Characterization of the genetic diversity in orange, and comparison of polymorphism in randomly-amplified microsatellites (RAMs), using polyacrylamide and agarose electrophoresis

Caracterización de la diversidad genética en naranja y comparación del polimorfismo de microsatélites amplificados al azar (RAMs) usando electroforesis de poliacrilamida y azarosa

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Abstract

We compared the efficiency of three methods of agarose and polyacrylamide electrophoresis (using the small tank of the DNA Sequencing System and the large OWL Sequi-Gen Sequencing Cell), for the detection of polymorphism in 21 accessions of orange (*Citrus sinensis*), using the primer CGA. The polyacrylamide gel gave better resolution of the PCR-amplified RAM products. This method allowed better detection of polymorphic DNA bands, facilitating the identification of genetic variability. The agarose electrophoresis may be more convenient in other applications, due to its low cost and easy implementation. The study of genetic diversity in orange using RAMs separated 51 accessions into seven groups with 0.75 similarity, and 0.25 heterozygosity, revealing low genetic polymorphism. The RAMs technique grouped the accessions into “Common or White”, “Navel” and “Pigmented or “Sanguine”.

Key words: *Citrus sinensis*, electrophoresis, agarose, polyacrylamide, RAMs.

Resumen

Se compararon las eficiencias de tres métodos de electroforesis en agarosa y poliacrilamida, usando la cámara pequeña de DNA Sequencing System y cámara grande OWL Sequi-Gen Sequencing Cell, en la detección del polimorfismo en 21 accesiones de naranja (*Citrus sinensis*) con empleo del cebador CGA. El gel de poliacrilamida dio mejor resolución de los productos amplificados vía PCR

producidos por RAMs. Este permitió una mejor detección de bandas de ADN polimórficas, lo que facilitó la identificación de la variabilidad genética. La electroforesis en agarosa puede ser más conveniente en otras aplicaciones, debido al bajo costo y fácil aplicación. El estudio de diversidad genética en naranja usando microsatélites RAMs diferenció 51 accesiones en siete grupos con 0.75 de similaridad y 0.25 de heterocigosidad, lo que revela bajo polimorfismo genético. La técnica RAMs permitió agrupar las accesiones en Comunes o Blancas, Navel y Pigmentadas o Sanguinas.

Palabra clave: *Citrus sinensis*, electroforesis, agarosa, poliacrilamida, RAMs.

Introduction

The genus *Citrus* (2n = 18), native to the southeast of Asia, and the Indo-Malayan archipelago (Avilán et al., 1989), contains the mandarin *Citrus reticulata*, lemon *C. medica*, and pomelo *C. maxima*, species which are the ancestors of today´s commercial varieties. Facultative apomixis predominates in *C. reticulata* and has been an important factor in the evolution of *Citrus*.

Citric fruits, grown in subtropical and tropical zones, are consumed each day by millions of people around the world. The orange, *Citrus sinensis* L. Osbeck, is the most representative and recognizable species of this group. The orange has its origin in South East Asia, and its hybrid nature appears to originate in a cross between the mandarin *Citrus reticulata* and the pomelo *Citrus grandis* L. Osbeck (Davies & Albrigo, 1992; Nicolosi et al., 2000).

The characterization of germplasm banks, genetic variation and the improvement of oranges and other *Citrus* species, has not been successfully carried out due to traits related to the reproductive biology of these species, for example high interspecific fertility, apomictic reproduction, polyembryony, a large juvenile phase, and a scarcity of polymorphic DNA markers (Bretó et al., 2001; Corazza-Nunes et al., 2002).

The genetic improvement of the citruses using conventional methods is limited due to their genetic and reproductive characteristics. The citruses have a complex reproductive system, with many cases of sterility, and of inter- and auto-incompatibility, apomixis, elevated heterozygosity and the majority of the species present a prolonged juvenile period. Additionally, the modes of inheritance of the majority of agronomic traits of interest are not known (Grosser y Gmitter, 1990). Biotechnology techniques, such as tissue and cell culture, and molecular biology have helped plant breeders to overcome such problems. Additionally, hybridization through protoplast fusion and genetic transformation have contributed significantly

to remove these limitations (Mendes-da-Glória et al., 2000). Molecular techniques have permitted the development of DMA markers associated with traits of agronomic interest, in addition to the development of a genetic linkage map for the citruses, which have proved useful in undertaking early selection of progeny with desired traits in traditional breeding programs (Mendesda- Glória et al., 2000). The molecular markers such as Restriction Fragment Length Polymorphisms (RFLP), Random Amplified DNA (RAPD), microsatellites and isozymes have been developed in order to study the genetic diversity and domestication, and establish molecular maps, characterize varieties, and assist in breeding programs. Microsatellite markers are simple to use, being codominant, and having high polymorphism, and may be employed to reveal the genetic diversity of Citrus at interspecific, intraspecific and intrapopulation levels. Genetic characterization using microsatellites has been published in *C. limon* (Golein et al., 2005), *C. sinensis* (Ahmad et al., 2003; Novelli et al., 2006), *C. limonia x P. trifoliata* (Kijas et al., 1997) and *C. reticulata* (Koehler-Santos et al., 2003).

The molecular markers known as RAMs are useful for measuring the genetic diversity in plants and animals with the power to discriminate between families and species (Muñoz et al., 2008). They sample the same variation base in individuals, and allow the selection of concrete regions within the DNA molecule for specific studies. The number of detectable polymorphisms is theoretically unlimited. Finally, they allow the analysis of both expressed and non-expressed information (Mahuku et al., 2002). This method is feasible in small labs, in terms of equipment and costs, and does not require previous knowledge of the DNA sequences nor the use of radioactive isotopes (Hantula et al., 1996). The markers obtained by RAM may be used equally for population level studies (Hantula, et al., 1996).

Different methods have been used for the separation and detection of amplified DNA fragments. Electrophoresis with polyacrylamide gels with radioactivity and silver staining was the first method used for PCR fragment analysis (Cipriani et al., 1999). Electrophoresis in agarose gels is an alternative method to polyacrylamide that is cheaper and more easily performed (Morgante et al., 2001). Recently, new methods have been developed based on the automatic sequencer with capillary electrophoresis (Aranzana et al., 2003; Ahmad et al., 2004). However, a comparison of efficiency of these methods for separation and analysis of DNA fragments, and their implications in the evaluation of diversity and genetic relationships has not been conducted.

The aim of this work was to apply the RAM technique to study the genetic diversity of orange (*Citrus sinensis*), and compare the polymorphism generated by different electrophoresis methods in polyacrylamide and agarose.

Materials and methods

The molecular characterization of the genetic diversity of the orange *C. sinensis* surveyed 51 of the 54 accessions of the germplasm bank of the Colombian Corporation for Agricultural Investigation (Corpoica), Palmira. For the comparison between the three electrophoresis methods 21 accessions were evaluated (Box 1). The molecular characterization was undertaken in the National University of Colombia, Palmira campus. DNA was extracted according to the protocol of La Dellaporta *et a*l (1983).

Cuadro 1. Accesiones de naranja *Citrus sinensis* L. del Banco de Germoplasma de Corpoica utilizadas para la caracterización molecular con microsatélites RAMs.

Nombre	Sitio de recolección	Tipo de material
Jaffa*	U. California-Riverside	Introducido
Valencia 1-D-E*	U. California-Riverside	Introducido
Valencia Cutter*	Indio-California	Introducido
Galicia*	Finca Galicia-Palmira	Variedad agricultor
Indian River*	U. California-Riverside	Introducido
Joppa*	U. California-Riverside	Introducido
Salerma*	El Bolo-Palmira	Variedad agricultor
Cuban Queen*	U. California-Riverside	Introducido
Valencia Olinda*	Indio-California	Introducido
Sanguinella	Cartagena-Colombia	Introducido
Salustiana*	U. California-Riverside	Introducido
Shamoutti	California	Introducido
Frost Washington	Indio-California	Introducido
Nativa de Chocó*	Juribidó-Chocó	Variedad Agricultor
Old Vini*	U. California-Riverside	Introducido
Lue Gim Gong*	U. California-Riverside	Introducido
Morocco Blood*	California	Introducido
Star Calyx*	C.I.T. Ospina-Antioquia	Introducido
Valencia Frost*	Indio-California	Introducido
Weldon*	C.I.T. Ospina- Antioquia	Introducido
Du Roi*	C.I.T. Ospina- Antioquia	Introducido
Rico*	U. Pto. Rico-Mayaquez	Introducido
Valencia Campbell*	Indio-California	Introducido
Australian Navel*	U. California-Riverside	Introducido
Valencia Variegado	U. Pto. Rico-Mayaquez	Introducido
Wialua	C.I.T. Ospina-Antioquia	Introducido
Homosassa	C.I.T. Ospina-Antioquia	Introducido
Ruby Blood	U. California-Riverside	Introducido
Palmira Ruby	Granja Palmira-Palmira	Variedad Agricultor
Bessie	C.I.T. Ospina-Antioquia	Introducido
Lerma	Finca Lerma-El Bolo-Palmira	Variedad Agricultor
Nave Late	IVIA-Valencia-España	Introducido
Lane Late	U. California-Riverside	Introducido
Pera del Río	Río de Janeiro-Brasil	Introducido
Valle Washington	Granja Palmira-Palmira	Introducido
Valencia Olinda2	IVIA-Valencia-España	Introducido
Lima dulce	San Joaquín-Palmira	Variedad Agricultor
St. Michael	C.I.T. Ospina-Antioquia	Introducido
García Valencia	Finca García-Chodular-Palmira	Variedad Agricultor
Golden Nugget Navel	U. California-Riverside	Introducido
Mediterranean	U. California-Riverside	Introducido
New Hall	IVIA-Valencia-España	Introducido
Enterprise	Desconocido	Introducido
Navelina	IVIA-Valencia-España	Introducido
Cafetera N°1	Zona Cafetera	Variedad Agricultor
Moro Blood	U. California-Riverside	Introducido
Margarita	Mompox	Variedad Agricultor
Valencia Costa Rica	Costa Rica	Introducido
Atwood Navel	U. California-Riverside	Introducido
ICA-Hamlin Nucelar 7	Desconocido	Variedad Mejorada
Pineapple	U. California-Riverside	Introducido

* Accesiones utilizadas para la comparación entre los tres métodos de electroforesis evaluados.

Total DNA was visualized on 0.8% agarose gels, stained with Ethidium Bromide, in a Maxicell Primo EC-340 Electrophoresis Gel System. DNA concentration was determined through construction of a dilution curve with DNA from the Lambda

bacteriophage from an initial concentration of 20 ng/µl to final concentrations of 20, 40, 60, 80 y 100 ng/µl. Quantified DNA was diluted in HPLC water to a total volume of 100 µl at 10 ng/µl and was stored at -20 °C.

For RAM analysis seven polymorphic primers were used (Technologies Inc) (Bonilla et al., 2004; Mahuku et al., 2002; álvarez et al., 2003) (Box 2). In order to standardize the conditions of each of the primers, a mix of reagents was prepared in a sterile microcentrifuge tube (1.5 ml) to a final volume of 25 µl (Box 3).

Cuadro 2. Cebadores utilizados en la técnica de microsatélites RAMs.

Cebador	Secuencia (5'a 3')*
CCA	$DDB(CCA)_5$
CGA	$DHB(CGA)_5$
GT	$VHV(GT)_7G$
AG	$HBH(AG)_7A$
CT	$DYD(CT)_7C$
TG	$HVH(TG)_7T$
CA	$DBDA(CA)_7$

*Las siguientes designaciones se usan para los sitios degenerados: H (A ó T ó C); B (G ó T ó C); V (G ó A ó C) y D (G ó A ó T).

Cuadro 3. Mezcla para la amplificación de ADN de los 51 materiales de naranja *Citrus sinensis* (L) incluidos en el estudio.

Coctel	Volumen (µl)
Buffer Taq (1x)	2.5
dNTPs (0.2mM)	4.0
Cebador (2mM)	2.0
$MgCl_2$ (1.5mM)	2.5
ADN (10ng)	5.0
Taq Polimerasa ()	0.2
H_2O	8.8
Total	25

PCR amplification was carried out in a PTC 100 Programmable Thermal Controller (MJ. Research, Inc). Initial denaturation was at 95 °C for 5 min; denaturation at 95 °C for 30 s, annealing at a temperature of 50 °C (primer AG & CA), 55 °C (primer CCA-TG-CT) and 58 °C (primer GT-CGA) for 45 s, followed by an extension period of 72 °C for 2 min, 37 cycles from denaturation to extension, and finally an extension period at 72 °C for 7 min.

For the comparison of electrophoresis methods 21 of the 51 accessions of orange used (Box 1) were evaluated with the primer CGA. The PCR products were separated in 1.2% agarose gels at 90volts for 2h and 30min in a Maxicell Primo EC-340 Electroforesis Gel System. Electrophoresis was also conducted in 7% polyacrylamide gels at 150 v, in a small tank of the DNA Sequencing System FB-SEQ-3545 de Fisher Biotech. The PCR products were also separated in a sequencing chamber (OWL-Sequi-Gen Sequencing Cell) in 4% polyacrylamide gels (29:1 acrilamide-bisacrilamide), under denaturing conditions (urea 5M). To each PCR product 10 µl of denaturing buffer was added (95% Formamide, 0.025% Bromophenol blue, 0.025% Xylene cyanol). This mix was denatured at 95 °C for 5 min, and 5 µl of the mix was run on a gel at de la 120 Watts, 1600 V, for 2 h. The staining was performed using silver salts.
A binary matrix of absence (0) and presence (1) of bands was generated. Genetic similarity between individuals was calculated using the similarity coefficient of Ne & Li (1979) also known as DICE (1945) (Sneath & Sokal, 1973). Cluster analysis was performed using UPGMA, and a dendrogram was generated using the statistical packet NTSYS (Numerical Taxonomy System for Personal Computer, version 2.02 PC). In order to evaluate the genetic diversity, the unbiased heterozygosity and the proportion of polymorphic loci was estimated using the statistical packet TFPGA (Tools For Population Genetic Analyses, version 1.3, 1997). The value of unbiased F was determined with a confidence interval of 95%.

Results and discussion

Comparison of electrophoresis methods

Electrophoresis in 1.2% agarose gels, and in polyacrylamide gels were effective in separating the amplified fragments. The first method revealed a lower level of polymorphism compared with the other two methods (Box 4). The genetic differentiation coefficient (Fst) was 0.20, with a standard deviation (SD) of 0.04, confirming the existence of an intermediary level of genetic differentiation.

Agarose gels allowed a rapid electrophoresis, but with a limited resolution, as the bands tended to be diffuse and expanded. This is a consequence of the size of the pores, which cannot be controlled (Westermeier, 1997). However, this method has the advantage of a wide range of separation for fragments with high molecular weight (100 bp to 50 Kbp) (Fernández-Tresguerres, 2003). This type of electrophoresis is preferred for the resolution of DNA fragments resulting from the PCR amplification using RAPD primers (Williams et al., 1990). However, when various fragments of DNA of similar sizes are generated, as occurs frequently in the DNA ‘fingerprinting’, agarose does not have sufficient resolution (Caetano-Anollés, 1991).

The number of bands obtained using the primer CGA in polyacrylamide gels was 31. The percentage of polymorphic loci was 16.13%, with a heterozygosity of 0.09 (Box 4). In comparison with agarose gels, a greater number of bands was obtained as the polyacrylamide allows a greater resolution and separation of the PCR products, and, thus greater values of heterozygosity. Although the value of Fst was gr4eater in agarose (0.23), it reveals intermediate genetic diversity.
When the PCR fragments were separated in the large OWL tank, a greater number of bands were obtained (53), with 74% polymorphism, an average expected heterozygosity of 0.33 and an Fst of 0.09 (Box 4). Polyacrylamide is a tool frequently employed in gel electrophoresis, being chemically inert with uniform properties, and rapidly prepared and reproducible. It forms relatively non-ionic, transparent gels with mechanical stability insoluble in water, which allow a good visualization of bands over a prolonged period. Additionally, it has the advantage that, on varying the concentration of polymers the size of the pore can be modified (Campbell, 1995). Differences between the three methods are important, as methods with greater resolving power allow the detection of greater differences.
It is possible to use the polyacrylamide electrophoresis method to identify allelic variation between cultivars at a finer scale. However, the detection is more expensive in terms of equipment and analysis, uses more toxic reagents and requires more time and experienced personnel.

Generally, in a polyacrylamide gel, in a small chamber, the primary, dark bands often coalesce with the secondary bands, giving faint bands, particularly those of lower molecular weight. In contrast, polyacrylamide gel bands in the large chamber were sharper, of a greater size and uniform width, and the primary and secondary bands could be distinguished relatively easily. The primary bands presented a good resolution in agarose, but the secondary bands were not clear. The fluorescent background of ethidium bromide significantly reduced the clarity of the DNA fragments, especially faint of secondary bands.

The use of polyacrylamide in the large tank permitted a better identification of polymorphic bands than the other methods used, with single bands being resolved into two bands. Additionally, this process does not use toxic compounds such as ethidium bromide, which is mutagenic. The electrophoresis was more rapid and with greater tolerance to elevated run temperatures, thus allowing the use of higher voltages (100 Watt). The polyacrylamide gel grows during staining, making it easier to distinguish the bands and calculate polymorphism. Additionally, it may be stored for longer periods without distortion of the run front. An important advantage of the polyacrylamide gels is that they are chemically inert, transparent and stable over a wide range of pH, temperature and ionic force.

Agarose and polyacrylamide gels may be employed in a wide range of sizes, thicknesses, and porosities that allow electrophoresis to be carried out under different configurations. Selections depend principally on the size of the fragments to be separated.

Vertical polyacrylamide gels are the method most effective at separating small fragments of DNA (5 to 500 bp). Resolution power was so high that it was possible to separate DNA fragments differing by 1bp, such as occurs in DNA sequencing. The main limiting features of the polyacrylamide gels are the neurotoxicity of acrylamide and the difficulty in preparation and handling. This study showed that the polyacrylamide gel gives a better resolution for products amplified using RAMs. This method enabled rapid electrophoresis of DNA fragments and facilitated better detection of polymorphic DNA bands, thus facilitating the identification of genetic variation existing in natural populations. Thus, the analysis of PCR products in agarose electrophoresis should be matched with polyacrylamide electrophoresis due to its lower precision levels. The agarose method may be used in other studies due to its low cost and ease of use.

In agreement with the results, the RAM technique may be a useful tool to characterize the genetic diversity of different accessions of the genus *Citrus*. Additionally, it is a technique that does not require previous information, it uses a primer, it is reproducible for the size of the primer, and can differentiate between species, and intra-specific variation, detecting relationships between biological groupings and groups formed by the technique. Finally, it has a low cost, and is easily implemented (Muñoz et al., 2008).

Study of genetic diversity with electrophoresis in the sequencing tank.

The seven RAM primers generated 176 bands, ranging from 32 (CCA) to 55 (TG). The number of polymorphic loci varied between 18 and 34 for the primers CT and CGA, respectively (Box 5). The analysis using the de Nei-Li coefficient, at a level of similarity of 0.75, differentiated the group of accessions into seven groups (Figure 1). **Group 1** was comprised of the common oranges ‘Jaffa’, ‘Indian River’, ‘Cuban Queen’, ‘Salustiana’, with spherical, flattened or ellipsoidal fruits of medium or large size, without a navel, and with coloration from yellow-orange to intense orange. Some varieties contain numerous seeds. Group 2 contained the majority of the ´navel´ oranges, with pathenocarpic fruits that lack seeds, a trait that contributes to the accessions genetic similarity. The principal use for navel fruits is fresh consumption, with a small quantity being used in industry, as the juice content is high, and the presence of limonene gives a bitter taste. Within group 2 were also found some accessions of ´common´ oranges: ‘Pera del Río’, ‘Lima Dulce’ and ‘Enterprise’, amongst others, showing the existence of relationships amongst the

orange varieties evaluated. In groups 3 to 6, a combination of different types of ´white´, ´Valencia´ or ´common´ oranges were found, showing the continual exchange of material between cultivators. The pigmented oranges (blood orange) were differentiated from the rest of the evaluated accessions (**group 7**) by the presence of anthocyanines in the pulp, and sometimes in the epidermis. The blood oranges show more benefits than the common oranges, containing more betacarotene, and tasting slightly of cherries or raspberries.

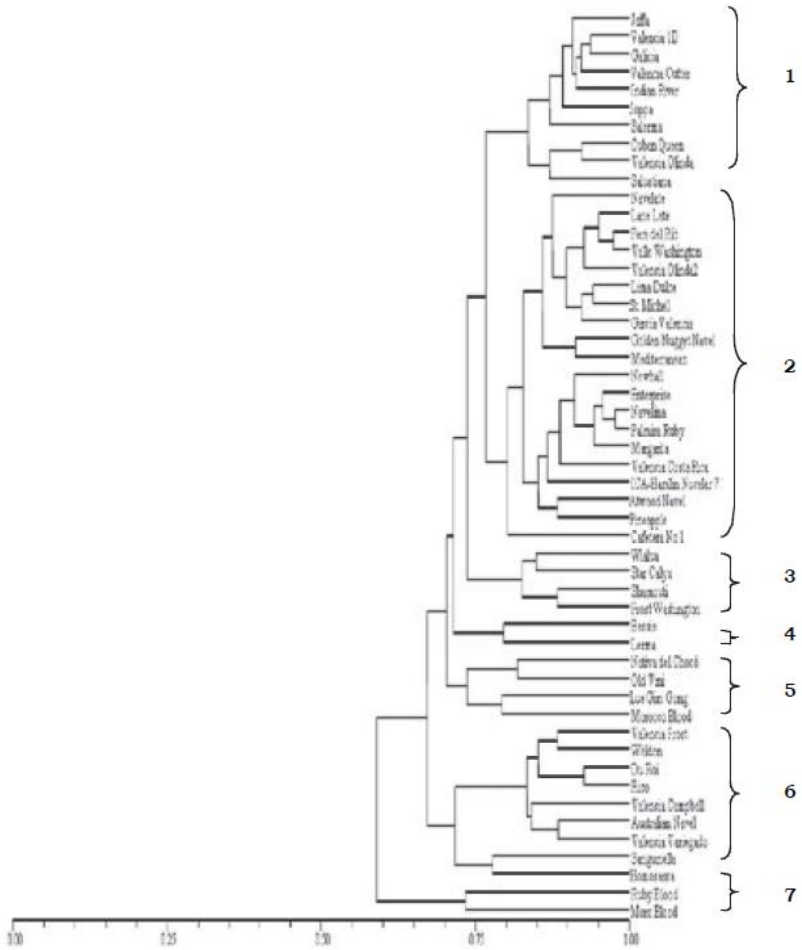

Figura 1. Dendrograma de la estructura genética de 51 accesiones de *Citrus sinensis* basado en el Coeficiente de Nei-Li, y calculado de los datos combinados de los siete cebadores microsatélites RAMs.

The expected heterozygosity (He) for the total population was 0.25, revealing a low genetic variability. The primer TG gave the greatest support to the genetic variation (Box 6). Novelli et al (2006) developed and characterized polymorphic microsatellite markers fo the sweet orange, *C. sinensis* L, and found values of He between the range 0.49 and 0.57 with a mean of 0.50, observed heterozygosity

(Ho) between 0.88 and 1.00 and mean of 0.99, being high values in comparison with those seen in the present study.

Cuadro 5. Cebadores RAMs utilizados para la evaluación de la diversidad genética en *Citrus sinensis*.

Cebador	No. Loci polimórficos	% Loci polimórficos (95%)
CA	23	73.9
CGA	34	73.5
CT	18	55.6
CCA	28	25.0
AG	26	58.2
TG	27	96.2
GT	20	30.0
Población total		64.8

Cuadro 6. Heterocigosidad promedio estimada (He) y porcentaje de loci polimórficos para los siete cebadores RAMs evaluados en 51 accesiones de naranja *Citrus sinensis*.

Cebador	He estimada	% Loci polimórficos (95%)
CA	0.28	73.9
CGA	0.27	73.5
CT	0.21	55.6
CCA	0.09	25.0
AG	0.31	58.2
TG	0.34	96.2
GT	0.15	30.0
Población total	0.25	64.8

Tapia et al. (2005) evaluated 63 mandarin cultivars (*Citrus* spp.) using morphological markers and AFLP (Amplified Fragment Length Polymorphism) for 20 quantitative and 10 qualitative traits of the leaves, flowers and fruits. The best AFLP primer combinations were Mse + CAG with Eco + ACA, and Mse + CAA with Eco + AGG, giving a total of 109 bands with 86% polymorphism. Both the morphological and the molecular markers showed a high degree of variation amongst the individuals analyzed, indicating that this is an important source of variability for genetic improvement. Although the comparison of the morphological and molecular results using the Mantel test did not show a significant correlation (r = 0.31), both techniques seem complementary for the characterization of mandarins.

Golein et al (2005) reported the isolation and characterization of seven polymorphic microsatellite loci in *Citrus*. These markers produced between 4 and 9 alleles per locus (with an average of 6.14) in 32 cultivars of *C. limon* evaluated. The values of mean observed heterozygosity were between 0.43 and 0.72. The levels of polymorphism found in the study suggested that these microsatellite loci could be an important tool for genetic studies in *Citrus*.

In the present study, the coefficient of genetic differentiation (Fst) obtained from evaluating the 51 orange accessions with seven RAM microsatellites was 0.07, with a standard deviation of 0.01 (Box 7). According to Wright (1978) values between 0 and 0.05 show little genetic differentiation, between 0.05 and 0.15 moderate, and greater than 0.25 large differentiation. Taking this into consideration, the Fst value found in this study shows an intermediate level of genetic differentiation.

Cuadro 7. Diferenciación poblacional y estadístico Fst para 51 accesiones de naranja con los siete cebadores RAMs evaluados.

Cebador	Fst	SD
CA	0.11	0.03
CGA	0.09	0.02
CT	0.04	0.04
CCA	0.11	0.02
AG	0.01	0.01
TG	0.05	0.02
GT	0.09	0.02
Población total	0.07	0.01

The low genetic differentiation found in *C. sinensis*, similar to that in the genera *Fortunella* and Poncirus, is due to the fact that each of the numerous cultivated forms has been propagated from a single progenitor via the asexual processes of grafting, cuttings, and nuclear embryony, thus assuring the maintenance of the same genetic constitution in the progeny that form the horticultural variety (cultivar) or clone (Webber, 1943).

The selection of cultivated forms has pursued an improvement in traits useful to man, such as those related with fruit quality (taste, juice, sugar and acid content, pulp texture, degree of aspermia, epidermis color, size and shape), and the behavior and characteristics of the tree (vigor, productivity, spineless form). Consequently, there exists a tendency towards uniformity in the domestication process of each species, and for this reason, amongst certain varieties there are certain similarities in what are referred to as the most visible traits, with the result that it is difficult to distinguish them (Webber, 1943).

It is known that the sweet oranges have a narrow genetic base, and that variability may be produced by diverse factors such as hybridization, mutation, and the type of reproduction. The low intraspecific diversity contrasts with the high variability in important agronomic traits such as maturation period, size and fruit color (Webber, 1943).

Conclusions

- The electrophoresis using polyacrylamide gels in the OWL Sequi- Gen Sequencing Cell gave the best PCR product resolution, facilitating the detection of polymorphisms amongst the 21 accessions of the orange *C. sinensis* evaluated.
- The Nei-Li similarity values and the heterozygosity revealed a low genetic polymorphism, which may be due to the vegetative propagation of the species that will tend to perpetuate similar genotypes across various generations.
- The technique of RAMs allowed the grouping of different accessions of orange according to reported varieties (‘common´ or ‘white´, ‘Navel’ and ‘Pigmented’ or ‘Blood´ oranges), indicating that this tool is useful for evaluating genetic diversity in evaluated oranges, and may be useful for other *Citrus* species.

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