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MICROSATELLITE CHARACTERIZATION OF GUAVA (*Psidium guajava* L.) GERMPLASM COLLECTION IN CUBA

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ABSTRACT. Guava (*Psidium guajava* L.) is one of the most economically important fruit in Myrtaceae. Cuban breeding program has been limited to the selection and introduction of genotypes with promising agronomic characteristics, but studies focussed on genetic diversity organization has not been made, which is very important for the identification of potential parents for breeding program. The utilization of microsatellite markers for guava accession identification and germplasm characterization was the main objective of this work. A total of 34 different alleles ranging from three to seven were detected and the average number of putative alleles per locus was 4.57. Heterozygosity values ranged from 0.08 to 0.54 with 0.38 as the total average for this parameter. Except two genotypes, all the accessions were differentiated as a result of the molecular analysis and six diversity groups were detected, showing an acceptable level of genetic variability in the collection assayed. The high number of common alleles detected suggests that most of the analyzed plant material shares a common genetic ancestry. The microsatellites evaluated will play an important role in the identification of guava accessions representing an essential genepool for *ex situ* maintenance. Furthermore, molecular genotyping detected here will allow the efficient selection of parents for future guava breeding programs.

RESUMEN. El guayabo (*Psidium guajava* L.) es uno de los frutales más importantes económicamente dentro de Myrtaceae. El programa de mejoramiento en Cuba ha estado limitado a la selección e introducción de genotipos con características promisorias, desde el punto de vista agronómico, pero no se han realizado trabajos encaminados a conocer cómo está organizada la diversidad genética existente, lo cual es de gran importancia para la identificación de progenitores con potencial para ser utilizados en el programa de mejoramiento. El objetivo del presente trabajo consistió en la utilización de marcadores moleculares del tipo microsatélites, para la identificación de accesiones y caracterización del banco de germoplasma existente. Se detectaron de tres a siete alelos por locus para un total de 34, con un promedio de 4.57 alelos por microsatélite. Los valores de heterocigosidad variaron de 0.08 a 0.54, con un valor promedio de 0.38. Los resultados del análisis molecular permitieron diferenciar todas las accesiones estudiadas excepto dos de ellas, así como la formación de seis grupos de diversidad genética, mostrando un nivel aceptable de variabilidad genética en la colección evaluada. El alto número de alelos comunes detectado sugiere que la mayoría del material vegetal analizado comparte ancestros comunes. Los microsatélites evaluados jugarán un papel importante en la identificación de accesiones de guayabo que representen una colección de trabajo para el mantenimiento *ex situ*. La caracterización genética mediante SSR permitirá, además, seleccionar eficientemente parentales para futuros trabajos de mejora en el cultivo.

Key words: *Psidium guajava*, genetic markers, genetic variation

Palabras clave: *Psidium guajava*, marcadores genéticos, variación genética

INTRODUCTION

Guava (*Psidium guajava* L.) is an indigenous fruit crop of the American tropical area, where it exists in wild as well as cultivated forms (1). The fruit is an excellent source of vitamin C, a moderately good source of calcium, a fair source of phosphorus and a good source of iron (2).

Usually, the easiest assessment of genetic variation is throughout morphological or phenotypic measures. However, morphological traits are often influenced by environmental conditions, which can affect the estimation of genetic variation and relatedness (3).

During the last decades, classical methods to evaluate genetic variation have been complemented by molecular techniques (3, 4, 5). There is a great potential for the application of genetic markers to tropical, subtropical and indeed all perennial fruit crops (6, 7, 8, 9). Breeding of most fruit species can be complicated by factors including self-incompatibility, apomixis, dioecy, seedlessness, embryo maturity, heterozygosity, and long juvenile periods (10).

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Molecular markers have been exploited in some Myrtaceae members (11, 12, 13). Specifically in guava, various studies begin to arise (14, 15, 16, 17, 18, 19, 20), focussed on cultivar identification and germplasm biodiversity evaluations. Also, the first genetic linkage map with the association of different QTLs (Quantitative Trait Loci) was already reported in this crop (21).

A Simple Sequence Repeat (SSR) DNA marker, usually known as microsatellites, consists of tandemly repeated and often identical core units, containing from two to five nucleotides and represents a significant portion of higher eukaryote genomes (22). The most important value of microsatellites arises from their multiallelic nature, codominant transmission, ease detection by PCR, relative abundance, extensive genome coverage and the small amount of starting DNA required (23, 24, 25).

The objectives of the present work are: i) to detect more suitable primer combinations for cultivar identification, ii) to detect the most important genotypes to preserve for genetic variation in the gene pool, and iii) to characterize guava accessions included in the germplasm collection of this crop in Cuba, using species-specific microsatellites.

MATERIALS AND METHODS

Plant material. Cultivars and accessions used in this study are listed in Table I and form part of the Myrtaceae germplasm collection located in Alquízar Experimental Station (Havana province, Cuba) under the auspices of IIFT (Tropical Fruit Research Institute). The accessions originally came from three different sources: (i) foreign cultivars; (ii) plants prospected in different localities throughout the country; and (iii) selected genotypes segregated from open-pollinated seeds, mainly from cultivars “N6”, “Suprema Roja”, “Indian Pink” and “Perú Roja”. The genotypes included in this study are the most employed in guava breeding program, due to their economically important characteristics.

DNA isolation. Total genomic DNA was extracted from leaves by a modification (26) of the CTAB method (27). The integrity and concentration of isolated DNA was determined by electrophoresis in 0.7 % agarose gel and compared to 1Kb DNA ladder.

DNA marker analysis. Seven microsatellites isolated from guava (*Psidium guajava* L.) were used for biodiversity characterization, using the 34 accessions listed in Table I. The primer combinations used were mPgCIR05, mPgCIR09, mPgCIR10, mPgCIR11, mPgCIR15, mPgCIR16 and mPgCIR19, and the PCR reactions were performed using the reported protocol (28). After the reactions, mixtures were processed for analysis by polyacrylamide gel electrophoresis (PAGE) on sequence gels, adding sequencing loading buffer and denaturation by heating at 94ºC. Aliquots of 3µl were loaded onto a 6 % sequencing gel, run in 1X TBE buffer, pH 8.9 at 40W. After the run, the gel was fixed in 10 % acetic acid, washed with water, dried and exposed to X-rays film at room temperature for one to three days.

Data analysis. Some genetic parameters were determined by the use of GENEPOP (29). Also, allele classification was done according to the principles suggested by Perera *et al.* (30); in this study, a common allele was defined as the one that was present in at least one accession at a greater frequency than 1 %; a rare allele as the one that never occurs at a higher frequency than 1 %; a widespread allele as the one which was present in more than 12 accessions; a sporadic allele as the one present between 2 and 12 accessions and a localised allele as the one present in only one accession. The genetic similarity based on SSR polymorphism data was calculated considering major bands (alleles) as polymorphic units. Thus, autoradiograms were visually scored for the presence (1) or absence (0) of bands. Based on the distance matrix selected, the Jacard coefficient and the un-weighted pair group arithmetic mean analysis (UPGMA) were used to produce a dendrogram by NTSYS-pc software package Exeter Software, Setauket, USA (31).

Table I. Guava accessions studied by SSR molecular characterization

Number	Designation	Origin	Number	Designation	Origin
1	BG 76-19	Local	18	EEA 18-40 (Enana Roja Cubana)	Local
2	BG 73-8	Local	19	Ibarra	Local
3	Cotorrera	Local	20	EEA 14	Local
4	BG 73-6	Local	21	Indonesia blanca	Indonesia
5	BG 76-18	Local	22	BG 76-23	Local
6	Belic L-217	Local	23	N6	Florida
7	BG 76-11	Local	24	Belic L-207	Local
8	Belic L-120	Local	25	Dario 18-2	Local
9	BG 76-10	Local	26	Belic L-97	Local
10	Belic L-99	Local	27	BG 76-13	Local
11	Suprema Roja	Florida	28	BG 76-15	Local
12	BG 76-12	Local	29	Microguayaba	Local
13	BG 76-16	Local	30	Belic L-213	Local
14	Dario 19-2	Local	31	BG 76-21	Local
15	EEA 6-19	Local	32	Peru Roja	Local
16	Belic L-98	Local	33	Belic L-205	Local
17	BG 76-8	Local	34	EEA 1-23	Local

RESULTS AND DISCUSSION

The present study showed the molecular characterization of Cuban guava (*Psidium guajava* L.) germplasm by species-specific microsatellites. The majority of guava genotypes showed two equal alleles in their respective SSR profiles. Out of a total of 238 amplification profiles (34 genotypes x 7 primer pairs) scored in this study, 146 (61 %) showed a single allele, which is in correspondence with the self-pollinating behaviour of guava, and 88 (37 %) showed two different alleles, agreeing with the 35-40 % of outcrossing reported for this crop (32). This result provides a heterozygous, open-pollinated seedling population, with an adequate genetic variation for selection of desirable commercial types (1).

The seven guava-specific microsatellite primer pairs amplified a total of 34 different alleles ranging from 3 to 7. The average number of putative alleles per locus was 4.57. The same number and average of alleles were detected during the development of these microsatellites in guava using more primer combinations (28). A similar number has been reported for other fruits such as peach (33), grapevine (34), coconut (35) and members of guava family (36). On the other hand, no more than two bands/accessions were displayed, a result that confirms the diploid condition (2n=22) of this crop (32).

Both, the allele number and classification according to their frequencies and distribution are shown in Table II. A total of 24 alleles were classified as common ones, out of which 10 were widespread and 14 sporadic. No common localised alleles were found. Common widespread alleles account for 29 % of the total detected in the germplasm evaluated (Table II), it indicating that various sampling strategies would easily sample these alleles. The high frequency of this type of allele may be attributed to the origin of the accessions evaluated. Similar results were obtained in mango (*Mangifera indica* L.) using microsatellites (37). The number of common alleles detected by mPgCIR09 and BmPgCIR16 primer pairs suggests their utility to identify similarities between guava accessions.

Table II. Allele classification attending to their frequencies and distribution

Primers	No. A	Common allele			Rare allele		
		CWA	CSA	Total	RSA	RLA	Total
mPgCIR05	4	2	1	3	1	0	1
mPgCIR09	7	1	4	5	1	1	2
mPgCIR10	4	1	2	3	1	0	1
mPgCIR11	6	1	2	3	2	1	3
mPgCIR15	5	1	2	3	1	1	2
mPgCIR16	5	2	2	4	1	0	1
mPgCIR19	3	2	1	3	0	0	0
Total	34	10	14	24	7	3	10

A: Allele; CWA: Common widespread allele; CSA: Common Sporadic allele; RSA: Rare Sporadic allele; RLA: Rare localised allele

On the other hand, 10 alleles were classified as rare, from which seven were sporadic and three localised ones. Rare alleles are probably low in adaptative value (30); however, they can be important for breeding and conservation purposes.

Therefore, the aim of collection strategies should be to collect at least one copy of each allele occurring with a frequency of at least 0.05. The present study detected 10, which represents 29 % from the overall, it indicating the utility of these primer combinations to trace all types of alleles. From all, mPgCIR09, mPgCIR11 and mPgCIR15 could be the best candidates to search for rare alleles in guava germplasm; while mPgCIR19 primer combination can not detect this type of allele (Table II).

A rare-localised combination is considered as the most difficult type of alleles to capture in any kind of sampling strategy (30). The seven guava SSR combinations traced three rare-localised alleles (Table II). This suggests the possibility to use them to differentiate guava varieties based on their individual allele pattern. Similar results were obtained in the characterization of grapevine cultivars by microsatellites (34).

Expected and observed genotype number; observed homozygote/heterozygote number; heterozygosity and identification percentage are shown in Table III. For all the SSR primer combinations, the observed genotypes were lower than the expected ones and ranging from 3 to 12. The best results of identification percentage/primer combination were yielded using mPgCIR09, mPgCIR16 and mPgCIR11 primer pairs that discriminated 35.29; 26.47 and 20.59 % of the studied accessions respectively. The primer combination mPgCIR19 showed the lowest identification percentage (8.82 %). This parameter was low in general, compared to the results obtained for avocado, a cross-pollinated crop, using the same molecular marker (38). Once more, the autogamous nature of guava was the major reason for this behaviour. Nevertheless, mPgCIR09 appears to be the most suitable primer combination for guava accession fingerprinting.

Table III. Identification percentage, heterozygosis and homozygote/heterozygote proportion for each SSR primer evaluated

L	IP*	GN (obs)	GN (exp)	THON (obs)	THEN (obs)	H/locus
mPgCIR05	11.76	4	10	19	14	0.4242
mPgCIR09	35.29	12	28	15	18	0.5455
mPgCIR10	17.65	6	10	31	3	0.0882
mPgCIR11	20.59	7	21	25	9	0.2647
mPgCIR15	17.65	6	15	16	16	0.5000
mPgCIR16	26.47	9	15	21	13	0.3824
mPgCIR19	8.82	3	6	19	15	0.4412

L: Locus; IP: Identification percentage; GN (obs): Number of observed genotype; GN (exp): Number of expected genotype; THON (obs): Total number of observed homozygote; THEN (obs): Total number of observed heterozygote; H: Heterozigosity
(*) Fraction between band pattern number obtained per primer pair and total number of varieties expressed in percentage

Observed heterozygosity values varied from 0.08 to 0.54 and 0.38 was the total average, which is very close to the 0.42 detected with more primer combinations during the development of these microsatellites in guava (28). All SSR loci showed from medium to low levels of gene diversity (heterozygosis) (Table III). This result could be due to the high homozygote number present in almost every SSR primer combination, which confirms guava self-pollinating behaviour (40). Similar values were obtained using SSR to investigate genetic diversity and population genetic structure in coconut (*Cocos nucifera* L.) (34). Also, differences in heterozygosis and gene diversity between tall and dwarf coconuts were detected due to breeding habit using SSR (22, 41). In this sense, the combinations mPgCIR09, mPgCIR15, mPgCIR19 and mPgCIR05 used here could be useful to detect heterozygote patterns in guava accessions.

A high percentage of alleles was shared by the majority of the accessions (data not shown). This is not surprising attending to the presence in guava germplasm of various open-pollinated descendants from a few cultivars such as "N6", "Suprema Roja", "Indian Pink" (not included) and "Perú Roja". Similar results were obtained during the identification of *Feijoa sellowiana* accessions by RAPD markers and the study of coconut populations (12, 30).

Accessions showing from one to three cultivar-specific markers ("Darío 19-2"; "Belic L-98"; "BG 76-23"; "Belic L-205" and "Microguayaba") could be attractive genotypes for conservation targets, due to the presence of rare-localized alleles. "Microguayaba" (#29) also showed the lowest number of common alleles.

The polymorphism detected by SSR on the accessions assayed is shown in Figure 1. Among the 34 guava cultivars tested, 32 showed a unique pattern using the total of primer sets whereas two cultivars can not be identified because of genotype similarities ("Ibarra" and "N6"). The number of accessions showing unique banding pattern suggests the potential for identification of guava accessions by microsatellite markers. Similar results were obtained in mango (*Mangifera indica* L.) (37).

The dendrogram obtained showed six main groups (Figure 1) and two single clustering accessions ("BG 76-8" and "Microguayaba"): *Group I*: including "Cotorrera", a wild genotype, with other local accessions such as "BG 76-19"; "BG 76-23" and "Belic L-98"; *Group II*: formed by a set of local accessions such as "BG 76-18"; "EEA 14"; "BG 76-13"; "Perú roja", "Dario 18-2"; "Belic L-99"; "Dario 19-2" and two dwarf cultivars ("Enana Roja Cubana" or "EEA 18-40" and "EEA 1-23"); *Group III*: containing a variety from Florida ("Suprema Roja") and local accessions such as "BG 73-8"; "BG 76-16"; "BG 76-11"; "BG 76-10"; "Belic L-205"; "BG 73-6"; "BG 76-12" and "Belic L-97"; *Group IV*: with a set of "Belic" accessions, such as "Belic L-217"; "Belic L-207"; "Belic L-213" and "Belic L-120"; *Group V*: with the two local accessions "EEA 6-19" and "BG 76-21" and *Group VI*: containing foreign varieties such as "N6" and "Indonesia Blanca" and the local accessions "Ibarra" and "BG 76-15". The similitude values between groups varied from 14 to 37 %, it indicating the presence of the six groups mentioned above, while accessions BG 76-8 and Microguayaba only showed a similarity coefficient of 20 %.

The two single clustering accessions "BG 76-8" and "Microguayaba" were homozygote for all loci studied. The external position in the cluster confirms their differences in relation with the other genotypes evaluated. The location of "Microguayaba" (#29) is also in correspondence with the low genetic similarity value observed between this accession and the majority of the materials assayed (data not shown). The same results were obtained by AFLP analysis and by quantitative morphoagronomic characters (19) and confirm the preliminary classification of this genotype as the subspecies *Pumila* of *Psidium guajava* L. (19, 21).

Another important result was the association of Enana Roja Cubana "EEA 18-40" and "EEA 1-23" (Fig. 1). The relationship between these two dwarf cultivars is not surprising, attending to their common origin from open-pollinated seeds of "Indian Pink", which is in agreement with previously results obtained by AFLP (19).

Nevertheless, the relatedness of "Perú Roja" and their descendants by open-pollinated seeds "BG 76-13" and "BG 76-15" were not observed by SSR as were by AFLP analysis (19). The association only remained between "Perú Roja" and "BG 76-13". The origin from different male parents could be the explanation for the absence of association within "Perú Roja" (#32) and "BG 76-15" (#28), which is reflected in a different allele distribution. This can not be detected by AFLP markers due to its dominant nature and should be the principal difference with SSR results (42).

It is a well known phenomenon that plant genetic resource collections suffer to a certain degree from misnaming. Therefore, it is a continuous task to eliminate mistakes in order to maintain reliable collections (43). The molecular characterization by AFLP of this germplasm shed some doubt on the current classifications of "Belic L-213" and "Belic L-207" (#24) done during the establishment of this collection, because they clustered together by qualitative morphoagronomical traits as well as by molecular data (19). On the contrary, SSR analysis showed similar results between "Belic L-207" (#24) and Belic L-217 (#6), which eliminates the possibility of misnaming and confirms the existence of three different but genetically very closed accessions.

A similar problem was observed between cultivars "Ibarra" and "N6" (Figure 1). More SSR markers should detect differences between these two cultivars, attending to a previous study with AFLP in the same germplasm (19), which confirms the presence of two different but highly related genotypes. The same conclusion was reported during the study of apples (*Prunus persica*) with microsatellites (33). The narrow association of these two cultivars enhances the hypothesis of a possible origin by natural mutation of "Ibarra" from "N6".

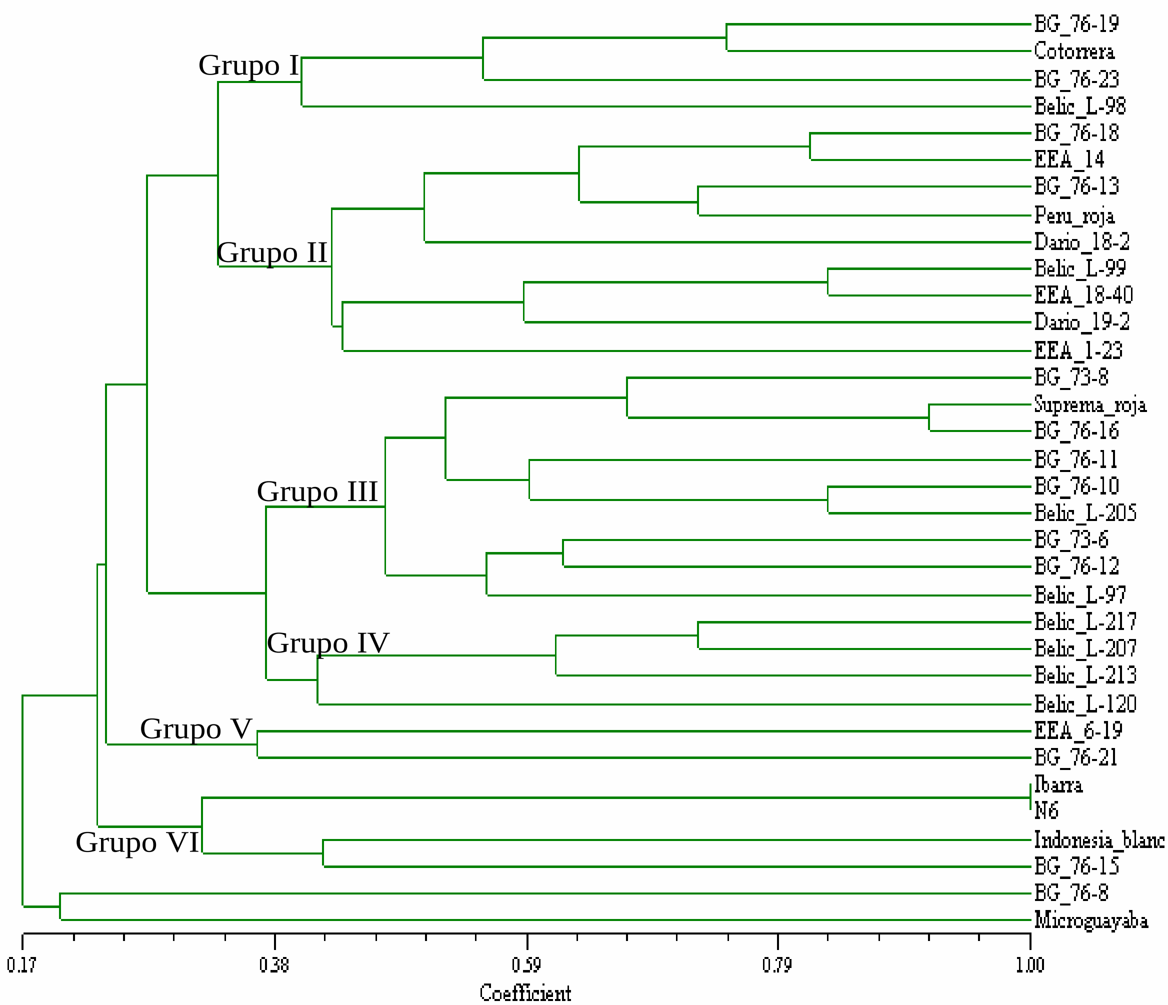

Figure 1. Genetic relationships for guava (*Psidium guajava* L.) accessions. The dendrogram was constructed for the 34 genotypes with the Jacard coefficient by UPGMA cluster analysis on the basis of 34 polymorphic SSR markers

The level of information generated suggests that these microsatellite loci can become an important tool for genetic studies in guava (*Psidium guajava* L.). Similar results were obtained in citrus (44) and coconut (*Cocos nucifera* L.) (39).

In summary, the molecular characterization of guava germplasm by microsatellites allowed the following conclusions: i) the primer combinations evaluated are suitable for guava fingerprinting based on their allele pattern, confirming the discriminatory capacity of SSR markers; ii) cultivars presenting rare alleles as well as wild genotypes represent an important genepool for conservation purposes; iii) the acceptable level of diversity detected and the number of groups formed will allow the efficient selection of parents for guava breeding programs, and iv) the correspondence between different molecular markers is a very important element to detect misnaming errors.

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