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GENOTYPIC IDENTIFICATION OF SWEET ORANGE TREE ACTIONS THROUGH RANDOMLY AMPLIFIED MICROSATELLITES (RAMs)

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Abstract: Citrus fruits are produced in subtropical and tropical zones, and their fruits are consumed by millions of people around the world. In Mexico, sweet orange is the most important citrus species cultivated, mainly because of the area planted (340 thousand ha^{-1}) and production (average yields of 13.95 ton ha^{-1}), with a value of more than 10.18 billion pesos. The objective of this work was to identify genetically by Random Amplified Microsatellites (RAMs), the accessions of sweet orange (*Citrus sinensis* L.) stored in the INIFAP Citrus Germplasm Bank at the General Terán Experimental Field. Twenty-eight accessions were collected (two replicates per accession established under greenhouse conditions). Genetic identification was carried out with the Random Amplified Microsatellites (RAMs) technique, using the CA primer, which was selected because it can generate up to 26 polymorphic fragments. The identity of the two replicates was confirmed in 25 accessions; however, differences were found between the two replicates of the Moro, Campbell O.L. and Rio Grande Navel Nuc varieties. These results allow inferring the possibility that the differences recorded between the replicates of these three accessions may be due to the mutation phenomenon, a characteristic that can be used in breeding programs to generate new varieties of commercial interest.
Keywords: Germplasm bank, Genetic variability, Mutation.

INTRODUCTION

Citriculture represents a very important economic segment in Mexican agriculture, since it generates an economic flow of more than US$375 million (González *et al*., 2020). In recent years, the area cultivated with citrus in the country has increased by 8%, registering 620,000 ha in 2021 compared to 569,914 ha in 2010. The most important species in Mexico is the sweet orange, mainly because of the area planted (340,000 ha^{-1}) and production (average yields of 13.95 ton ha^{-1}), with a value of more than 10.18 billion pesos (Rivera *et al*., 2020). In plant germplasm banks, the materials housed need to be characterized, and their introduction and existence as part of the collection must be documented. When there is interest in starting a genetic improvement program with outstanding materials, the need arises to generate information on the genetic value of the germplasm banks and working collections, which is why genetic identification is required to identify duplicates, detect genes of interest and then associate them with their agronomic characteristics and generate information that represents genetic diversity and maximizes the resources to conserve and use them (Martínez, 2013). This information is vital to avoid duplication and confusion in the pool, as well as to identify promising introductions for selection processes, genetic improvement or agroindustrial processes (Morillo *et al*., 2010), 2010) The characterization of germplasm banks and breeding of different *Citrus* species has not been as successful as that obtained in breeding programs for annual crops, mainly due to characteristics related to the reproductive biology of these species, e.g., high interspecific fertility, apomictic reproduction, polyembryony, numerous cases of sterility, inter- and self-compatibility, high heterozygosity, a prolonged juvenile phase, and the scarcity of polymorphic DNA markers (Ganopoulos *et al*., 2015). Simple Sequence Repeats (SSR), are considered ideal markers for genotype discrimination; these in their Random Amplified Microsatellites (RAMs) modality are useful for measuring genetic diversity in plants and animals, with discriminatory power between families and species and also intraspecifically. This technique has been a valuable and accurate tool for the assessment of genetic diversity in citrus and related genera (Hynniewta, *et al*., 2014). The objective of the study was to identify genetically by Ran-

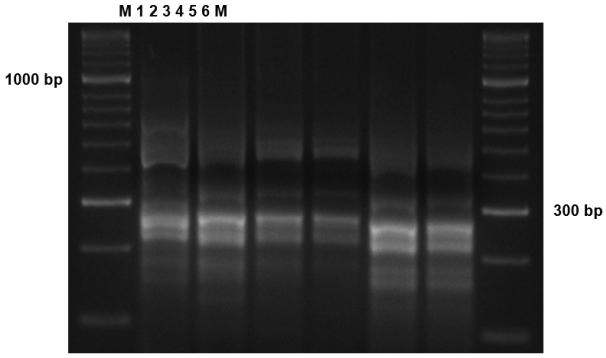
dom Amplified Microsatellites (RAMs), the accessions of sweet orange (*Citrus sinensis* L.) stored in the INIFAP Citrus Germplasm Bank at the General Terán Experimental Field.

MATERIALS AND METHODS

Healthy leaves were collected from 28 sweet orange accessions conserved in the Germplasm Bank of the Campo Experimental General Terán (Table 1). DNA extraction was performed using the CTAB methodology modified by Almeyda *et al*. (1981). Genetic identification was performed using the Random Amplified Microsatellites (RAMs) technique, initially 6 primers were evaluated and finally the CA primer (5'-DBDACACACACACACACACACACACACACACA-3') was selected based on the fact that all previously evaluated samples amplified and that it can generate up to 26 polymorphic fragments (Gallegos *et al*., 2017). PCR reactions were performed in a thermal cycler (BioRad Thermal Cicler), which, were prepared in a final volume of 25 µl containing: PCR Buffer (1X), $MgCl_2$ (4.0 mM), dNTP's (0.2 mM), CA Initiator (0.4 µM), Taq™ DNA-polymerase (1 U) and DNA (50 ng). The amplification program used in the thermal cycler was: one cycle at 95° C for 5 min, 35 cycles at 94 ° C for 30 sec, 50° C for 30 sec, 72° C for 2 min and a final cycle at 72° C for 10 min. Fragments amplified in the PCR reactions were fractionated on 2% agarose gels for one hour thirty minutes at 100 V. The gels were stained with GelRed® dye and analyzed in an ultraviolet light transilluminator.

RESULTS AND DISCUSSION

The RAMs methodology used allowed to consistently establish the identity as well as the difference between the two replicates of 28 sweet orange accessions collected for this work. The identity between the two replicates was corroborated for 25 of the 28 accessions evaluated (data not shown). However, in the case of the replicates of the accessions corresponding to the varieties Moro, Campbell O.L. and Rio Grande Navel Nuc, differences in the electrophoretic profile were detected (Figure 1-1 and 1-2; 1-3 and 1-4; 1-5 and 1-6).



Amplification of genome fragments of three sweet orange varieties by Random Amplified Microsatellites (RAMs) using the CA primer. Lanes M: Molecular Weight Marker HiperLadder™ 100bp; Lanes 1 and 2: Moro variety; Lanes 3 and 4: Campbell O.L. variety; Lanes 5 and 6: Rio Grande Navel Nuc variety.

The genetic difference found among the replicates in three of the 28 varieties of sweet orange evaluated, allows us to infer that these replicates correspond to different germplasm, and/or that it is feasible that the differences recorded may be due to external factors that modified the genetic structure of these varieties. This modification may be associated with the event known as mutation, which causes changes or modifications in the genomic sequence of living organisms and such changes occur due to errors in cell division or exposure to certain environmental conditions. This

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Accession No.	Variety or Common Name	Tree No.	Condition
1	Queen	1	Greenhouse
1	Queen	2	Greenhouse
2	Hamlin O.L.	1	Greenhouse
2	Hamlin O.L.	2	Greenhouse
3	Parson Brown	1	Greenhouse
3	Parson Brown	2	Greenhouse
4	Early L.R.	1	Greenhouse
4	Early L.R.	2	Greenhouse
5	Hamlin L.R.	1	Greenhouse
5	Hamlin L.R.	2	Greenhouse
6	Moro	1	Greenhouse
6	Moro	2	Greenhouse
7	Salustian	1	Greenhouse
7	Salustian	2	Greenhouse
8	Mars	1	Greenhouse
8	Mars	2	Greenhouse
9	Tarocco	1	Greenhouse
9	Tarocco	2	Greenhouse
10	Pineapple	1	Greenhouse
10	Pineapple	2	Greenhouse
11	San Miguel L.R.	1	Greenhouse
11	San Miguel L.R.	2	Greenhouse
12	Jaffa	1	Greenhouse
12	Jaffa	2	Greenhouse
13	Valencia	1	Greenhouse
13	Valencia	2	Greenhouse
14	Campbell O.L.	1	Greenhouse
14	Campbell O.L.	2	Greenhouse
15	Chain	1	Greenhouse
15	Chain	2	Greenhouse
16	Fisher navel	1	Greenhouse
16	Fisher navel	2	Greenhouse
17	Washington navel	1	Greenhouse
17	Washington navel	2	Greenhouse
41	Valencia Frost	1	Greenhouse
41	Valencia Frost	2	Greenhouse
42	Valencia Cutter	1	Greenhouse
42	Valencia Cutter	2	Greenhouse
56	Cara Cara navel	1	Greenhouse
56	Cara Cara navel	2	Greenhouse
57	Carter navel	1	Greenhouse
57	Carter navel	2	Greenhouse
58	Leng navel	1	Greenhouse
58	Leng navel	2	Greenhouse
59	Dream navel	1	Greenhouse

59	Dream navel	2	Greenhouse
60	Atwood navel	1	Greenhouse
60	Atwood navel	2	Greenhouse
61	Rio Grande navel Nuc.	1	Greenhouse
61	Rio Grande navel Nuc.	2	Greenhouse
62	Washington Frost navel	1	Greenhouse
62	Washington Frost navel	2	Greenhouse
63	Fukumoto navel	1	Greenhouse
63	Fukumoto navel	2	Greenhouse
65	Valencia Midknigth	1	Greenhouse
65	Valencia Midknigth	2	Greenhouse

Table 1. Sweet orange accessions conserved under greenhouse conditions in the Citrus Germplasm Bank of the Campo Experimental General Terán (CEGET).

is not uncommon, since most of the currently known commercial genotypes of C. sinensis, C. paradisi and C. limón do not constitute biologically defined species since many cultivars within each group originated from somatic mutations (Gómez *et al*., 2020). Under this precept, in the world there are more than nine million hectares planted with citrus and all edible species such as mandarins, oranges, grapefruits and lemons resort to apomixis, an exceptional characteristic in the plant kingdom, whose origin was a mutation 1.6 million years ago, and passed from species to species (Wu *et al.,* 2018). Another important aspect to consider is the environmental condition to which perennial species are exposed as is the case of citrus, under this context eventually morphological or maturity differences have been recorded between fruits of the same tree and such variations have been associated with mutations due to radiation, and from the material that presents differences with the mother tree, new varieties with characteristics of agronomic or commercial interest have been generated. Thus, history records chance discoveries such as the clementine mandarin (Citrus clementina Hort. Ex Tan.), a natural hybridization discovered by the Frenchman Clement Rodier at the beginning of the 20th century. Subsequently, in 1953, from a spontaneous mutation that occurred in a C. clementina tree, clemenules emerged, one of the most consumed mandarin varieties in Spain (Cubillo, 2013). The diversification of sweet orange is also a consequence of the occurrence of somatic bud mutations (Aubert, 2001). The results obtained in this study with the RAM's technique have allowed us to determine differences between and within sweet orange accessions, which undoubtedly reflects genetic diversity among these materials that could be exploited and used in genetic improvement programs. However, it is important to recognize that it is necessary to compare in adult trees the morphological characteristics that corroborate the difference found between the replicates in the three varieties of sweet orange, since the germplasm included in the study are young trees without flower and fruit production.

CONCLUSIONS

The Random Amplified Microsatellites (RAMs) technique was efficient for the genotypic identification of 25 varieties, as well as the existing difference between the replicates of three sweet orange varieties conserved in the Citrus Germplasm Bank of the General Terán Experimental Field. This information can be used for studies and to support genetic improvement programs of this species.

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