Determination of fruit origin by using 28S rDNA fingerprinting of fungi communities by PCR-DGGE: an application to Physalis fruits from Egypt, Uganda and Colombia

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Determination of fruit origin by using 28S rDNA fingerprinting of fungi communities by PCR-DGGE: an application to Physalis fruits from Egypt, Uganda and Colombia.

Abstract — Introduction. Consumption of Physalis fruits is quite recent and the products are poorly known among consumers. This plant has been known for a long time as an ornamental in Europe, for at least 200 years. Traceability is increasingly becoming important across the agri-food industry; however, at the present time, the traceability of this fruit is only documentary. In case of doubt or fraud, no standardized analysis can determine the geographical origin of the fruits. **Materials and methods**. In order to discover the relationship between the fungal communities of the fruit and their geographical origins, 28S rDNA-PCR-DGGE was used to analyze the variation in fungal communities in three species of Physalis fruit (*Physalis ixocarpa* Brot., *Physalis pruinosa* L. and *Physalis peruviana* L.) from Egypt, Uganda and Colombia. **Results**. Denaturing Gradient Gel Electrophoresis (DGGE) fingerprints of 28S ribosomal DNA (28S rDNA) analyzed by multivariate analysis could distinguish different fruit origins by their fungal communities. **Conclusion**. We propose the PCR-DGGE method as a new traceability tool which provides fruit in general, and Physalis in particular, with a unique barcode for each country by using 28S rDNA fingerprinting of fungi.

Egypt / Uganda / Colombia / *Physalis* sp. / DNA fingerprinting / provenance / microbial flora

Détermination de l'origine géographique des fruits en utilisant les empreintes génétiques de l'ADNr 28S des communautés de moisissures obtenues par PCR-DGGE : application aux fruits de Physalis provenant d'Égypte, d'Ouganda et de Colombie.

Résumé — Introduction. La consommation des fruits de Physalis est tout à fait récente et ces produits sont mal connus par les consommateurs bien que cette plante soit utilisée comme plante ornementale en Europe depuis au moins 200 ans. La traçabilité devient de plus en plus importante dans l'industrie agro-alimentaire. Cependant, à l'heure actuelle, la traçabilité est seulement documentaire. En cas de doute ou de fraude, aucune analyse normalisée ne permet de déterminer l'origine géographique des fruits. **Matériel et méthodes**. Afin d'établir la relation entre les communautés de moisissures des fruits et leurs origines géographiques, les ADNr 28S ont été analysés par PCR-DGGE pour étudier la variation des communautés de moisissures sur trois espèces de fruits de Physalis (*Physalis ixocarpa* Brot., *Physalis pruinosa* L. et *Physalis peruviana* L.) provenant d'Égypte, d'Ouganda et de Colombie. **Résultats**. Les empreintes des ADN 28S ribosomiques obtenues par DGGE et interprétées par analyse multivariée pourraient permettre de distinguer l'origine géographique des fruits par leur communauté de moisissures. **Conclusion**. Nous proposons cette méthode de PCR-DGGE comme un nouvel outil de traçabilité apte à fournir aux fruits en général, et aux Physalis en particulier, un code barre unique pour chaque pays.

Égypte / Ouganda / Colombie / *Physalis* sp. / Empreintes génétiques de l'ADN / provenance / flore microbienne

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1. Introduction

Despite the central role of traceability in quality control within the manufacturing sector, traceability systems have been adopted on a significant scale only relatively recently. Uptake has largely been precipitated by the bovine spongiform encephalopathy (BSE) crisis. However, ongoing food scares, bio-safety concerns and consumer desire for products with specific attributes have ensured that traceability systems are becoming standard. It is broadly recognized that traceability is essential for good safety and quality control. As the tracing system becomes more precise, the quality problems of food safety can be identified and resolved. Furthermore, the ability to segregate and trace products is seen as the key to providing consumers with information and choices about the food they consume[1] and represents a central component of consumer confidence[2]. Meanwhile, the traceability and labeling of imported products to European countries remain compulsory requirements (EU regulation 178/2002). However, simple traceability systems have been used in the food industry. In view of the difficulties of installing these documentary systems in developing countries, and following products during processing, we proposed to identify and validate some pertinent biological markers which come from the microbial ecology of the food to assure their traceability during international trade. Currently, there are no existing analytical methods which permit the efficient determination of the origin of food or following it during international trade [1].

Physalis is an important genus of the Solanaceae family. It is native to tropical and subtropical America and is widely distributed throughout the world. The genus *Physalis*, established by Linnaeus in 1753, contains about 463 species, a hundred of which are well-known to humans and have more fanciful names: Uvilla in Colombia, Harankash in Egypt and Ntuntunwe in Uganda [2].

Physalis has the potential to become a commercial fruit of particular interest to the world's upscale food industry [3]. Physalis is included on the priority list of many governments' horticulture and fruit export plans. It is relatively unknown in domestic markets and remains an exotic fruit. It is exported from several countries including Colombia, Egypt, Zimbabwe, Kenya, South Africa and Southeast Asia, but Colombia stands out as one of the largest producers, consumers and exporters [4].

In order to determine the geographical origin of Physalis, it is necessary to find a precise and fast analytical method. It thus seems difficult to use fruit genomic markers to ensure its traceability. Moreover, the classification of Physalis varieties is very complex and no information exists on their genetic specificities. So, depending on the presence of various microorganisms in the external environment of the fruit (soil ecology, spoilage, insects, diseases), the relationship between the microbial ecology of fruits and their geographical origins could be assumed [5].

Applied molecular microbiology is a fast-moving area. One of the branches of this discipline is involved in the development of molecular methods for the identification and monitoring of microorganisms in natural ecosystems. Molecular methods are also characterized by rapidity and reliability. PCR-DGGE is usually employed to assess the structure of microbial communities in environmental samples without cultivation and to determine the community dynamics in response to environmental variations [6, 7]. PCR–DGGE has also been used as a tool for identification and typing of microbial entities [7–9].

Genetic fingerprinting techniques are able to provide a profile representing the genetic diversity of a microbial community

[1] Anon., Animals and biotechnology, Rep., Agric. Environ. Biotechnol. Comm. (AEBC), U.K., 2002, 88 p., available at www.aebc.gov.uk/aebc/pdf/animals_and_biotechnology_report.pdf.

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from a specific environment. Denaturing gradient gel electrophoresis (DGGE) is perhaps the most commonly used among the culture-independent fingerprinting techniques. It is based on the separation of polymerase chain reaction (PCR) amplicons of the same size but different sequences. PCR-DGGE of ribosomal DNA was introduced into microbial ecology by Muyzer *et al*. [10].

The purpose of our research was to create a "biological barcode" [11] based on the analysis of the DNA of fungal communities on the Physalis fruits. This method is based on the assumption that the microbial communities of the fruits are specific to a geographical area [12–14].

In order to create an analytical technique which can establish the linkage of fungal communities to the geographical origin and avoid the individual analysis of each fungal strain, our paper seeks to describe the role of the PCR-DGGE method to analyze all the fungi present on the fruit in a unique step. The acquired band patterns for the fungal communities of different species of Physalis fruits from different harvesting locations were compared and analyzed statistically to determine the Physalis' geographical origin. To the best of our knowledge, this is the second paper describing a PCR-DGGE method, that will permit the creation of a unique "biological barcode" for fruit in general, and Physalis in particular, for each country by using 28S rDNA fingerprinting of fungi.

2. Materials and methods

2.1. Fruit sampling

Mature fruits of Physalis (*P. ixocarpa* Brot., *P. pruinosa* L. and *P. peruviana* L.) were collected in a particular field in two different districts from three countries: Colombia, Egypt and Uganda. These districts were: Kilometer 5.5 Via Siberia, Cota Cundinamarca district of Sabana region and Usaquén district of Bogotá region in Colombia, species: *P. peruviana* L.; Qalyoubia district and Beheira district in Egypt, species: *P. ixocarpa* Brot. and *P. pruinosa* L., respectively; and Kawempe district and Mukono district in Uganda, species: *P. peruviana* L.

Kilometer 5.5 Via Siberia, Cota Cundinamarca, Sabana and Usaquén, Bogota, in Colombia are in the subtropical climate region, which is characterized by high rainfall (the rainiest months are April to November), a mild temperature range (the warmest month is January with temperatures up to 25 °C) and an elevation of 2640 m above sea level. But the climatic conditions in this region are irregular and quite variable due to the El Niño and La Niña climatic phenomena, which are responsible for very pronounced climatic changes.

In Egypt, Qalyoubia has a semi-desert climate, which is characterized by low rainfall, a high temperature range and sandy (light) soil, but Beheira has a Mediterranean climate, which is characterized by abundant rainfall, a low temperature range and clay (heavy) soil.

Uganda is at a high altitude: Kawempe's climate is tropical wet with temperatures ranging from 29 °C to 36 °C, which is characterized by very high rainfall, but Mukono has a tropical semi-wet climate with temperatures ranging from 21 °C to 25 °C, which is characterized by moderate rainfall.

The fruits were gathered to preserve their initial flora. They were collected in May 2008 directly on the tree using gloves and put in sterile bags. These bags were kept in a refrigerator then transferred by plane to CIRAD Montpellier (France) and the fungal genomic 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 genomic DNA extraction from Physalis fruit samples

For fungal genomic DNA extraction, we created a new protocol which takes into account the methods of Karakousis *et al*. [15] for fungal DNA extraction and El Sheikha *et al*. [14] for yeast DNA extraction.

Two fruits of Physalis were randomly taken and put in a sterile Stomacher bag

containing 6 mL peptone water then crushed by hand. The resulting suspension was placed into two Eppendorff 2-mL vials with 0.3 g of 0.5-mm diameter acid-washed glass beads 425–600 µm (Sigma, France). The mixture was vortexed vigorously for 30 min in a bead-beater instrument (Vortex Genie 2 SI-A256, USA), then centrifuged at 12,000 *g* for 15 min and the supernatant discarded. The cell pellet was resuspended in 300 µL of breaking buffer [2% Triton X-100 (Prolabo, France); 1% SDS (sodium dodecyl sulfate, Sigma); 100 mM NaCl (Sigma); 10 mM Tris pH 8.0; 1 mM EDTA pH 8.0 (Promega, France)]. Then, 100 µL of TE (10 mM Tris-HCl; 1 mM EDTA pH 8.0, Promega), 100 µL of lysozyme solution (25 mg·mL^{-1}, Eurobio, France) and 100 µL of proteinase K solution (20 mg·mL^{-1}, Eurobio, France) were added and incubated at 42 °C for 20 min. Then 50 µL of 20% SDS were added to each tube, and the tubes were incubated at 42 °C for 10 min. We added 400 µL of MATAB 2% (mixed alkyltrimethyl ammonium bromide, Sigma) 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 µL of phenol-chloroform-isoamyl alcohol (25:24:1, Carlo Erba, 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 µL 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 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 ultrapure water and stored at –20 °C until analysis. In order to check DNA extraction, an electrophoresis on agarose gel was done. Eight µL of the extracted DNA was loaded into 0.8% agarose gel in 1×TAE buffer (40 mM tris-HCl pH 7.4; 20 mM sodium acetate; 1.0 mM Na2-EDTA, Eppendorf, Germany) with a molecular weight ladder (16.21 kb) as reference (Supercoiled DNA ladder, Invitrogen, USA). After running at 100 V for 30 min, the gels were stained for 30 min with ethidium bromide solution (50 µg·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 analysis

The fungal universal primers U1 (5'- CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GTG AAA TTG TTG AAA GGG AA-3', Sigma) and U2 (5'-GAC TCC TTG GTC CGT GTT-3', Sigma) were used to amplify an approximately 260-bp fragment of the 28S rDNA gene [16–18]. 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, a 30-bp GC-clamp (Sigma) was added to the forward primer (the GC-clamp is underlined) [19]. PCR was performed in a final volume of 50 µL containing 2.5 µL DMSO, 0.4 µM of each primer, all the deoxyribonucleotide triphosphate (dNTPs) at 200 µM, 3 mM $MgCl_2$, 5 µL of 10 × 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 Na_2-EDTA), stained with ethidium bromide 50 µg·mL^{-1} in TAE 1 × and quantified by using a standard (DNA mass ladder 100 bp, Promega).

We analyzed 30 µL of the PCR products by DGGE, by using a Bio-Rad DcodeTM universal mutation detection system (Bio-Rad

Laboratories, USA). 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 30–40% 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

The individual lane of the gel images was straightened and aligned using ImageQuant TL software v2003 (Amesham Biosciences, USA). Banding patterns were standardized with two reference patterns included in all gels; *Mucor racemosus* DNA and *Trichoderma harzianum* DNA. This software permitted identifying 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 fungi in the populations. An individual discrete band refers to a unique "sequence type" or phylotype [20, 21]. This was confirmed by Kowalchuk *et al.*, who showed that co-migrating bands generally corresponded to identical sequences [22]. 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) [23]: $S_D = [2\ N_c / (N_a + N_b)]$, where N_a represents the number of bands detected in the sample A, N_b represents the number of bands in the sample B, and N_c represents the number of bands common to both samples. The similarity index was expressed within a range of 0 (completely dissimilar) to 100 (perfect similarity). Significant differences in fungal communities of Physalis were determined by factorial correspondence analysis using the first two variances, which described most of the variation in the data set.

3. Results

3.1. Efficiency of fungal genomic DNA extraction of different Physalis varieties

Eight µL of genomic DNA extraction of the fungal communities present on Physalis fruits was 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 was amplified by classic PCR using a protocol improved by El Sheikha *et al.* [24]. 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 (*figure 1*). Successful amplification permits one to continue to analyze these amplicons by the DGGE method.

3.3. DGGE pattern of fungal genomic DNA from Physalis varieties from different countries

On DGGE gel, the observed bands had sufficient intensities to analyze samples of fungal genomic DNA extracted from Physalis fruits from four different geographical areas (*figure 2*), so the total quantity of DNA deposited in the wells of DGGE gel was sufficient to consider that fungal genomic DNA

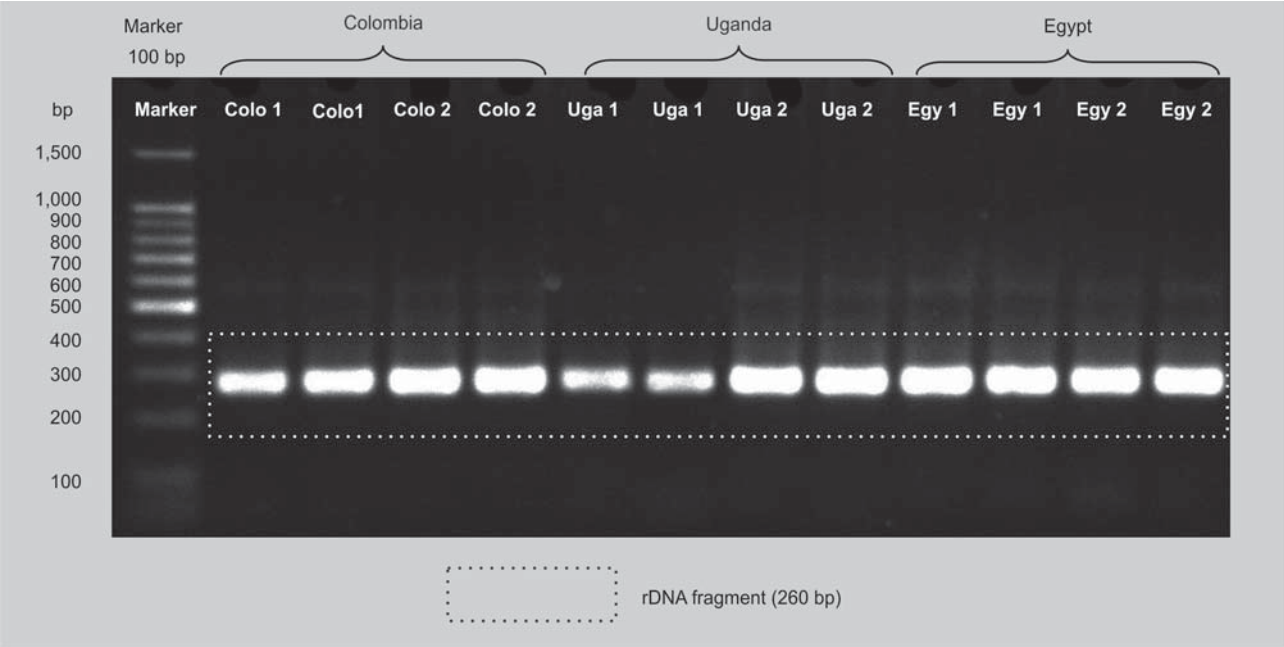

Figure 1.
Agarose gel for verification of PCR reaction of DNA extracted from different varieties of Physalis from three countries: Colombia, Uganda and Egypt (Colo: Colombia; Uga: Uganda; Egy: Egypt). 1, 2: two different districts. The duplicate of the sample represents another two individual fruits from the same district.

could be used as potential markers. The reference DNA of *Mucor racemosus* and *Trichoderma harzianum* indicates that DGGE was perfectly done. Each vertical lane represents a sample obtained from two individual fruits and each spot represents a species of fungi. For example, the lane Colo 1 represents the sample obtained from two individual fruits from the district 1 from Colombia and the duplicate of Colo 1 represents the sample obtained from another two individual fruits from the same location, but both of them processed in parallel. The duplicates of sample fingerprints were identical, which demonstrated that the samples were representative of the origin. Some spots appeared double or smeared because of the presence of single-strand DNA (ssDNA) [25].

The duplicates of PCR-DGGE patterns of Physalis fruits for each location were similar for each country and revealed the presence of six to eleven bands for each Physalis fruit (*figure 2*).

Factorial Correspondence Analysis (FCA) proved to be a useful statistical tool to compare the similarity of the fungal communities of Physalis fruit samples from the three different countries in the harvest season. For the fruit samples, the two variances described 83% among the fungal communities (*figure 3*). We can observe clearly three different groups for the three different countries.

4. Discussion

For economic reasons and for profitability, several batches of fruits of various origin or various cultivars could be mixed. It is thus very difficult to check their exact geographical origin. The most popular analytical methods which allow us to ensure the determination of origin are the barcode, spectroscopy, stable isotope, etc. [1]. Stable isotopes are the only ones which are referenced as a European regulation for wine origin determination [26]. The advantages of stable isotopes are that they are non-radioactive, do not decay, do not emit radiation and occur naturally in the environment. Stable isotopes are completely safe to handle and therefore there are few safety considerations to be addressed. But one of the main disadvantages of using stable isotopes is the capital cost of isotope ratio mass spectrometers required; these can cost in excess of

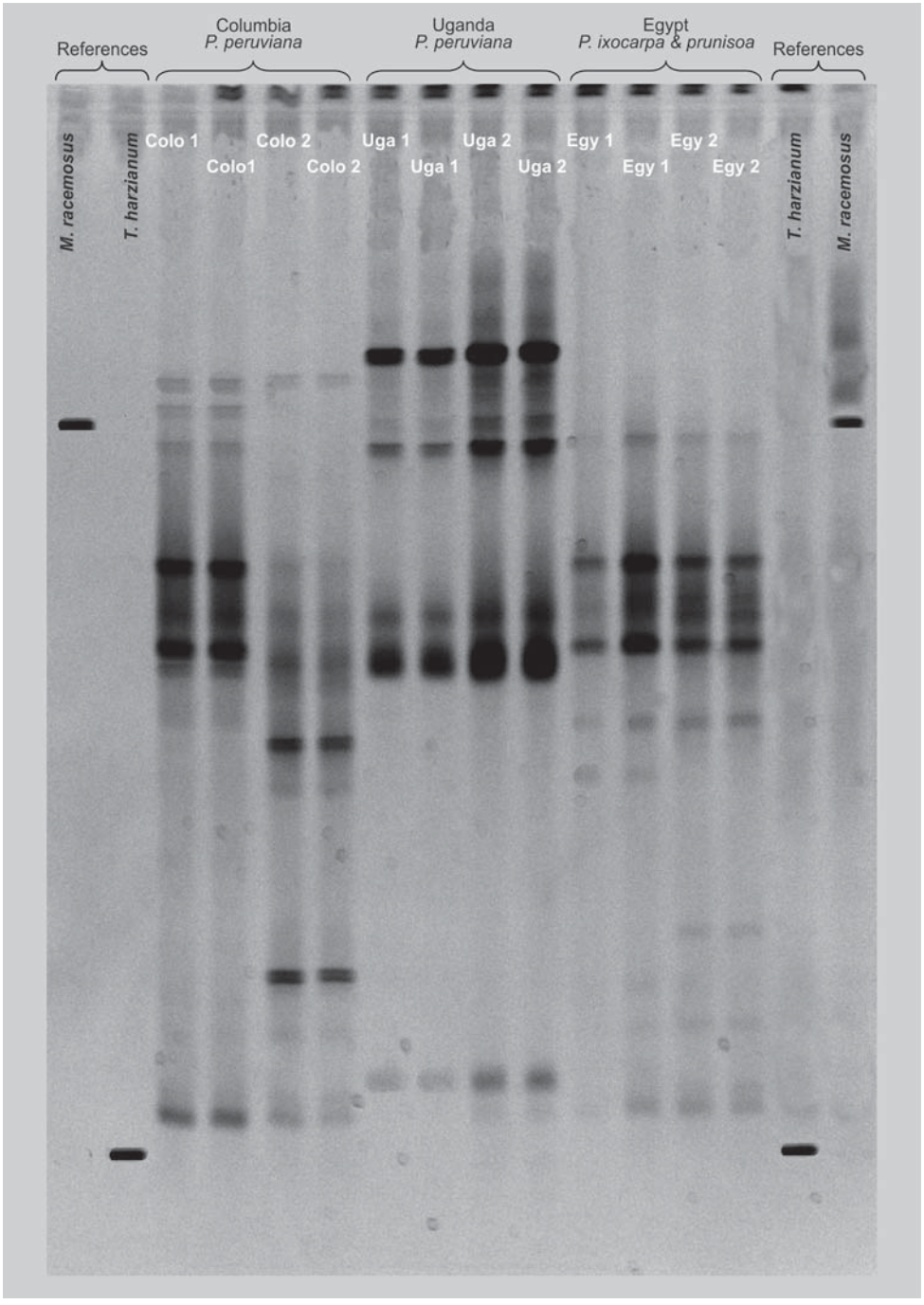

Figure 2.
PCR-DGGE 28S rDNA banding profiles of different varieties of Physalis from three countries: Colombia, Uganda and Egypt (Colo: Colombia; Uga: Uganda; Egy: Egypt). 1, 2: two different districts. The duplicate of the sample represents another two individual fruits from the same district.

100,000 USD and analysis costs for the sample more than 100 USD. Additionally, the equipment requires a temperature-controlled environment and highly skilled personnel to maintain and service the sensitive instrumentation[3]. In our study, we proved

[3] Anon., Manual for the use of stable isotopes in entomology, Int. At. Energ. Agency (IAEA), Austria, 2009, 79 p., available at http://www-naweb.iaea.org/nafa/ipc/public/IAEA_SI_Hi-Res_final.pdf (accessed on June 2009).

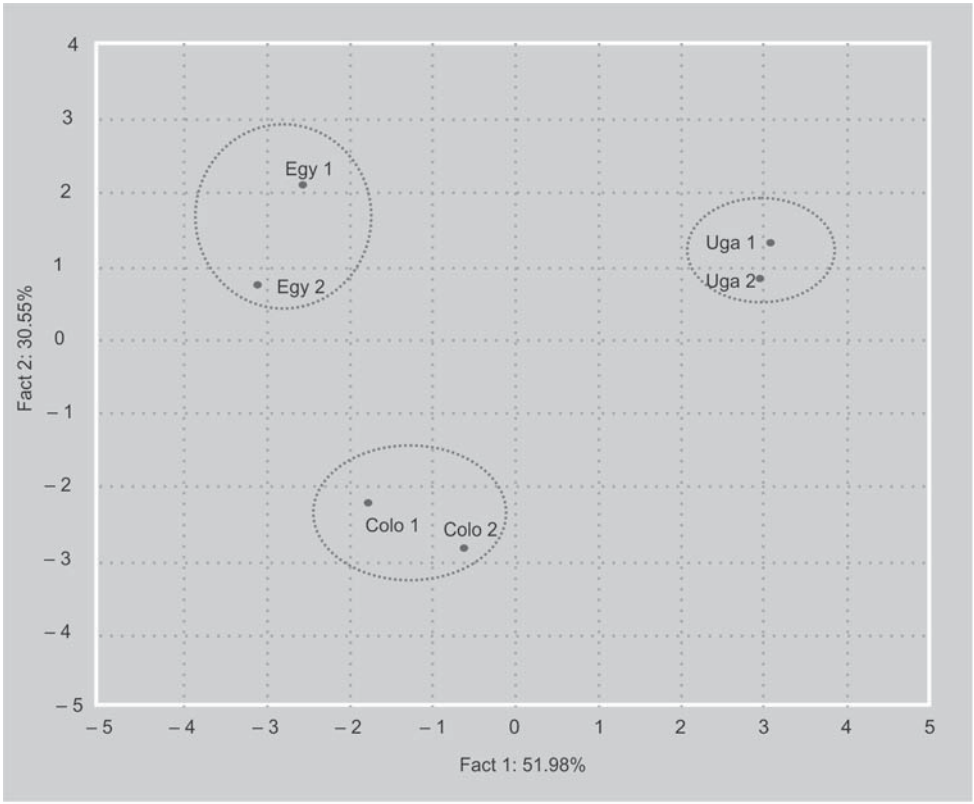

Figure 3.
Factorial variance analysis of 28S rDNA banding profiles of different varieties of Physalis from three countries: Colombia, Egypt and Uganda (Colo: Colombia; Uga: Uganda; Egy: Egypt). 1, 2: two different districts. The duplicate of the sample represents another two individual fruits from the same district.

that the PCR-DGGE pattern of the genomic DNA of fungal communities from Physalis fruits was strongly linked to the microbial environment of the fruits, which can be used as a new, cheap (10 USD) and fast traceability technique.

The PCR-DGGE approach has also been profitably applied to studying fungal communities that very often play an important role in food fermentation. Ben Omar and Ampe [27] and Ampe *et al.* [28] looked for fungi in pozol and cassava samples, respectively, during fermentation. The authors performed analyses by using PCR-DGGE amplicons of 18S rDNA. This 18S rDNA-PCR-DGGE was also used by Röling *et al.* [29] to identify fungi in vanilla beans during curing. Flórez and Mayo [30] used 26S rDNA to detect the fungal species in Cabrales cheese during manufacture and ripening. Three papers were published by our team that described the linkage between bacterial and yeast communities and the geographical origin of fruits [13, 14, 31], and one paper was published by our team that described the linkage between fungal communities and the geographical origin of fruits [24].

The acquired band patterns for the fungal communities of different species of Physalis fruits and different harvesting locations were compared and analyzed statistically to determine the Physalis geographical origin. The analysis of Physalis samples from different locations 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 districts. The type of processing system applied could also affect the microbial communities of Physalis. In the gel, some common bands appeared in all of the samples independently of the location or the variety. These bands could be common fungi for all of the Physalis.

In fact, when comparing the different locations of fruits sampled with the statistical analysis of the DGGE pattern throughout the study, we noted that we obtained a complete statistical correspondence between the geographical areas and the fungal communities. We can conclude that there were enough environmental differences between the districts where the Physalis were harvested to obtain a major effect on the fungal ecology, whereupon we could create a statistical link between the fungal populations and the geographical area.

5. Conclusions

The analysis of Physalis fungal communities by PCR-DGGE could be applied to differentiate geographical locations. We showed that the biological markers for the specific locations were sufficiently statistically different 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 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 barcode for each country. Furthermore, the diversity of Physalis varieties and the ecological study of fungi in many other products in which they occur provide another area for future study.

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Determinación del origen geográfico de los frutos, gracias al uso de huellas genéticas del ADNr 28S de comunidades de moho, obtenidas mediante PCR-DGGE: aplicación en los frutos de Physalis procedentes de Egipto, de Uganda y de Colombia.

Resumen — Introducción. El consumo de frutos de Physalis es totalmente reciente y los consumidores apenas conocen estos productos, a pesar de que esta planta se emplee como planta ornamental en Europa, desde hace al menos 200 años. La trazabilidad se está volviendo cada vez más importante en la industria agroalimentaria. Sin embargo en la actualidad, la trazabilidad sólo es documental. En caso de duda o de fraude, ningún análisis regularizado puede determinar el origen geográfico de los frutos. **Material y métodos**. Con el fin de relacionar las comunidades de moho de los frutos y sus orígenes geográficos, se analizaron los ADNr 28S mediante PCR-DGGE (reacción en cadena de la polimerasa- electroforesis en gel con gradiente de desnaturalización) para estudiar la variación de las comunidades de moho, en tres especies de frutos de Physalis (*Physalis ixocarpa* Brot., *Physalis pruinosa* L. y *Physalis peruviana* L.), procedentes de Egipto, de Uganda y de Colombia. **Resultados**. Las imprentas de los ADN 28S ribosómicos obtenidos por DGGE e interpretados mediante análisis multivariable podrían permitir distinguir el origen geográfico de los frutos por su comunidad de moho. **Conclusión**. Proponemos este método de PCR-DGGE como una nueva herramienta de trazabilidad, apta para proporcionar un código de barras único para cada país, a los frutos en general, y a las Physalis en concreto.

Egipto / Uganda / Colombia / Physalis sp. / huellas de ADN / origen / flora microbiana