Genetic diversity of parthenocarpic genotypes shrubby squash using molecular genetic markers

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Abstract

The production of squash (*Cucurbita pepo* L.) for vegetables or zucchini under cover presents difficulties due to the lack of pollination, for this reason, parthenocarpy is an alternative to generate greenhouse zucchini varieties, so efforts are currently being made in the search for genetic parthenocarpy that does not it requires the stimulation of pollination, nor by physiological factors in order that the fruit develops in a normal way until physiological maturity. A total of 46 parthenocarpic genotypes of round zucchini shrub type squash obtained at the Autonomous University Chapingo were studied. The objective was to characterize and identify the genetic diversity among the genotypes from the variability obtained by means of molecular genetic markers such as the microsatellites of the ISSR type (inter repeated simple sequences) and RAMP (microsatellite polymorphism amplified at random). It was used $(GATA)_4$ as an ISSR initiator and the combination of $(GATA)_4$ with four random sequence primers of the Operon series A (OPA05, OPA10, OPA13 and OPA19) for RAMP. With the two techniques, a total of 43 polymorphic *loci* were obtained, six *loci* amplified with ISSR and 37 *loci* with RAMP. The percentage of polymorphic *loci* for ISSR was 100%, while for RAMP it was 91.8% polymorphism, a high degree of genetic relationship was found between the partenocarpal genotypes. Five groups were formed for ISSR and four for RAMP, with gene distances between 0.72 to 1.

Keywords: *Cucurbita pepo* L., genetic analysis, microsatellites, RAMP.

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Introduction

The genetic diversity of the species of *Cucurbita* spp. in Mexico is broad, one of the species of this genus of greater economic importance worldwide is *Cucurbita pepo* L., because it presents both intra and inter specific variation in different types of characters and in those that have more reports is in morphological aspects, Méndez *et al.* (2010). The importance of the squash is due to its content of nutrients and the taste qualities of its fruit, which is consumed mainly as a vegetable or in mature form (Villanueva, 2007). The production of squash for vegetables or zucchini under cover presents difficulties due to the lack of pollination; however, it is an important and feasible economic alternative to develop (Manzano *et al*., 2010).

Currently, knowing the importance of the parthenocarpy for varieties of greenhouse zucchini, efforts are made in the search for parthenocarpy genetically determined that does not require the stimulation of pollination, or physiological factors in order that the fruit develops normally until maturity (Martínez *et al*., 2013).

The characterization and traditional identification of varieties has been based on the use of morphological and agronomic characters. However, this method has restrictions, since its expression may be subject to environmental or phenological factors, therefore, identification and characterization methods based on the use of molecular genetic markers have proven to be more efficient and in most the cases, overcome the limitations of traditional methods, these markers provide a valuable tool to establish an accurate genotyping of cultivars and are used internationally for these purposes (Azofeifa, 2006).

The emergence and use of molecular genetic markers gave new dimension to studies of genetic diversity, with advantages over other types of markers as they are not affected by the environment and are generally selectively neutral, therefore, they evolve mostly as result of mutations (Rajwant *et al*., 2011).

Based on the previous context, the objective of the present study was to identify genetic diversity by means of molecular markers ISSR (inter repeated simple sequences) and RAMP (random amplification of microsatellite polymorphism: combination of RAPD (polymorphisms in DNA amplified at random) + ISSR) in parthenocarpic genotypes of bush squash type round zucchini.

Materials and methods

Vegetal material

The plant material was selected from a population with a broad genetic base of parthenocarpic plants of bush squash, 46 genotypes from the free recombination of progeny with diallel crossing (method IV of 1989), of seven experimental varieties type round zucchini. This population was developed *ex profeso* in the Genetic Improvement Program of the Plant Breeding Department of the Autonomous University Chapingo, Mexico.

Molecular analysis

The molecular analysis was carried out in the Plant-Pathogen Interaction Physiology Laboratory (FPP) at the Postgraduate School, Mexico. The genomic DNA of 46 genotypes was extracted from young, healthy and lyophilized leaves with the protocol modified by Rojas *et al*. (2003), known as the combined method: MLO and CTAB 3%.

DNA quality was verified by electrophoresis in 1.2% (w/v) agarose gels stained in a solution of ethidium bromide (10 mg ml^{-1}) and by means of an ultra-low volume spectrophotometer (NanoDrop ND-1000 Thermo Scientific® V3.7) the absorbance (260/280) was determined in a range of 1.33 to 1.94, the DNA concentration was 202.3-512.1 ng μL^{-1}.

The polymerase chain reaction (PCR) was carried out for the ISSR with an initiator $(GATA)_4$ and for RAMP, four random sequence primers of the Operon series A were used (OPA: 05, 10, 13, 19) combined with $(GATA)_4$, these primers were synthesized by the Institute of Biotechnology of the National Autonomous University of Mexico (UNAM). The selected initiators are the result of standardization of the protocol and they were the ones that presented polymorphism and marked considerable differences in the banding, among the genotypes.

The volume and concentration of each reagent in the reaction mixture for the PCR was adjusted to 25 µL as the final volume, which contained: 2.5 µL of Buffer [10 X], 1.5 µL of $MgCl_2$, [30 Mm μL^{-1}], 0.5 µL of Dntp's [2.5 Mm μL^{-1}], 2 µL of $(GATA)_4$ [10 pmol μL^{-1}], 0.2 µL of Taq DNA-polymerase (Amplificasa®) [5 U μL^{-1}], 4 µL of genomic DNA [20 ng μL^{-1}] and 14.05 µL of sterile deionized water, in the case of ISSR, while for RAMP, 2 µL of each random primer was added per reaction (OPA05, OPA10, OPA13 and OPA19) [10 pmol μL^{-1}] and the water was adjusted to 12.05 µL, the combinations of each RAMP were as follows: OPA05+$(GATA)_4$, OPA10+$(GATA)_4$, OPA13+$(GATA)_4$ and OPA19+$(GATA)_4$. The DNA was placed individually to each tube according to the genotype number of squash to be characterized.

An APOLO® thermocycler was used (Instrumentation ATC 201, series 00449), thermocycling conditions for ISSR consisted of a pre-denaturation cycle of 1 min at 94 °C, 40 cycles from 40 to 94 °C, 1 min at 40 °C and 1 min at 72 °C, followed by a cycle of 8 min at 72 °C and a period at 4 °C of final extension, while for RAMP the conditions consisted of a pre-denaturation cycle of 2 min at 94 °C, 35 cycles of 1 min at 94 °C, 2 min at 50 °C and 3 min at 72 °C, followed by a 10 min cycle at 72 °C and a final extension period at 4 °C.

The amplification products obtained from the ISSR and RAMP were separated by means of electrophoresis in a 2.5% (w/v) agarose gel at 85 volts, for a period of 4.5 h. The gels were stained with ethidium bromide and the bands were visualized under ultraviolet light in a photodocument (model Gel Doc 2000, BIO RAD®), the photo of the gel was captured with the program Quantity One 4.0.3. The 1 Kb molecular weight marker (Invitrogen®) was used on both sides of the agarose gel.

Statistical analysis

The statistical analysis was carried out separately for each of the molecular techniques. In the conglomerate analysis, genetic distances were calculated with the simple mating coefficient, Sokal and Sneath, (1963) and a similarity dendrogram was obtained by means of the arithmetic average

method, unweighted by pairs between groupings, type UPGMA (Adams *et al*., 2000). The robustness was tested by means of 1 000 resampling in the matrices of genetic distances with α= 0.05, the reliability of the results was verified by means of a Mantel test (1967). As parameters of genetic diversity were considered: the percentage of polymorphic *loci* and the rank of polymorphism in base pairs (bp), the genetic distances of similarity and the range of genetic distances (RDG) between groups and genotypes. The NTSYSpc computer package version 2.21 h was used (Rohlf, 2009).

Results and discussion

Analysis of polymorphism

A total of six amplified *loci* were produced, which represented 100% of the polymorphism generated by the initiator $(GATA)_4$ used for the ISSR and with a molecular weight range in the amplification of polymorphic *loci* from 506 to 1 636 bp (Table 1). These results indicate that the squash parthenocarpic genotypes have little genetic differentiation, due to the low number of *loci* detected with the ISSR; in this regard, Vigoroux *et al*. (2002), mentions that cultivated species have undergone a strong selection pressure directed to genes that control some genomic regions, while the genes that do not have selection pressure maintain similarity with their wild relatives, the selection that the human being has made the species cultivated during its domestication and its genetic improvement have reduced the excess variation especially in the genes for which said species had allelic variation, while other *loci* experience modification of the diversity according to the natural effect, in this sense Restrepo and Vallejo (2008), in a work with collections of *Cucurbita moshata* and with polymorphism markers in the length of amplified fragments (AFLP), mention that the levels of genetic variation or higher heterozygosity are due to the distance between populations and to the anthropic exchanges that occurs in this species.

Table 1. Number of *loci* amplified by the ISSR primer and for RAMP in 46 parthenocarpic squash genotypes (*Cucurbita pepo* L.).

Initiator	Sequence (5'-3')	P. amplified		Polymorphism (%)	Monomorphic *loci* (%)	Rpm (pb) of *loci*
		Tts	Plmfcs			
Microsatellites (ISSR)						
$(GATA)_4$	GATAGATAGATAGATA	6	6	100	0	1636-506
RAMP (RAPD + ISSR)						
OPA05	AGGGGTCCTG + GATAGATAGATAGATA	7	7	100	0	1018-298
OPA10	GTGATCGCAG + GATAGATAGATAGATA	8	6	75	25	3054-506
OPA13	CAGCACCCAC + GATAGATAGATAGATA	12	11	91.7	8.3	3054-506
OPA19	CAAACGTCGG + GATAGATAGATAGATA	10	10	100	0	2036-396
Average		37	34	91.8	8.2	

Tts= totals; Plmfcs= polymorphic; Rpm= molecular weight range; pb= base pairs.

Pradeep (2002) mentions that ISSRs frequently amplify 25 to 50 bands in a single reaction and allow the detection of polymorphism among individuals of the same population, evaluation of genetic diversity, as well as the distinction and identification of intraspecific varieties (particularly in species with economic importance), among others.

For RAMP, 34 *loci* of 37 amplified totals were polymorphic representing 91.8% and with an average of 8.5 *loci* per initiator. The amplification range was between 298 and 3 054 bp. The combinations in which less genetic variability was identified among the genotypes was with the combinations OPA10+$(GATA)_4$ and OPA13+$(GATA)_4$ with 25% and 8.3% monomorphic *loci*, respectively (Table 1), Wu *et al*. (1994) reported between 10 and 20 polymorphism per initiator in acrylamide gels in Arabidopsis ecotypes using the RAMP technique, which exceeded the expectations of the use of this technique with respect to other alternatives, in addition to which it mainly favors the amplification of microsatellites, without excluding the amplification of RAPD.

Valadez-Moctezuma *et al*. (2005), mention that with the RAMP technique it is possible to identify new profiles that share some fragments, from the profiles obtained independently with the RAPD and microsatellite techniques, also indicate that the RAMP analyzes provide a new alternative to analyze and make studies of genetic diversity practically in any genome; these results support this research because they corroborate the differences found between the ISSR polymorphism, with respect to RAMP, in the squash parthenocarpic genotypes, so the RAMP technique turned out to be very informative.

Cluster analysis

The consensus tree or dendrogram of genetic distances of similarity, obtained from the robustness of the clusters by 1 000 resampling generated five groups with ISSR and four groups with RAMP. These groups were defined mainly by the genetic variability that exists between the genotypes (Figure 1 and 2), Méndez *et al*. (2010) in a previous work characterized the same partenocarpal genotypes, with morphological and agronomic variables, in which they found three groupings.

The groups obtained in the dendrogram for ISSR show genotypes 3, 15, 16, 20, 25, 28, 33, 38, 47, 51, 82, 84, 97, 103, 106 and 107 in group I, with a RDG from 0.781 to 1, group II is composed of genotypes 5, 13, 14, 21, 30, 35, 45, 48, 62, 63, 70, 90, 91 and 105 (RDG from 0.802 to 1), while that group III is composed of genotypes 19, 26, 29, 41, 44, 56, 57, 87, 93 and 108 (RGD: 0.849 to 1), group IV is made up of genotypes 23, 69 and 88 with similarity genetics of 1, while group V is composed of genotypes 73, 83 and 96, this group was found at greater genetic distance with respect to the other groups (0.469), the RDG among the genotypes was 0.761 to 0.849 (Figure 1).

These results show agreement with Méndez *et al*. (2010), so that the groups formed with ISSR presented the following characteristics: group I is defined by plants with long internodes, round fruits of intermediate weight, thin skin, a lot of seed and small, in groups II and V, predominate plants with short internodes, elongated fruits with low weight, thin skin, little seed and small. The group IV presents plants with intermediate internodes, round, heavy fruits, thick rind, a lot of seed and large.

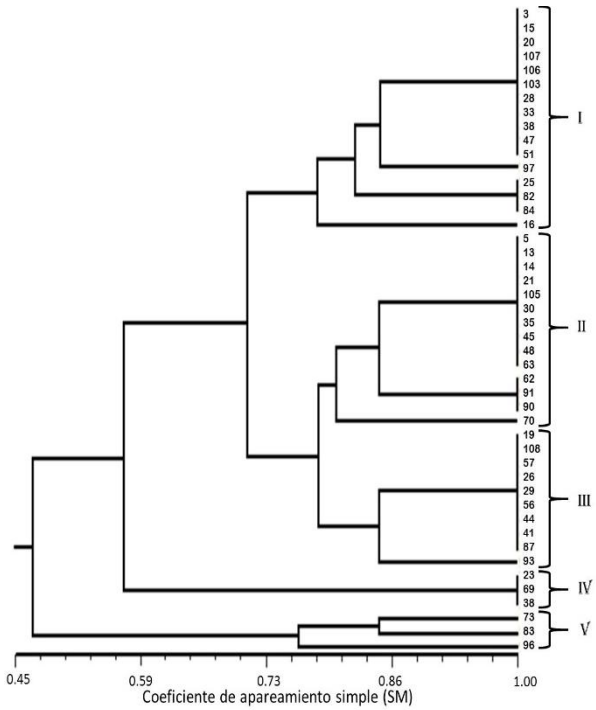

Figure 1. Average dendrogram constructed from 1 000 combinations of resampling obtained by the simple mating coefficient (SM) of the ISSR $(GATA)_4$ amplified products for 46 squash parthenocarpic genotypes.

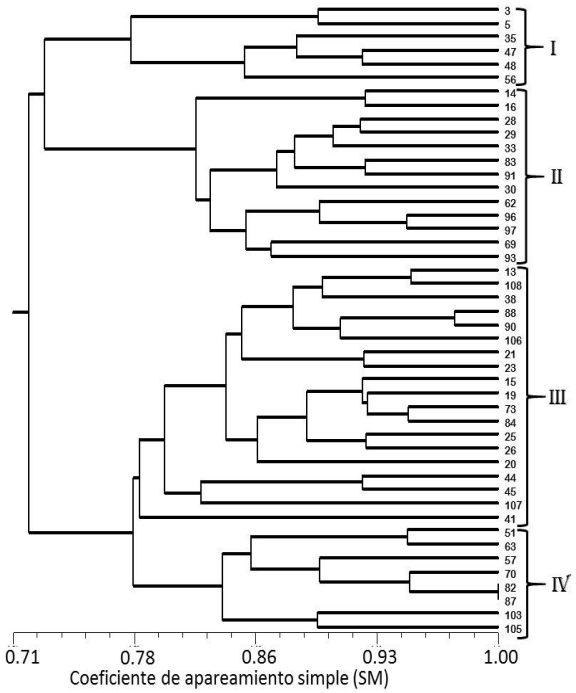

Figure 2. General dendrogram constructed from 1 000 combinations of resampling, using the simple mating coefficient (SM) of RAMP amplified products (OPA05, 10, 13 and 19)+$(GATA)_4$) for 46 parthenocarpic squash genotypes.

While, with the results obtained with RAMP the groups were formed as follows: group I, genotypes 3, 5, 35, 47, 48 and 56, RDG from 0.729 to 0.919, group II conformed by genotypes 14, 16 , 28, 29, 30, 33, 62, 69, 83, 91, 93, 96 and 97, RDG from 0.720 to 0.945, group III composed of 13, 15, 19, 20, 21, 23, 25, 26, 38 , 41, 44, 45, 73, 84, 88, 90, 106, 107 and 108, RDG from 0.782 to 0.974 and group IV with genotypes 51, 57, 63, 70, 82, 87, 103 and 105, the latter group contains the most genetically related genotypes (RDG: 0.8352 to 1) (Figure 2). The morphoagronomic characteristics reported by Méndez *et al*. (2010) for these genotypes, are defined as follows: for groups I and II, plants with short internodes, elongated fruits with low weight, thin skin, little seed and small predominate. In group III, plants with long internodes prevail, round fruits of intermediate weight, thin peel, a lot of seed and small.

Group III and IV (ISSR and RAMP, respectively), did not show a predominance of some morphological and agronomic characteristics among the genotypes. In this sense, Demey *et al*. (2008), showed that the characterizations generated by morphological descriptors of those made by molecular markers, are usually independent responding in each case to different rules and evolutionary pressures.

The groups formed in RAMP presented greater stability than the groupings that the analysis of the ISSR showed, in addition there was a greater number of concordances with the groups presented by Méndez *et al*. (2010), so the relationships found with the molecular markers used in this work indicate the genetic condition in which squash genotypes are found for their future use in the improvement and search for parthenocarpy. However, Sánchez *et al*. (2000), mention that studies with molecular markers are not associated with phenotypic differentiation and the way in which empirical and scientific improvement has been made.

Demey *et al*. (2008) in maize works, have suggested to combine information of different nature for diversity analysis because it allows to analyze all the variability and obtain a more precise analysis, better distribution and definition of the classification of the populations and the resolution of the results is better.

The results of the comparison between matrices of genetic distances without resampling and the average matrix of 1 000 resampling, both for ISSR and RAMP were corroborated with the Mantel test (1967), which identified high, positive and significant correlation for both molecular techniques (r= 0.999), with 99.9% congruence between genetic distances and clusters of genotypes, about Beyene *et al*. (2005), mention that significant correlations indicate that the data sets reflect the same pattern of genetic diversity and validate the use of these data to perform statistical analyzes of different types, such as clusters on different populations.

Conclusions

It was confirmed that there is genetic diversity among the 46 parthenocarpic genotypes of round zucchini squash, by means of genetic markers ISSR and RAMP.

The molecular marker ISSR, with $(GATA)_4$ amplified six loci with 100% polymorphism; while the combination of $(GATA)_4$ and each of the Operon decamers (OPA05, OPA10, OPA13 and OPA19) in the RAMP technique, amplified 37 total loci with 91.8% polymorphism.

The genetic distances between the genotypes were very close to one (0.72 to 1), for which genetic identification was identified between the parthenocarpic genotypes of studied squashs, with five groups for ISSR and four for RAMP.

The molecular techniques ISSR and RAMP, used in this study, were appropriate for the characterization of the genetic diversity of the squash parthenocarpic genotypes and confirmed the diversity that is reported in the literature.

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