

ARTICLE

Genetic diversity of Cuban pineapple germplasm assessed by AFLP Markers

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Abstract - The Cuban pineapple germplasm collection represents the genetic diversity of pineapple cultivated in that country and includes other important genotypes obtained from the germplasm collections in Brazil and Martinique. The collection has previously been characterized with morphological descriptors but a molecular characterization has been lacking. With this aim, 56 six genotypes of *A. comosus* and one of *Bromelia pinguin* were analyzed with a total of 191 AFLP markers. A dendrogram that represents the genetic relationships between these samples based on the AFLP results showed a low level of diversity in the Cuban pineapple collection. All *Ananas comosus* accessions, being the majority obtained from farmers in different regions in Cuba, are grouped at distances lower than 0.20. Molecular characterization was in line with morphological characterization. These results are useful for breeding and conservation purposes.

Key words: molecular markers, accessions, Ananas.

INTRODUCTION

Pineapple (*Ananas comosus* (L) Merr) is an important crop for many countries in Central and South America as well as in the Asia-Pacific region. Most of the pineapple production is based on a few leading cultivars, such as Smooth Cayenne and MD2. In Cuba, Spanish cultivars are predominant.

Pineapple germplasm is maintained in several collections around the world. The most important are the collections maintained by EMBRAPA/CNPMPF, in Cruz das Almas, Brazil, by CIRAD-FLHOR, in Martinique, and the USDA collection, in Hawaii. These collections have been partially characterized with morphological descriptors (Leal et al. 1986, Duval and Coppens d'Eeckenbrugge 1993, Ferreira and Cabral 1993, Duval et al. 1996). Cuba maintains a small but still important collection, since it represents the genetic diversity of the cultivated pineapple in that country. Most of the genotypes in the Cuban pineapple germplasm collection at the Bioplants Center (Ciego de Ávila, Cuba) have been obtained from farmers or through exchanges with other collections (Isidrón et al. 2003).

Aradhya et al. (1994) and De Wald et al. (1992) have previously used isozyme polymorphism in the genus *Ananas* to clarify taxonomical aspects. However, the scope of these studies is limited due to the low number of markers. Later, Duval et al. (2001) and Duval et al. (2003) used RFLP markers and chloroplast genotypes or genoma to study genetic diversity in *Ananas*. Three hundred and one accessions including all *Ananas* species and the related species *Pseudoananas sagenarius* were tested. This technique revealed a higher level of polymorphism since 41 % of the probes were polymorphic. Based on these studies, Coppens d'Eeckenbrugge and Leal (2002) have proposed a simplification for the pineapple classification. In this new classification, the seven *Ananas* species proposed by Smith and Downs (1979) are downgraded to the level of five botanical varieties of *A. comosus*.

AFLP markers (Vos et al. 1995) have also been widely used to study diversity in several species such as *Arabidopsis* (Breyne et al. 1999), coffee (Coulibaly et al. 2003), *Rubus* (Marulanda et al. 2007) and *Jatropha curcas* (Santos et al. 2010). The technique has also been employed to identify varieties in pineapple (Leal et al.

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1986) and carry out classical genetic mapping studies (Carlier et al. 2004). Kato et al. (2004), Paz et al. (2005) and Tapia Campos et al. (2005) proved its suitability for germplasm evaluation.

In this work, AFLP markers were used to assess the genetic diversity of the Cuban pineapple germplasm collection that represents both commercially cultivated material grown in Cuba and germplasm used in ongoing breeding programs.

MATERIALS AND METHODS

Fifty-six genotypes, including fifty-five genotypes of *Ananas comosus* and one of *Bromelia pinguin*, belonging to the Cuban National Pineapple Germplasm Collection at the Bioplants Centre (Ciego de Ávila, Cuba), were analyzed (Table 1). The names used in this article correspond with common names used locally. The species were indicated according to the last classification by Coppens d'Eeckenbrugge and Leal (2002).

Table 1. Pineapple cultivars and relatives analyzed in the study

	Genus	Species	Fruit type/leaf spinness	Cultivar
1	Ananas	comosus	Red Spanish typical ¹ /spiny	Española Roja Pinareña
2	Ananas	comosus	Red Spanish typical/spiny	Española Roja Nozerán
3	Ananas	comosus	Red Spanish typical/spiny	Española Roja Colorada del Caney
4	Ananas	comosus	Red Spanish typical/spiny	Española colorada de Ramón
5	Ananas	comosus	Red Spanish typical-big fruit/spiny	Cabezona
6	Ananas	comosus	Red Spanish typical/spiny	Española Roja Jíbara del Caney
7	Ananas	comosus	Red Spanish typical/spiny	Española Caney
8	Ananas	comosus	Red Spanish typical/spines only on one border	Española Roja un borde liso
9	Ananas	comosus	Red Spanish typical/spiny	Española Roja M 35
10	Ananas	comosus	Red Spanish typical/spiny	Española Roja M 25
11	Ananas	comosus	Red Spanish typical/spiny	Española Roja Enana
12	Ananas	comosus	Red Spanish typical/very few spines	Española Roja P3R5
13	Ananas	comosus	Smooth Cayenne typical ² /Only at the tip	Cayena lisa de Oriente
14	Ananas	comosus	Smooth Cayenne typical/Only at the tip	Cayena de Martinica
15	Ananas	comosus	Smooth Cayenne typical/Only at the tip	Cayena lisa Serrana
16	Ananas	comosus	Smooth Cayenne typical/spiny	Cayena lisa Barón de Rosthshilt
17	Ananas	comosus	Smooth Cayenne typical/Only at the tip	Cayena de Kenia
18	Ananas	comosus	Smooth Cayenne typical/Only at the tip	Cayena de México
19	Ananas	comosus	Smooth Cayenne typical/Only at the tip	Cayena de Panamá
20	Ananas	comosus	Smooth Cayenne typical/Only at the tip	Cayena de Rep. Dominicana
21	Ananas	comosus	Smooth Cayenne typical/Only at the tip	Cayena de Sao Tomé y Príncipe
22	Ananas	comosus	Smooth Cayenne typical/Only at the tip	Cayena de Ecuador
23	Ananas	comosus	Smooth Cayenne typical/Only at the tip	Cayena de Hawaii
24	Ananas	comosus	Smooth Cayenne- big fruit/No spines	Mocaena
25	Ananas	comosus	Red Spanish typical-big fruit/spiny	Puerto Rico
26	Ananas	comosus	Cylindrical, 1-1,5 kg. White flesh/No spines	Primavera
27	Ananas	comosus	Pyramidal. 1-5 kg. White flesh/spiny.	Piña Blanca del Caney
28	Ananas	comosus	Cylindrical, 1-1,5 kg. White flesh/No spines	Pomare
29	Ananas	comosus	Comosus var. bracteatus	Small, not edible/spiny
30	Ananas	Comosus var. bracteatus	Small, not edible/spiny	Branco
31	Ananas	Comosus var. bracteatus	Small, not edible/spiny	Var Típico
32	Bromelia	pinguin	Small ovoid, not edible/spiny	Piñuela Karata
33	Ananas	comosus	Cylindrical 1-1.5kg/spiny	Jupi

to be continued...

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34	Ananas	comosus	Red Spanish typical/spiny	Española Roja(1)
35	Ananas	comosus	Red Spanish typical/spiny	Española Roja(3)
36	Ananas	comosus	Red Spanish typical/spiny	Española Roja(8)
37	Ananas	comosus	Red Spanish typical/spiny	Española Roja(9)
38	Ananas	comosus	Red Spanish typical/spiny	Española Roja (10)
39	Ananas	comosus	Red Spanish typical/spiny	Española Roja(12)
40	Ananas	Comosus	Red Spanish typical/spiny	Española Roja(12A)
41	Ananas	Comosus	Red Spanish typical/spiny	Española Roja(15)
42	Ananas	Comosus	Red Spanish typical/spiny	Española Roja (18)
43	Ananas	Comosus	Smooth Cayenne typical	Cayena de Francia 921
44	Ananas	Comosus	Similar to Smooth Cayenne/few spines	Híbrido cxe 202
45	Ananas	Comosus	Similar to Smooth Cayenne/few spines	Híbrido cxe 203
46	Ananas	Comosus	Similar to Smooth Cayenne/few spines	Híbrido cxe 287
47	Ananas	Comosus	Red Spanish typical/spiny	Piña criolla
48	Ananas	Comosus	Smooth Cayenne typical/Only at the tip	Cayena Melba
49	Ananas	Comosus	Red Spanish typical/spiny	Española Roja (I-122)
50	Ananas	Comosus	Red Spanish typical/spiny	Española Roja
51	Ananas	Comosus	Red Spanish typical/spiny	Española Roja (I-126)
52	Ananas	Comosus	Red Spanish typical/spiny	Española Roja (I-127)
53	Ananas	Comosus	Red Spanish typical/spiny	Española Roja (I-128)
54	Ananas	Comosus	Red Spanish typical/spiny	Española Roja (I-129)
55	Ananas	Comosus	Red Spanish typical/spiny	Española Roja (I-130)
56	Ananas	Comosus	Red Spanish typical/spiny	Española Roja (I-131)
57	Ananas	Comosus	Similar to Smooth Cayenne/few spines	Híbrido 1339

¹ Red Spanish Typical- fruit weight around 2 kg, pale yellow flesh with pleasant aroma; squarish in shape. Well adapted for shipping as fresh fruit to distant markets. Spiny leaves.

² Smooth Cayenne typical- Fruit weight between 2.5-3 kg, pale yellow to yellow flesh. Cylindrical in shape and with high sugar and acid content. Well adapted to canning and processing. Leaves with spines only at the tip.

Leaf samples were collected and stored at -20 °C until DNA extraction. DNA isolation was based on the protocol described by Kobayashi et al. (1998) with several modifications. It was used 250 mg of fresh young leaf tissue, which was grinded in liquid nitrogen and resuspended by vortexing in 650 µL of extraction buffer (50 mmol L⁻¹ Tris-Cl, pH 7.5, 20 mmol L⁻¹ EDTA, pH 8.0, 0.3 mmol L⁻¹ NaCl, 2 % sarcosyl, 0.5 % SDS [Sodium Dodecyl Sulfate] and 4.8 mol L⁻¹ urea). Then, an equal volume (650 µL) of phenol-chloroform-isoamyl alcohol (25:24:1, v:v:v) was added. After centrifugation at 12 000 rpm for 15 min, at room temperature in a tabletop centrifuge, the DNA in the liquid phase was precipitated by adding 0.8 volume of isopropanol at room temperature and centrifuging for 10 min at 12 000 rpm. The pellet was then washed with 70 % ethanol and the DNA was resuspended in 50 µL of DNAase water free, containing 10 µg mL⁻¹ of Rnase A. The quality and integrity of DNA was checked in a 0.8 % agarose gel. DNA concentration was visually estimated by using a series DNA of standards.

AFLP analysis was carried out according to Vos et al. (1995). The EcoRI+A (5'-AGACTGCGTACCAATTC/A-3'), and the MseI+A (5'-GACGATGAGTCCTGAGTAA/A-3') oligonucleotide primers were used for the preamplification step. This step was followed by a second selective amplification step using three selective nucleotides. The EcoRI primer was kept constant with the selective nucleotides AAT whereas the Mse I primer varied with addition of an extra AG, TG, GT or CC.

Autoradiograms were analyzed visually and scored as 1= presence of band, 0= absence of band. Genetic similarity was calculated using the NTSYS-pc software (Rolf 2000) using the simple matching coefficient (SMC) (Skroch et al. 1992). Given two samples *i* and *j*, SMC is the sum of the number of AFLP bands that are present in both samples plus the number of AFLP bands absent in both samples, divided by the total number of possible bands in the study. This coefficient varies between 0-1. Genetic distances were calculated using the formula: Distance=1-SMC. Cluster analysis was based on distance

matrices using the unweighted pair group method with arithmetic averages (UPGMA) and relationships between samples were graphically presented as dendrograms.

RESULTS AND DISCUSSION

A total of 191 scorable AFLP bands were generated with four primer combinations (Table 2). The levels of polymorphism of the collection were: 87.4 % polymorphism considering both the genera *Ananas* and *Bromelia*, and 64.3 % considering only *Ananas comosus*. The use of the four primer combinations allowed differentiating all genotypes one another. Indeed, it was possible to differentiate the somaclones from Española Roja pinareña M-35 and P3R5 obtained by Pérez (2008). These somaclones were also differentiated previously by Pérez et al. (2008) using AFLP.

In this study the average number of bands per primer combination was 44.6. The number of bands per enzyme/probe combination informed by Duval et al. (2001) using RFLP markers was 7.9. Tapia et al. (2005) obtained 5.9 bands per primer combination using ISSR markers and 6.83, using RAPD markers. Bejaj et al. (2003) compared RAPDs, AFLPs and SSRs in terms of their informativeness and efficiency in a study of genetic diversity and relationships among 32 olive cultivars grown in Italy and Spain. SSRs presented a higher level of polymorphism and greater information content, as assessed by the expected heterozygosity, than AFLPs and RAPDs.

The highest genetic distance (0.58) was found between *Bromelia pinguin* and the rest of the accessions. All *Ananas comosus* accessions were grouped at distances lower than 0.20. Two main clusters can be identified in the dendrogram (Figure 1), one includes Red Spanish genotypes and hybrids from Red Spanish and Cayenne and the other consists mainly of Smooth Cayenne genotypes. According to Isidrón et al. (2003), the Red Spanish accessions are the most cultivated in Cuba. They are barrel-like fruits, with spiny leaves and are very well adapted to the Cuban climate conditions. On the other hand, Smooth Cayenne genotypes are characterized by

their cylindrical fruits and leaves with spines only at the tip. They are less adapted to Cuban climate conditions and more susceptible to soil diseases (Isidrón et al. 2004). Similarly, there are some other small genotypes clusters, including other less related cultivars. Although a similar study has not been done with morphological markers, the partial characterization carried out with this kind of markers, the results obtained with isozyme markers and the data obtained from curators allow us to make some comparisons with the molecular analysis.

The levels of polymorphism detected in the present study were acceptable taking into consideration the low genetic diversity of the collection. Paz et al. (2005), using AFLP markers, obtained 98.2 % of polymorphism on 39 genotypes of the Mexican pineapple germplasm collection. This is superior to the 87.4 % obtained by the Cuban collection. However, the Mexican collection includes three genotypes of *Bromelia* (two more than the Cuban). On the other hand, Duval et al. (2001), using RFLP markers, found 94.4 % polymorphism among 294 accessions belonging to five *Ananas* species, and 74.7 % among 167 accessions of *Ananas comosus* (according to the classification by Smith and Downs [1979]). However these results are not directly comparable to this work since here a much narrower collection of germplasm was analyzed. Another work was done in pineapple using isozyme markers (De Wald et al. 1992) but its scope is very limited due to the low level of polymorphism and the number of loci being identified. The levels of polymorphism are in close relation with the species and the number of genotypes being evaluated. For instance, using AFLP markers Tang et al. (2003) obtained 88.5 % polymorphism among 125 genotypes of *Hibiscus tileaceus*, while Zong et al. (2003) reached only 55 % polymorphism among 156 accessions of *Vigna angularis*.

The AFLP technique allowed to separate clearly the wild species *Bromelia pinguin* and *Ananas* genotypes from the rest of the genotypes of that genus. Likewise, almost all Red Spanish genotypes were separated from Cayenne's genotypes. Among Red Spanish genotypes some data support the robustness of the molecular analysis. For instance, Española M-35 is a somaclone of Española Roja Pinareña,

Table 2. AFLP Primer combinations, number of markers and levels of polymorphism

Primer combination	Number of markers	Total number of polymorphic markers	Polymorphism (%)	Number of polymorphic markers in <i>Ananas</i>	Polymorphism (%)
Eco-AAT, Mse-AAG	46	36	78.2	17	37.0
Eco-AAT, Mse AAC	43	37	86.0	29	67.4
Eco-AAT, Mse-AGT	50	46	92.0	40	80.0
Eco-AAT, Mse ATG	52	48	92.3	37	71.1
Total	191	167	87.4	123	64.3

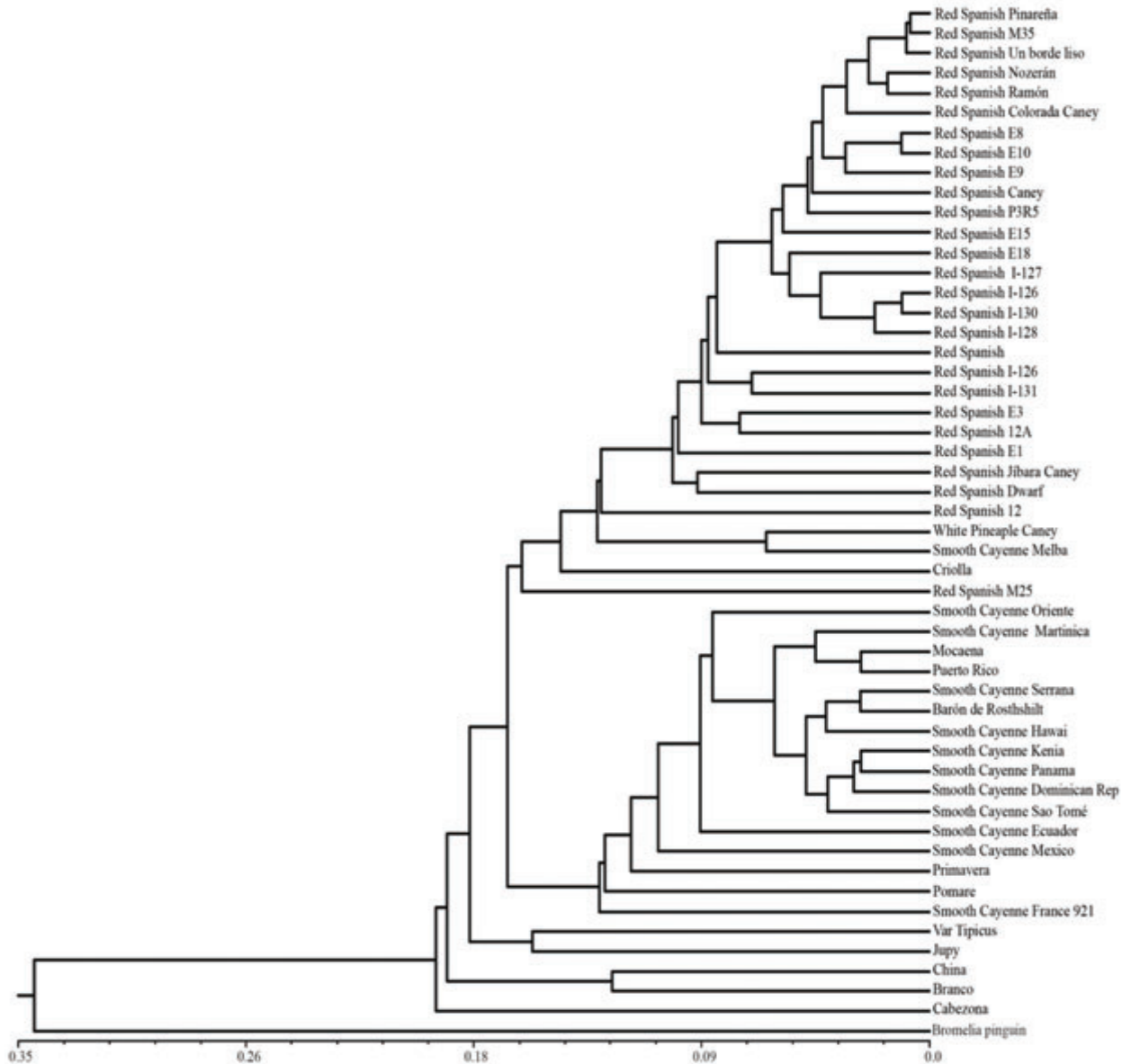


Figure 1. Dendrogram showing the diversity of the Cuban pineapple germplasm collection. Accessions are presented with cultivar names.

which is morphologically very similar to it; both genotypes are located very close to each other in the dendrogram. The other somaclone of Española Roja Pinareña (Española Roja P3R5), with certain morphological differences, such as the absent of spines on the leaf margins and a different agronomical behavior (Pérez 2008), is located more distant from it in the dendrogram, but as a member of a little *cluster* that includes Española Roja Pinareña.

Española Roja (8), Española Roja (9) and Española Roja (10) are morphologically very similar and were collected in the same region and are grouped in a small cluster at dis-

tances less than 0.05. Also, a group of genotypes collected in La Isla de la Juventud (Española Roja (I-127), Española Roja (I-126) Española Roja (I-130) Española Roja (I-128), Española Roja (I-129) and Española Roja (I-131) are clustered together at a distance less than 0.08.

The Hybrids (CxE) 203 and (CxE) 287 were located in an intermediate position between Cayenne and Red Spanish genotypes, in the second cluster, in which its male progenitor (Española Roja pinareña) is included. However, the hybrid (CxE) 1339, with its female progenitor Cayena lisa Serrana, grouped with the Cayennes' cluster.

The Spanish genotypes are the most cultivated in Cuba, which, taking into account the grouping of these genotypes in the dendrogram, permits to state that the genetic diversity of the cultivated pineapple in Cuba is very low. Most of the other genotypes present in the collection are not cultivated in Cuba.

Although AFLP is a very efficient technique, the molecular analysis randomly explores a number of loci and compares them, but an important part is unexplored. There could be some differences in this unexplored part of the genome, which could be in a locus of agronomical importance. Therefore, it is important to complement the molecular analysis with morphological data.

The information presented here could serve as the basis for the organization and development of the Cuban pineapple germplasm collection. For the collection to be more representa-

tive of the broad spectrum of pineapple germplasm, it should be enriched with more cultivated and non-cultivated varieties since most of genotypes in this collection are Red Spanish or Cayenne's cultivars. Other collections previously characterized with morphological descriptors (Leal et al. 1986, Ferreira and Cabral 1993, Duval et al. 1996), such as those from Brazil, Martinique and Hawaii or with molecular markers such as the Brazilian (Duval et al. 2001) and the Hawaiian collections (Kato et al. 2004) showed more diversity.

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Diversidade genética do germoplasma cubano de abacaxi avaliada por marcadores AFLP

Resumo - A coleção cubana de abacaxi representa a diversidade genética de abacaxis cultivados no país e inclui importantes genótipos das coleções do Brasil e da Martinica. A coleção foi previamente caracterizada com descritores morfológicos, faltando ainda a sua caracterização molecular. Com este objetivo foram analisados 56 genótipos de *Ananas comosus* e um de *Bromelia pinguin* com 191 marcadores AFLP. O dendrograma baseado nos resultados do AFLP e que representa as relações genéticas entre esses genótipos revelou baixa diversidade genética da coleção cubana de abacaxi. Todos os genótipos de *Ananas comosus*, as quais foram obtidos de agricultores das diversas regiões de Cuba, foram agrupados em distâncias menores de 0.20. Observou-se que a caracterização molecular da coleção está de acordo com a classificação morfológica feita previamente. O resultado é útil para os propósitos de melhoramento e conservação.

Palavras-chave: marcadores moleculares, acessos, Ananás.

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