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Molecular characterization of Cuban endemism *Carica cubensis* Solms using random amplified polymorphic DNA (RAPD) markers

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ABSTRACT

The objective of this work is to present an appropriate set of RAPD (random amplified polymorphic DNA) markers using single and multiplex PCR analysis suitable for the characterization of the endemic Cuban species *Carica cubensis* and the establishment of genetic relationships with the cultivated species *Carica papaya*. RAPD markers presented a high level of polymorphism. In addition, the incorporation of more than one RAPD primer in the PCR analysis increased the number of obtained bands and the polymorphism of these bands. A total of 73 RAPD bands were detected (45 of them polymorphic) with the nine RAPD markers assayed using single and multiplex PCR analysis. Results demonstrated a reduced genetic variability within the tested *Carica cubensis* accessions. The observed clustering in this species could be better explained according to geographic proximity and can indicate the similar precedence of the isolated studied populations. *C. cubensis* seem to be subspecies of *C. papaya* adapted to the environmental conditions of the mountains of Cuba or a endemic species close to *C. papaya*. The implications of these results in the creation of effective germplasm core collection in *Carica* species have been also discussed.

Keywords: Carica Species; Germplasm; Molecular Markers; RAPDs; Breeding

1. INTRODUCTION

Carica is a genus of family Caricaceae originating from Central and South America including more than forty different species being *Carica papaya* L. the most important species from the economically and agronomy point of view and the most cultivated species in these areas of Central and South America [1,2]. Cuba can be considered an area of putative endemism [3,4] of these species according to the origin and dispersion of the different accessions analyzed. In this sense, one of these examples of related species is *Carica cubensis* Solms syn. *Carica prosoposa* L., an endemic fruit tree species from Cuba considered a papaya endemism in preliminary studies [5]. This specie was described at the first time by Solms and Grafen in 1889 [6] indicating the presence of the specie in forested areas of Cuba since Baracoa to Portero of St. Andre.

Carica cubensis is a shrub from 2 to 4 meters with a thick trunk and branches and leaves spongy no alternated and terminals. As well as *Carica papaya*, this species is at the same time dioecious with female and male plants, monocious with female and male flowers in the same plant, and polygamous with hermaphrodite flowers [7,8]. Flowers are white to yellow-green and can have three sex forms male (in the case of male plants) and female and hermaphrodite (in the case of female plants) **Figure 1**. Only the two last types give fruits. The fruit with a size around 80-100 grams is smaller than the cultivated papaya and contains an enzyme (papaine) with intensive digestive action that can be used for medicinal purpose against dyspepsia disease and with antitumoral properties [5,9]. *Carica cubensis* is a wild related species of the cultivated papaya species (*Carica papaya*) with a lot of potential from the agronomic and breeding point of view. Some authors, however, included this *Carica cubensis* species inside the *Carica papaya* species. This species can be particularly useful in marginal lands, where they have been selected to withstand stress conditions and where they contribute to sustainable production with few inputs.

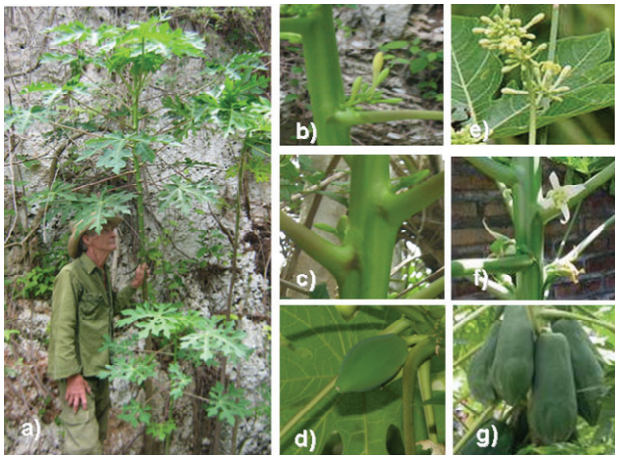

Figure 1. General overview of the *Carica cubensis* shrub (a) and detail of racine male flower, female or hermaphrodite flower and globose fruit in *C. cubensis* (b, c, and d respectively) and *C. papaya* (e, f, and g respectively).

Traditionally, characterization and identification of variability in the fruit species such as *Carica cubensis* has been based on morphological descriptors [5]. Molecular marker technology offers several advantages over the sole use of traditional descriptors and has become an essential tool for the study and conservation of the fruit species. DNA marker technology offers great advantages for the characterization of these rare fruit germplasm [10]. The more recent utilization of PCR-based markers has increased the opportunities for DNA characterization of populations in a wider range of species [11]. In this sense, random amplified polymorphic DNA (RAPD) markers based on the PCR amplification of random locations in the genome [12,13] can be a suitable marker to the first molecular analysis of species such as *Carica cubensis* [14]. RAPD markers utilize random primers that amplify random DNA sequences in the genome. This results in differential amplification of regions that vary in primer site sequence resulting in polymorphic amplification products usually analyzed as presence/absence. These markers have been previously assayed in the molecular characterization of *Carica papaya* cultivars [15-18] and some wild related species [14,19,20], the identification of flower sex types [8,21-25] and development of genetic linkage maps [26]. In addition, to improve the capacity of the molecular characterization assays using these PCR-based markers, multiplex PCR, a variant of the PCR in which more than one target sequence is amplified using more than one pair of primers are being assayed [27-29].

The objective of this work is to present an appropriate set of RAPD markers suitable for the molecular characterization and establishing of genetic relationships in the endemic Cuban species *Carica cubensis* using single and multiplex PCR analysis and to establish the genetic relationships with the cultivated species *Carica papaya*.

2. MATERIAL AND METHODS

2.1. Plant Material

Young well expanded leaf samples from 18 individual *Carica cubensis* trees growing in isolated clusters in natural populations were collected at the tropical mountains of Cordillera Habana Matanza, around 260 m altitude, in three different locations (Lomas Escaleras de Jaruco, Recria and Lomas Francisco Javier) between the cities of Jaruco and San José de las Lajas in the province of La Habana (Northwest of Cuba) **Figure 2**. In addition, three known genotypes of *Carica papaya* were included in the study **Table 1**. Leaf samples were lyophilized before DNA extraction in a Pharma Lyophilizer (Pharma Biotek, Chennai, India) and stored at room temperature.

2.2. DNA Extraction

Lyophilized leaves were used for DNA isolation using the procedure described by Doyle and Doyle [30] with some modifications. 40 mg of healthy leaf blade was ground, without use of liquid nitrogen, in 2 ml tube containing 750 µl of worm (65ºC) CTAB extraction buffer. Homogenate was incubated in a water bath at 65ºC for 15 min, mixed with an equal volume of 24:1 chloroform: isoamyl alcohol and centrifuged at 6,000 g (20 min). The upper phase was recovered and mixed with an equal vo-

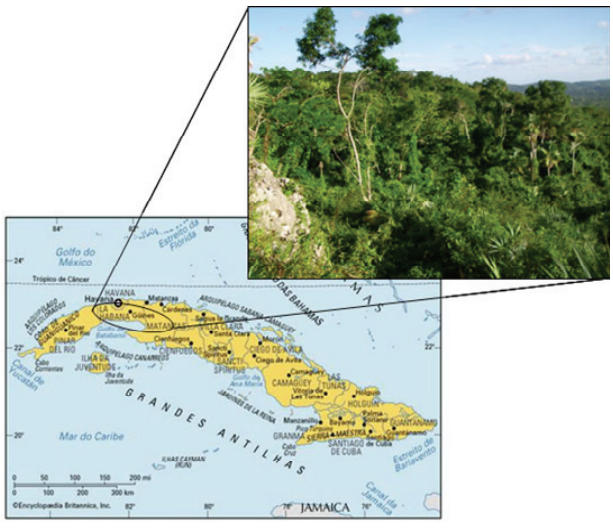

Figure 2. Location of natural populations of *Carica cubensis* collected in Northwest of Cuba and general overview of the region.

Table 1. *Carica cubensis* and *Carica papaya* accessions assayed.

Accession	Species	Origin	Sex of the plant
"Lomas Escaleras-1"	*Carica cubensis*	Seedling collected in Lomas Escaleras	Plant without flower
"Lomas Escaleras-2"	*Carica cubensis*	Seedling collected in Lomas Escaleras	Plant without flower
"Lomas Escaleras-3"	*Carica cubensis*	Seedling collected in Lomas Escaleras	Female
"Lomas Escaleras-4"	*Carica cubensis*	Seedling collected in Lomas Escaleras	Plant without flower
"Lomas Escaleras-5"	*Carica cubensis*	Seedling collected in Lomas Escaleras	Plant without flower
"Lomas Escaleras-6"	*Carica cubensis*	Seedling collected in Lomas Escaleras	Plant without flower
"Recria-1"	*Carica cubensis*	Seedling collected in Recria area	Plant without flower
"Recria-2"	*Carica cubensis*	Seedling collected in Recria area	Plant without flower
"Recria-3"	*Carica cubensis*	Seedling collected in Recria area	Plant without flower
"Recria-4"	*Carica cubensis*	Seedling collected in Recria area	Plant without flower
"Recria-5"	*Carica cubensis*	Seedling collected in Recria area	Plant without flower
"Recria-6"	*Carica cubensis*	Seedling collected in Recria area	Plant without flower
"Lomas Francisco-1"	*Carica cubensis*	Seedling collected in Lomas Fco Javier	Male
"Lomas Francisco-2"	*Carica cubensis*	Seedling collected in Lomas Fco Javier	Male
"Lomas Francisco-3"	*Carica cubensis*	Seedling collected in Lomas Fco Javier	Female
"Lomas Francisco-4"	*Carica cubensis*	Seedling collected in Lomas Fco Javier	Plant without flower
"Lomas Francisco-5"	*Carica cubensis*	Seedling collected in Lomas Fco Javier	Female
"Lomas Francisco-6"	*Carica cubensis*	Seedling collected in Lomas Fco Javier	Male
"Papaya"	*Carica papaya*	Open pollination of cultivar "Papaya"	Female
"Maradol Roja"	*Carica papaya*	Cuban commercial cultivar	Plant without flower
"1500"	*Carica papaya*	Cuban commercial cultivar	Plant without flower

lume of isopropanol at –20°C. The nucleic acid pellet was washed in 400 µl of 10 mM NH4Ac in 76% ethanol, dried, resuspended in 50 µl of TE, incubated with 0.5 µg of RNase-A at 37ºC for 30 min to digest RNA, and quantified using a Biophotometer (Eppendorf, Barcelona, Spain).

2.3. Random Amplified Polymorphic DNA (RAPD) Marker Application

Nine RAPD universal primers (OPA-07, OPB-07, OPN-14, OPR-15, OPR-16; OPW-12, OPW-13, OPY-13 and OPZ-17) purchased from Operon Biotechnologies (Huntsville, USA) were assayed performing single (one primers) and multiplex (combination of two or three primers) PCR analysis **Table 1**. Amplifications were carried out in 20 µL total volume containing 1 × Buffer, 1 mM $MgCl_2$, 0.16 mM of dNTP, 0.4 µmol of each primer, 1.0 unit of *Taq* DNA polymerase (New England Biolabs, Ipswich, USA), and 4 ng templates DNA. The amplification program consisted of a step of DNA melting of 4 min at 94°C, followed by 35 cycles of 94°C for 1 min, 35°C for 1 min, and 72°C for 1 min, and a final elongation step of 72°C for 10 min. Amplified products were resolved in 2% agarose gels stained using Gel Red Nucleic Acid Gel Sating® (Biotium, Hatwad, CA, USA) and visualized with UV transmitted light. A 1 Kb DNA Ladder (Invitrogen Life Technologies, Barcelona, Spain) was used as molecular size standard. RAPD amplifications were repeated at least twice in order to check the reproducibility of bands.

2.4. Data Analysis

Polymorphic alleles were scored as present or absent (1/0). DNA band scoring was analyzed using GeneTools gel analysis software of SYNGENE (Beacon House, Nuffield Road, Cambridge, UK). The average polymorphic information content (PIC) was calculated for RAPD markers across assay units by applying the formula given by Powell *et al.* (1996). Mean character difference distances were calculated for all pairwise comparisons with the MEGA4 test (http://www.megasoftware.net/) [31], which was used to construct UPGMA dendograms [32] depicting the phenetic relationship among the different accessions. Relative support for the branches in

each dendrogram was assessed with 2000 replicates of UPGMA bootstrap.

3. RESULTS AND DISCUSSION

The nine RAPD primers (OPA-07, OPB-07, OPN-14, OPR-15, OPR-16; OPW-12, OPW-13, OPY-13 and OPZ-17) assayed generated polymorphic and reproducible patterns in the *Carica papaya* and *C. cubensis* assayed genotypes. In addition, to improve the capacity and polymorphism of the molecular characterization assays using RAPD markers different multiplex PCR using more two or three RAPD primers have been assayed. Results showed that the incorporation of more than one RAPD primer in the PCR analysis increased the number of obtained bands and the polymorphism of these bands. The number of RAPD bands detected by each primer depended on species, primer and the single or multiple PCR analysis performed. A total of 73 RAPD bands were detected (45 of them polymorphic) with the nine RAPD markers assayed in *C. cubensis* using single and multiplex PCR analysis with a size range between 105 and 3600 bp.

The total number of bands varied from 3 (for OPN-14) to 7 (for OPW-12 and OPZ-13/OPW-12/OPN-14 primer combination), with an average of 4.8 bands per analysis. In addition, the mean PIC score over all loci was 0.70, ranging from 0.33 in OPN-14 to 0.85 in OPZ-13/OPW-12/OPN-14 primer combination **Table 2**. Even using lyophilized leaf samples results showed a high yield and good quality DNA with good results after the PCR analysis **Figure 3**. This fact is very important taking in account that the most crucial factor for the application of RAPD technology is the DNA quality and concentration [33,34].

In the case of *C. papaya* polymorphism was lower due to the reduced number of accessions assayed (only three) although proportionally higher than the results obtained in *C. cubensis* in which we assayed 18 accessions. A total of 64 RAPD bands were detected (28 of them polymorphic) with the nine RAPD markers assayed in *C. cubensis* using single and multiplex PCR analysis with a size range between 105 and 3600 bp. The total number of bands varied from 0 (for OPN-14, OPW-13, OPY-13) to 3 (for OPB-07, OPY-13/OPB-07/OPA-07 and OPW-13/OPR-16/OPR-15 primer combination, with an average of 4.2 bands per analysis. In addition, the mean PIC score over all loci was 0.33, ranging from 0.00 in OPN-14, OPW-13 and OPY-13 to 0.63 in OPW-13/OPR-16/OPR-15 primer combination **Table 2**.

RAPD analysis showed higher number of bands (abundance) and higher polymorphism in comparison with other markers as has been described in papaya by Jobin-Décor [19] and other tropical species such as cacao [35] or coffee [36]. The low heterozygosities values showed in this study can be a consequence of the low number of *Carica cubensis* accessions assayed which can also be genetically close according with the geographic proximity of the samples collected. For all the loci studied the expected heterozygosity was greater than the observed heterozygosity, implying the presence of null alleles or a deficit of heterozygotes due to non-random mating. The presence of common RAPD alleles indicated the closer genetic distance between the cultivated papaya and *C. cubensis* in comparison with other wild papaya species before studied including *C. cauliflora*, *C. pubescens*, *C. parviflora* or *C. quercifolia* [14, 19,20]. *C. cubensis* seem to be subspecies of *C. papaya* adapted to the environmental conditions of the mountains of Cuba or a Cuban endemic species close to *C. papaya* as was indicated previously by Leon and Alain [5].

Phenetic (taxonomy) relationships among *Carica cubensis* accessions were analyzed with several UPGMA dendograms **Figure 4**. Relationships moderately sup

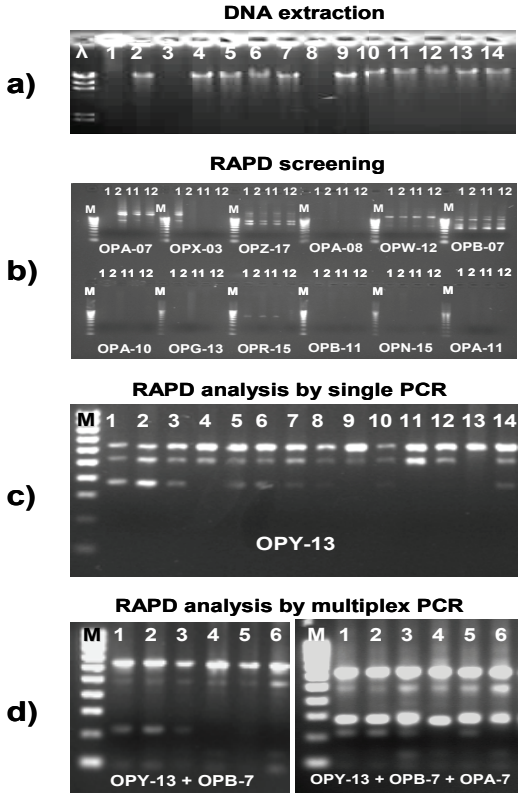

Figure 3. Agarose gel showing the DNA extraction of *Carica* samples (a); the first screening of RAPD markers in some genotypes (b); and the application of RAPD markers in all the genotypes using simple (c) and multiplex (d) PCR analysis. Λ DNA quantification marker HindII from Invitrogen. M DNA ladder 1 Kb plus from Invitrogen.

Table 2. Universal primers (decamers) purchased from Operon Biotechnologies used as RAPD markers molecular characterization of the *Carica cubensis* accessions assayed.

	Carica cubensis			
Marker	**Number of bands**	**Polymorphic bands**	**Size (bp)**	**PIC**
OPA-07	4	2	500-800	0.58
OPB-07	5	3	490-5490	0.83
OPN-14	3	1	970-2170	0.33
OPR-15	4	3	560-1110	0.83
OPR-16	5	3	750-3200	0.71
OPW-12	7	4	700-3250	0.75
OPW-13	4	2	500-1400	0.55
OPY-13	3	1	376-755	0.31
OPZ-17	6	4	400-915	0.76
OPY-13/OPB-07	4	3	95-755	0.79
OPY-13/OPB-07/OPA-07	5	3	95-1046	0.82
OPZ-13/OPW-12	6	4	500-2500	0.83
OPZ-13/OPW-12/OPN-14	7	5	300-3600	0.85
OPW-13/OPR-16	5	4	750-3500	0.80
OPW-13/OPR-16/OPR-15	5	3	750-3500	0.80

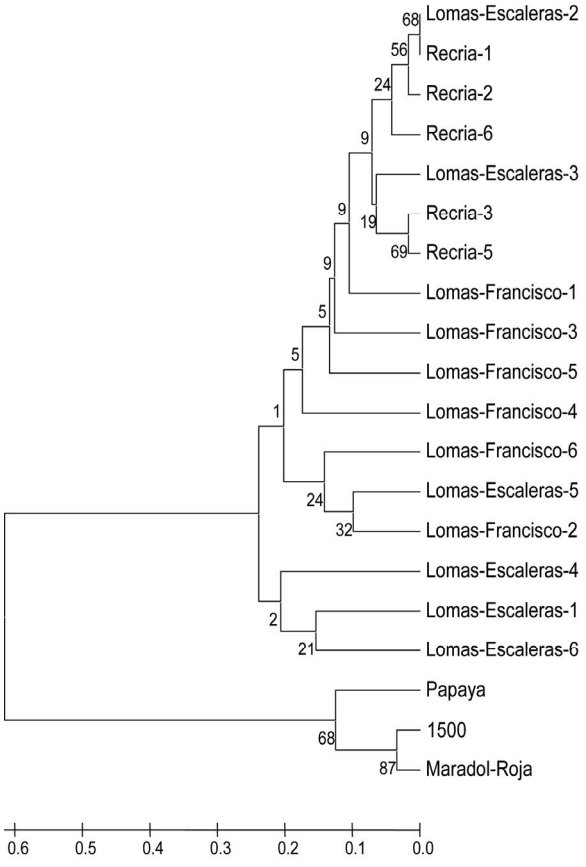

Figure 4. Dendograms obtained by UPGMA cluster analysis based on mean character differences among the *Carica cubensis* and *Carica papaya* accessions assayed using RAPD markers by single and multiplex PCR anlaysis. Numbers in the branches represent bootstrap values.

ported established two groups in relation to the two different studied species. In addition, bootstrap values of UPGMA dendogram obtained with the utilization of RAPDs were slightly higher. In the case of *C. cubensis* in the most of cases accessions studied from a location are closer than accessions from other locations. This clustering is explained by geographic proximity and can indicate the similar precedence of the isolated studied populations. However, no clustering and RAPD markers association was observed in our samples according to the sex characterization as has been before observed in *C. papaya* species [8,21-23]. These results also can support both establish hypothesis *C. cubensis* as a subspecies of *C. papaya* or a Cuban endemic species close to *C. papaya*.

One of the goals of conservation programs in these rare fruit species such as *Carica cubensis* is to characterize and maintain existing level of variation and genetic resources [10]. Genetic resources not only provide the required raw material for suitable genetic crop improvement, but offer a unique gene combination to ensure adaptability and productivity [37,38]. Designing of core collection using suitable DNA markers involves an appropriate use of diversity, offering breeders an opportunity to work with a manageable number of accessions. *C. cubensis* is a better adapted species to the Cuban conditions and could also be a source of new genes for low temperature resistance or disease resistance including the Papaya Ring Spot Virus (PRSV) [39,40]. Other important characteristic of this species is the smaller fruit (around 100 grams) which will be a new good commercial trait.

Designing of core collection involves an appropriate use of diversity, offering breeders an opportunity to work with a manageable number of accessions. Therefore, the availability of an optimized protocol for DNA characterization to estimate the genetic diversity and to ensure genetically representative non-redundant samples is of great interest [41]. In this sense, universal RAPD markers can be a most suitable in the molecular characterization of *C. cubensis* accessions and to construct efficient core collections as has been described by Bortolini [42] in white clover complementary to the application of more recent developed markers in *C. papaya* of dominant SPAR (Single primer amplification reaction) [17] and SCAR (sequence characterized amplified region) markers [25], and codominant SSR (Single sequence repeat) markers [43,44].

Other additional advantages of RAPD markers are that do not require labour intensive and expensive, no genomic/cDNA libraries are need for development of probes and very small quantity of DNA is need in the PCR reactions even using lyophilized leaf samples.

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