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RESEARCH

Analysis of genetic polymorphism among 14 acerola (*Malpighia emarginata* D. C) accessions revealed by RAPD markers

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ABSTRACT

Acerola (*Malpighia emarginata* D. C), known as Barbados cherry, is a tree cultivated in tropical and subtropical areas throughout the world, showing a high degree of polymorphism among genotypes. In this research, the genetic relationship of 14 acerola accessions was assessed using PCR-Randomly Amplified Polymorphic DNA (RAPD) markers. Fifteen UBC random primers (University of British Columbia, Vancouver, Canada), were used for screening and those able of amplification were selected for scoring the monomorphic and polymorphic bands generated, using the PyElph software for gel images analysis. Genetic diversity among acerola accessions was computed using the Numerical Taxonomy and Multivariate Analysis System (NTSYS) version 2.10e. Seven out of the fifteen random primers amplified, and 78 (70.91 %) bands were polymorphic, which were used for genetic discrimination among different accessions, their genetic similarity being established using the Jaccard's similarity coefficient. Those with the highest genetic similarity and cophenetic correlation value (0.88401) were selected. UPGMA dendrogram based on cluster analysis of combine genetic similarity coefficients discriminated the acerola accessions evaluated into 10 different clades. Six of them (Anabel, Camila, Kevin, Javier, Lisset and Víctor) were shown as the most divergent accessions and, therefore, the most promissory parents for further acerola crossbreeding studies. RAPD-PCR was confirmed as effective for the assessment of the genetic relationship among the analyzed acerola accessions. These results provide the methodology require to further characterize the diversity of the acerola genetic resources stored in the gene bank at the Tropical Fruit Culture Research Institute of Cuba.

Keywords: molecular markers, RAPD-PCR, genetic diversity, variability, acerola, *Malpighia emarginata* D. C

RESUMEN

Análisis del polimorfismo genético entre 14 accesiones de acerola (*Malpighia emarginata* D. C) revelado mediante marcadores RAPD. La Acerola (*Malpighia emarginata* D. C), es un árbol cultivable de las áreas tropicales y subtropicales del mundo, la cual presenta un alto grado de polimorfismo entre sus genotipos. En el presente trabajo, la relación genética entre 14 accesiones de acerola se investigó mediante marcadores de ADN polimórficos amplificados aleatoriamente por PCR (RAPD-PCR). Amplificaron siete de los 15 cebadores aleatorios UBC (University of British Columbia, Vancouver, Canada) escogidos, los que se seleccionaron para análisis posteriores. Las bandas monomórficas y polimórficas generadas se analizaron con el programa PyElph y la diversidad genética entre las accesiones se computó con el sistema NTSYS (Numerical Taxonomy and Multivariate Analysis System), versión 2.10e. Setenta y ocho bandas fueron polimórficas (70.91 %), las que se emplearon para la diferenciación genética. El análisis de semejanza, mediante el coeficiente de similitud de Jaccard, mostró el mayor valor de correlación cofenética (0.88401), y el dendograma UPGMA diferenció las accesiones de acerola estudiadas en 10 grupos diferentes. Las accesiones Anabel, Camila, Kevin, Javier, Lisset y Víctor fueron las más divergentes, y se seleccionaron como los parentales más promisorios para futuros estudios de cruzamiento de acerola. Los resultados presentados reafirman la efectividad del RAPD-PCR para la evaluación de la diversidad genética entre accesiones de acerola y constituyen el punto de partida para la caracterización de la diversidad de las fuentes genéticas de acerola del banco de germoplasma del Instituto de Investigaciones en Fruticultura Tropical de Cuba.

Palabras clave: marcadores moleculares, RAPD-PCR, diversidad genética, variabilidad, acerola,

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Malpighia emarginata D. C

Introduction

Acerola (*Malpighia emarginata* D. C), known as Barbados cherry or West Indian cherry is a tree taxonomically classified as class Magnoliopsida, order Malpighiales, family Malpighiaceae, genus *Malpighia*, and species M. emarginata D.C [1]. Its origin is not exactly determined, but the acerola tree is likely native to Caribbean and Antillean islands [2]. This medium sized evergreen shrub is predominately allogamous and outcrossing rate vary between families [3].

The acerola crop is currently cultivated in tropical and subtropical areas throughout the world, due to its high content of vitamin C [1]. It contains other nutrients, such as anthocyanins and some phytochemicals present in the pulp of the fruit, which contribute to its antioxidant capacity [4, 5].

In Cuba, the crop of acerola began in the decade

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of 1990 with the establishment of a small acerola collection in the gene bank at the Tropical Fruit Culture Research Institute [6]. However, a reduced number of acerola clones used for a long time can result in a major vulnerability to pests and diseases. Therefore, the genetic variability preservation of acerola by implementing active germplasm banks is highly important for its biological preservation and genetic improvement [7]. In this regard, the analysis of genetic relationship among accessions of acerola becomes a key component for enhancing the future genetic resource usage of this crop [8].

For the aim of phenotypic characterization and contrary to conventional phenotype characterization markers, molecular markers are stable and detectable in all tissues, regardless of growth differentiation, development, pleiotropic effect, epistatic effects, and not determined by the plant growing environmental conditions [9]. Among them, the PCR-randomly amplified polymorphic DNA (RAPD) technique provides a PCR-based molecular marker that has been used in the molecular characterization of several medicinal plants [10-12]. Studies characterizing the genetic diversity of acerola have been performed in the last two decades [7, 13, 14], but no experiments have been conducted to determine the level of diversity of Cuban acerola accessions, despite the insufficient morphological variation among acerola cultivars. Therefore, this work was aimed to determine the genetic relationships of 14 acerola accessions by the RAPD molecular analysis method.

Materials and methods

Plant material and DNA extraction

Fourteen acerola (*Malpighia emarginata* D. C) accessions were tested: Lisset, Víctor, Helen, Miguel, Javier, Domingo, Camila and Vitico, collected in Cuba; Kevin, Tatica, Anabel and Amanda, collected in Brazil; and Antonio and Puerto Rico, of unknown origin. Leaves from each accession were sampled from the gene bank of the Tropical Fruit Culture Research Institute, located in Alquízar-Cuba (22°47'N and 82°31'W). Total genomic DNA was purified from 5 g of fresh young leaves, according to the method by Sreelakshmi *et al*. [15], with modifications [6]. DNA quality was checked by 1.5 % agarose gel electrophoresis and it was quantified with the aid of GeneQuant 100 spectrophotometer at 260 nm. The extracted DNA was diluted to a final concentration of 50 ng/µL in 1 × TE buffer and stored at –20 °C until use.

RAPD-PCR amplification

Initially, fifteen UBC (University of British Columbia, Vancouver, Canada) random primers were used for screening. The primers showing amplification were selected to study genetic diversity by RAPD analysis. PCR was carried out in 25 µL reaction volume containing 1× DreamTaq Buffer, 0.2 µM of each dNTP, 0.3 µM UBC primer, 1.0 U of DreamTaq DNA Polymerase and 40 ng of genomic DNA template. PCR amplifications were performed in a MJ Research PTC-100 Thermal Cycler with an initial step of 4 min at 94 °C, followed by 35 cycles, each consisting of 1 min at 94 °C, 1 min at 37 °C, 2 min at 72 °C and a final extension cycle for 5 min at 72 °C. The amplified PCR products were resolved by 1.5 % agarose gel electrophoresis with ethidium bromide staining, and visualized under ultraviolet light using an UV Transilluminator (Fisher Scientific, USA). The molecular size of the PCR products was estimated using the 100 bp DNA ladder (Promega, USA).

Data scoring and analysis

All monomorphic and polymorphic bands, generated by the seven UBC primers, were scored using the PyElph software for gel images analysis [16], on the basis of their presence (1) or absence (0) and assembled onto a binary data matrix. Amplification failure was scored as "9" as an indicator of missing data. The primer banding characteristics were: total number of band (TNB), number of polymorphic band (NPB), percent polymorphism (PP), polymorphic information content (PIC), band informativeness (IB) and resolving power (R_p). They were estimated according to the following formula: PP = NPBs/TNB generated by each primer; $PIC = 1 – (p^2 + q^2)$ for dominant markers, where p and q are the frequencies of present and absence bands respectively [17]; $IB = 1 – [2 \times (0.5 – P)]$, where P is the proportion of accessions containing the band. RP was calculated as $RP = \sum I_B$. The relationship between the RP of primers and the proportion of cultivars that each is able to distinguish was describe by the equation RP = 0.15 x + 1.78, where x is the number of genotypes identified [18].

The genetic diversity among acerola accessions was computed using the different modules of the Numerical Taxonomy and Multivariate Analysis System (NTSYS) software, version 2.10e (Exeter Software; New York, NY, USA) [19]. The similarity indices were calculated by employing the similarity for qualitative data measure included in the SimQual module of the software, based on Simple Matching, Dice and Jaccard coefficients. From these similarity indices, a sequential, agglomerative, hierarchical and nested (SAHN) clustering algorithm was performed, applying the Unweighted Pair Group Method of Arithmetic (UPGMA). Similarity matrices were compared by the Mantel test and the correlation with their corresponding phenograms with the MXCOMP and COPH modules of the NTSYS software, respectively. The phenograms generated from different coefficients were compared by the consensus fork Index (CIc) option, and the cut off line position on dendrogram was calculated using the mean of the similarity matrix data [20]. The best fitted coefficient of the similarity matrix was employed for the diversity analysis.

Results and discussion

RAPD analysis

Seven out of 15 UBC primers (Table 1) produced polymorphic, scoreable and reproducible bands, which were used to generate RAPD profiles of 14 acerola accessions. A representative DNA banding pattern, resulting from RAPD-PCR amplification with primer UBC-231, is shown in Figure 1. The assessment of genetic diversity demonstrates that autogamous or allogamous shrubby plants with a high level of allogamy, like acerola, exhibit a high degree of polymorphism [21].

Regarding the use of RAPD markers for evalua-

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ting genetic diversity of different plant germplasms, including acerola, it has demonstrated to detect a very high polymorphism level, in comparison with the most commonly used molecular markers for the assessment of plant genetic variability [22]. Despite, as far as we know, the genetic diversity of acerola has been achieved by other molecular markers than RAPD. Lima *et al*. [14] effectively discriminated 56 acerola clones from an Embrapa Tropical Agroindustry's germplasm collection with a 79.57 % of polymorphism, by using the Inter Simple Sequence Repeats (ISSR) technique [14]. Similarly, high levels of polymorphism (57.40 and 58.85 %) were detected by this type of markers among 96 acerola accessions collected in different states of Brazil [23]. Remarkably, the analysis of the genetic variability among 24 acerola genotypes showed that the polymorphism level detected by RAPD (90.8 %) was higher than that detected by the Simple Sequence Repeated (SSR) amplification (68 %) [7]. Furthermore, the amplified fragment length polymorphism (AFLP) technique provides a dominant marker with a high polymorphism level of detection, which has been successfully applied in genetics and breeding of various agricultural crops. Otherwise, no previous reports were found in the scientific literature reviewed, for evaluating the acerola genetic diversity by AFLP.

In this scenario, our results demonstrate that the RAPD technique can be suitable for detecting genetic variation within populations of acerola, showing 50 to 100 % polymorphism percentage, the lowest established using the UBC-230 primers and the highest with UBC-221 and UBC-228. Amplification failures were only produced by primer UBC-226 in samples of the acerola accession Amanda. Up to 110 bands were produced, with an average of 16 bands per primers and 200-1500 bp amplicon size range (Table 1). These bands were scored and performed on a binary data matrix. Of them, 32 bands (29.09 %) were monomorphic and 78 (70.91 %) polymorphic, the latter being able to discriminate all acerola accessions evaluated.

It was also found that some primers are more useful than others, their discriminatory power being determined by the PIC and R_p values, the highest the better [24]. In this regard, Lemos *et al*. set 0.5 as the PIC maximal value for dominant markers, and proposed a primer marker dominance classification based on PIC values, in low (0 to 0.10), medium (0.10 to 0.25), high (0.30 to 0.40), and very high (0.40 to 0.50) [18]. According to that classification, six out of the seven primers (Table 1) gave high and very high PIC scores, indicating they could be the most useful for detecting by RAPD the genetic diversity analysis of the 14 acerola accessions tested.

Also, our research show that the R_p values of the seven RAPD UBC primers ranged from 16.0000 (UBC-234) to 7.7143 (UBC-227), with a score of 10.0394 in average. The R_p scores of primers UBC-228, UBC-231 and UBC-234 (Table 1) were superior to the average R_p value. Therefore, according to R_p, these three primers could be considered the most suitable to discriminate the acerola accessions evaluated.

Genetic similarity rating among acerola accessions

The similarity matrices correlated by Mantel test showed the highest correlation value (0.99312) for Jaccard and Dice coefficients (Table 2). Similarly, the consensus index obtained from comparison among UPGAM phenograms was the highest (1.00000) for these coefficients. The cophenetic correlations amongst the similarity matrices and the cophenetic matrices of clusters displayed the highest value of 0.88401 for the Jaccard coefficient in comparison with Dice (0.84681) and Simple Matching (0.82938) [25], Hence, they were interpreted as a good fit for determining genetic variability. Altogether, the results demonstrated that the Jaccard's similarity coefficient as the best for deducing the genetic relationship among the acerola accessions tested (Table 2).

Cluster analysis

A pairwise comparison showed a high variabil-

Table 1. Primers sequences and PCR-Randomly Amplified Polymorphic DNA (RAPD) markers' banding characteristics of Acerola (*Malpighia emarginata* D. C) accessions

Primer code	Primer Sequence (5'-3')	TNB	NPB	PP	ASR (bp)	PIC	Ave Ib	Rp
UBC-221	CCCGTCAATA	13	13	100	300-1500	0.46	0.7253	9.4286
UBC-226	GGGCCTCTAT	11	7	64	300-1500	0.48	1.1619	8.1333
UBC-227	CTAGAGGTCC	9	6	67	300-1500	0.45	1.2857	7.7143
UBC-228	GCTGGGCCGA	15	15	100	300-1500	0.48	0.6762	10.1429
UBC-230	CGTCGCCCAT	18	8	50	200-1500	0.29	0.9821	7.8571
UBC-231	AGGGAGTTCC	19	10	53	200-1500	0.39	1.1000	11.0000
UBC-234	TCCACGGACG	25	19	76	300-1500	0.31	0.8421	16.0000
Total	–	110	78	–	–	0.41	0.9676	10.0394

TNB: Total number of bands. NPB: Number of polymorphic band. PP: Polymorphism percentage. ASR: Amplicon size range. PIC: Polymorphic information content. IB: Band informativeness. RP: Resolving Power.

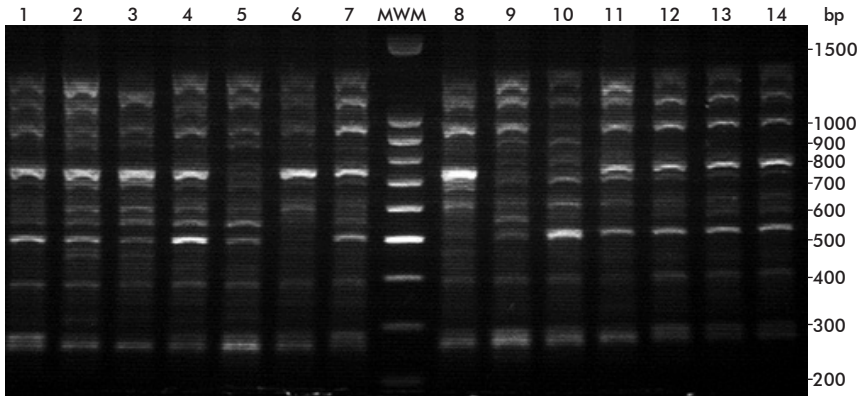

Figure 1. Random Amplified Polymorphic DNA (RAPD) profile for primer UBC-231, showing the genetic relationship among 14 Acerola (Malpighia emarginata D. C) accessions. Lanes: 1, Antonio; 2, Lisset; 3, Víctor; 4, Kevin; 5, Tatica; 6, Helen; 7, Miguel; G8, Javier; 9, Domingo; 10, Anabel; 11, Camila; 12, Amanda; 13, Vitico; G14, Puerto Rico. MWM: Promega 100 pb DNA ladder.

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Table 2. Mantel test correlates of similarity matrices, consensus indexes obtained after comparison among phenograms and similarity matrices correlates with cophenetic clusters of acerola (*Malpighia emarginata* D. C) accessions, by simple matching, Jaccard and Dice correlation coefficients

	Mantel test		Consensus indexes		Clustering modules of similarity (UPGMA)		
Coefficients	Simple matching	Jaccard	Simple matching	Jaccard	Simple matching	Jaccard	Dice
Jaccard	0.95545	–	0.66667	–	0.82938	0.88401	0.84681
Dice	0.95929	0.99312	0.66667	1.00000			

Consensus indexes were obtained by comparison among phenograms, by applying the Unweighted Pair Group Method of Arithmetic (UPGMA). Similarity matrices correlates with cophenetic matrices of clusters were computed by UPGMA module using MXCOMP (matrix comparisons) option of NTSYS to generate the cophenetic correlation coefficients.

ity among the 14 acerola accessions, establishing ten clades that were clearly defined by a cut-off line (0.62) on the phenogram (Figure 2). Acerola accessions Amanda, Vitico and Puerto Rico clustered together in the first clade, the first two the closest (0.87 similarity coefficient).

Domingo and Tatica accessions formed the second clade, and Helen and Miguel accessions the third. The acerola accessions Antonio, Anabel, Camila, Kevin, Javier, Lisset and Víctor clustered apart in individual clades. According to Vieira *et al*. [26], clades with just one accession identifies divergent genotypes. These bears high genetic variability, supporting the obtention of hybrid combinations with higher heterotopic effect, thereby allowing to develop superior genotypes in their subsequent segregating generations [27]. In this case, except for the Antonio accession, the last six were clustered apart from Helen, Miguel, Amanda, Víctor and Puerto Rico by a similarity coefficient of 0.39, achieving the lowest level of similarity, indicative of its divergence.

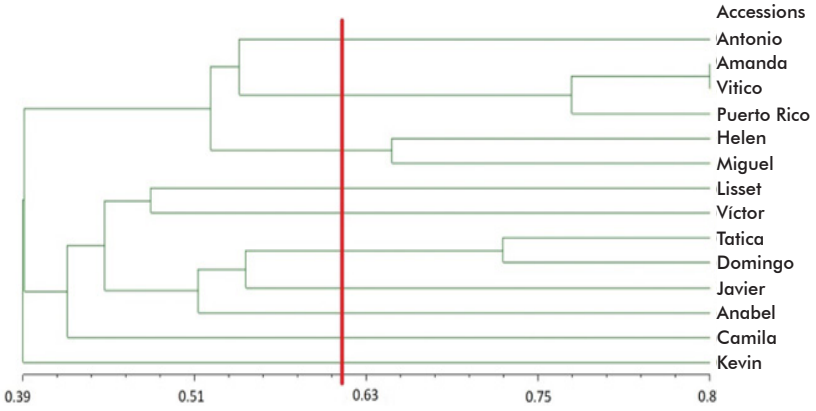

Figure 2. Phenogram of 14 acerola (*Malpighia emarginata* D. C) accessions by Unweighted Pair Group Method of Arithmetic (UPGMA) cluster analysis, using Jaccard's similarity coefficient. The analyzed binary data matrix for 78 RAPD loci was generated from sequences obtained by seven UBC primers. The mean of the similarity matrix data was used to calculate and draw the cut-off line position (0.62) in the phenogram, distributed in ten clusters.

Conclusions

Our results demonstrated that RAPD-PCR could be an effective technique for the assessment of the genetic relationship among acerola accessions. This becomes a starting point to further characterize the genetic variability of the acerola genetic resources available at the gene bank of the Tropical Fruit Culture Research Institute of Cuba. Among the 14 acerola accessions tested, Anabel, Camila, Kevin, Javier, Lisset and Víctor were the most divergent, being regarded as the most promissory parents for future acerola crossbreeding studies. Prospectively, these selected accessions should be genotyped, using a combination of other markers such as AFLP, ISSR and SSR, to corroborate their genetic diversity, and to develop and run a successful acerola breeding program in Cuba.

Conflicts of interest statement

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