands appear in all the varieties from the same country. The specific band for each variety can also be found. Many common bands were found in all the profiles within the same sampling origin.

The purpose of our study was to apply the PCR-DGGE method to analyzing the bacteria in clementine in order to create a technique to link bacterial communities to the geographical origin and avoid the individual analysis of each bacterial strain. The acquired band patterns for the bacterial species of different clementines were compared and analyzed statistically to determine the

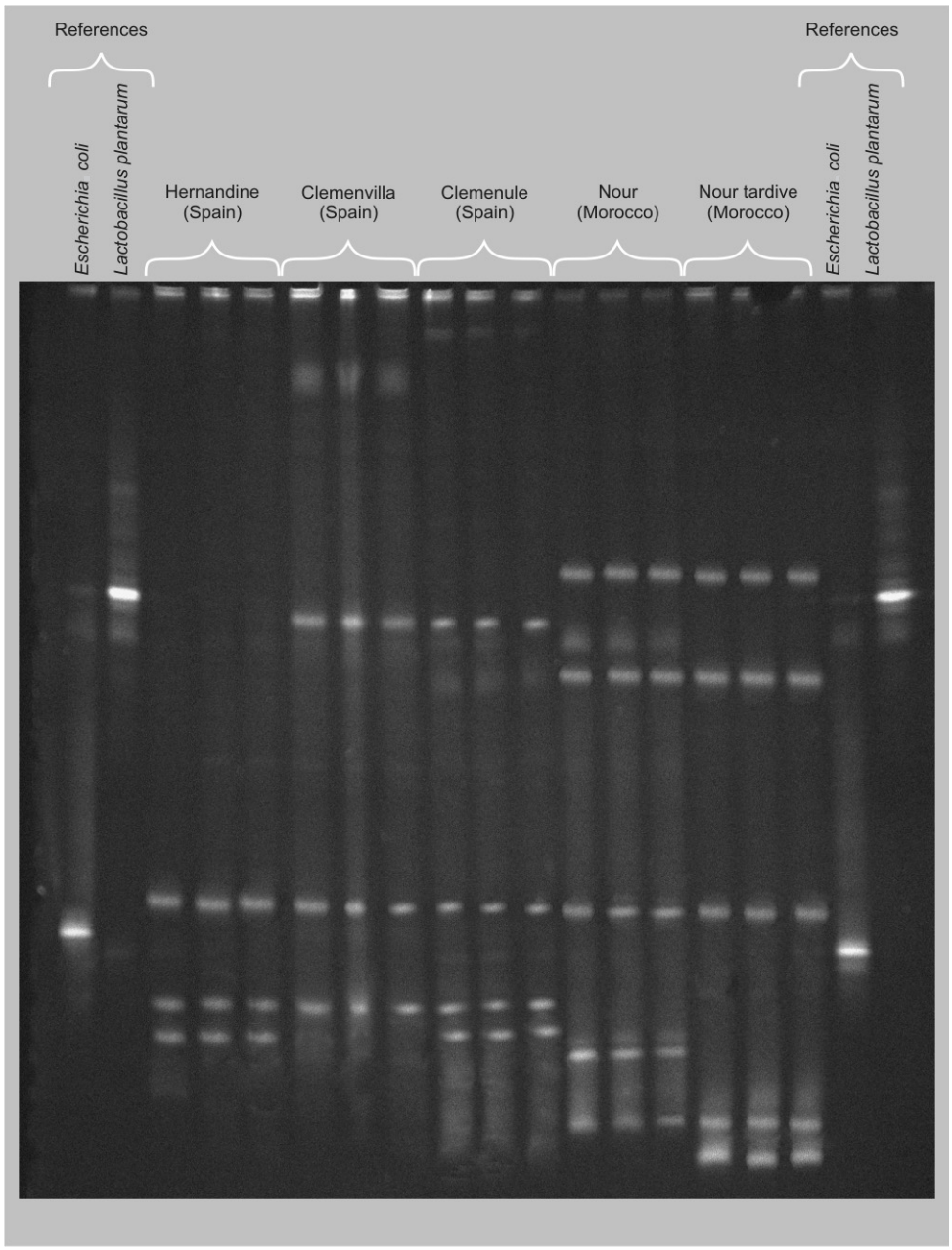

Figure 3.
PCR-DGGE 16S rDNA band profiles of different clementine varieties from Spain and Morocco.

clementine origin. In the next step, we will excise and identify the DGGE bands by sequencing in order to know more clearly about the bacterial community of the clementine.

The clementine samples came from two different countries, Spain and Morocco. With the statistical analysis of DDGE patterns, we can distinguish well the geographical origin of clementines. For the samples from the same countries, although they came from the same region, there are various varieties and they are supplied by different companies. We could say that they came from different cultivated farms. This fact could result in some minor variations in the DGGE profiles of the bacterial communities of the clementines in the same region.

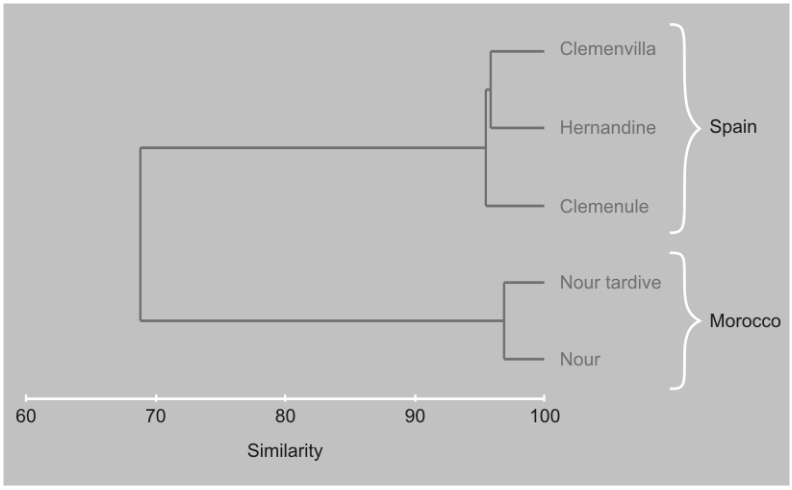

Figure 4.
Cluster analysis of 16S rDNA band profiles of different clementine varieties from Spain and Morocco.

It is thus possible to propose PCR-DGGE as a potential method to discriminate the origin of clementines from two different geographical areas.

PCR-DGGE has many advantages but also many disadvantages. Thus, concerning the limits of this method, it is necessary to point out that total DNA extraction includes some DNA other than bacterial DNA, as well as the DNA from the fruits. Although the primer sets gc-338f/518r are specific for bacteria, we found in only one paper [12] that it is possible to obtain some amplification of eukaryote DNA, *e.g.*, *Zea mays* chloroplast 16S rDNA. In addition, the size of the amplified sequences is 236 base pairs and the amplified sequences are generally hypervariable zones of the genome. We usually consider that two very genetically close microbes lead to distinct bands, but it is not excluded with a very weak probability that two different microbes give very close bands or even identical bands.

5. Conclusions

The PCR-DGGE method proved that it is possible to extract bacterial DNA in sufficient quantity on clementines that were imported in a real industrial chain and that had been treated by various insecticides and antimicrobial components (not identified). It was noted that the DGGE patterns of clementines from different areas were different, which signifies that bacteria can be used as markers of geographical origin. It is thus possible to propose this method as a potential method to discriminate the origin of clementines from two different countries and, inside the countries, to two or three different areas. The protocols used could still be improved to reach the true microbial diversity carried by citrus fruits. The following stage would consist of studying other microbial markers of origin such as yeasts and moulds with specific primers.

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Determinación del origen de frutos cítricos mediante el empleo de la imprenta del ADNr 16S de las comunidades bacterianas por PCR-DGGE: aplicación en las clementinas de Marruecos y de España.

Resumen — Introducción. El consumo de los cítricos y de su jugo se ha desarrollado fuertemente durante estas últimas décadas. Sin embargo hoy en día, el rastreo de esta filial consta únicamente en documentos. En caso de duda o de fraude, no existe ningún análisis normalizado que permita discriminar o determinar el origen geográfico del cultivo del fruto. **Material y métodos**. Se empleó un método de ecología bacteriana, el PCR-DGGE, con el fin de caracterizar la flora bacteriana de las clementinas importadas a Francia desde España y Marruecos, para mostrar que existe una relación entre las comunidades bacterianas de los frutos y sus orígenes geográficos. El principio se basa en la determinación de marcadores biológicos específicos de una localización predefinida. Se optimizaron los protocolos de extracción y de amplificación de los ADN microbianos. **Resultados**. El perfil de DGGE estudiado mediante un análisis multivariado permitió determinar los perfiles microbianos específicos de las diferentes regiones estudiadas. **Conclusión**. Proponemos emplear el método PCR-DGGE como un nuevo instrumento de rastreo que proporcionaría un código de barras único para los productos derivados de los cítricos y que permitiría relacionar los cítricos con su zona de cultivo original.

Marruecos/ España / *Citrus reticulata* / frutas / procedencia / proteínas microbianas / ADN / PCR / huellas genéticas ADN