AFLP Characterization of the Mexican Pineapple Germplasm Collection

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ABSTRACT. The Mexican pineapple germplasm collection represents the genetic diversity of cultivated pineapple [*Ananas comosus* (L.) Merr.] in that country and includes important genotypes from Hawaii, Ivory Coast, and Latin America. The collection has been partially characterized at the morphological level, but a molecular characterization has been lacking. With this aim, 39 genotypes of *A. comosus* var. *comosus* Coppens & Leal, two of *A. comosus* var. *bracteatus* (Lindl) Coppens & Leal, two of *A. comosus* var. *ananassoides* (Baker) Coppens & Leal, and three from the related genus *Bromelia* L. were analyzed with a total of 169 amplified fragment length polymorphism (AFLP) markers. A dendrogram representing the genetic relationships between these samples based on the AFLP results, showed a low level of diversity in the Mexican pineapple collection. In general, the molecular classification of the materials agreed well with the morphological classification. Several groups of genotypes showed distances of <0.03, whereas others thought to be similar based on morphological criteria were found to be distant. These results will allow more efficient use of the materials in the germplasm collection for breeding purposes and support the acquisition of genotypes that are scarce or lacking in the collection.

Pineapple is one of the most important tropical fruits and world production exceeded 14.6 million t in 2003 (FAO, 2004). Until recently, ~70% of pineapple production was based on a single cultivar, 'Smooth Cayenne', which monopolized the market. In the past few years, however, the cultivar DelMonte Gold (MD-2) has also become popular and captured a significant share of the fresh-fruit market (Coppens d'Eeckenbrugge et al., 1997). Reflecting this situation, most breeding programs are also centered on a few leading cultivars and neglect much of the germplasm diversity of the existing genetic pool. This omission is exacerbated by the incomplete characterization of pineapple germplasm and unsolved taxonomical complexities.

Several pineapple germplasm collections exist. The most important are the collection maintained by Empresa Brasileira de Pesquisas Agropecuarias/Centro Nacional de Pesquisas de Mandioca

y Fruticultura (EMBRAPA/CNPMF) in Cruz das Almas, Brazil, that of Centro Internacional de la Recherche Agricole–Département Productions Fruitières et Horticoles (CIRAD–FLHOR) in Martinique, and the U.S. Dept. of Agriculture collection in Hawaii. These collections have been partially characterized with morphological descriptors (Coppens d'Eeckenbrugge et al., 1997; Duval et al., 2001; Ferreira and Cabral, 1993; Leal et al., 1986). Other countries including Mexico have smaller but nonetheless important collections since they represent the genetic diversity of the cultivated pineapple in that particular country. Most of the genotypes in the Mexican pineapple germplasm collection at the Instituto Nacional de Investigaciones Forestales y Agropecuarias (INIFAP) experimental station at Papaloapan, Veracruz, Mexico, have been obtained from farmers or through exchanges with other collections (A. Rebolledo, personal communication).

Pineapple taxonomy has been seriously criticized (Coppens d'Eeckenbrugge et al., 1997; Duval and Coppens d'Eeckenbrugge, 1993) since the previously accepted taxonomic key was based on traits that depend on single genes or vary greatly with the environment. A thorough revision of this system has recently been proposed by Coppens d'Eeckenbrugge and Leal (2003). They suggest a simplified system where all pineapples are

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grouped under one genus that includes two species. The seven species previously accepted by Smith and Downs (1979) are now described as five botanical varieties of *A. comosus*. The classification proposed by Coppens d'Eeckenbrugge and Leal (2003) has been used throughout this report, but both old and new classifications are presented in Table 1 in order to avoid confusion. Within cultivated pineapple genotypes, attempts have also been made to classify germplasm into horticultural groups based on morphological characteristics; however, this may have led to the association of very distinct genotypes within a single group based on a few outstanding phenotypic traits (Duval and Coppens d'Eeckenbrugge, 1993; Duval et al., 1996).

Isozyme polymorphism has been used previously in the genus *Ananas* Mill. to clarify taxonomical aspects (Aradhya et al., 1994; De Wald et al., 1992; García, 1988). However, due to the low number of markers, the scope of these studies is limited. More recently, Duval et al. (2001, 2003) used RFLP markers and chloroplast genotypes to study genetic diversity in pineapple. Three hundred and one accessions covering the genus *Ananas* were tested. Compared to the results obtained with isozymes in *Ananas*, RFLP revealed a higher level of polymorphism since 41% of the probes were polymorphic.

The RFLP technique is extremely robust but expensive and time-consuming (Kochert, 1994). In the case of chloroplast markers, DNA from this organelle must be isolated increasing the complexity of the analysis and the data may not always be informative when individuals of a single species are analyzed. AFLP markers (Vos et al., 1995) have been widely used in other species to study diversity (Breyné et al., 1999; Coulbaly et al., 2003), identify cultivars (Dirlenwanger et al., 1998), and carry out classical genetic mapping studies. The possibility to detect low levels of polymorphism makes it the method of choice for intra-species analysis. The AFLP technique is also very reproducible with a high multiplex ratio, combining the robustness of the RFLP method with the efficiency and simplicity of polymerase chain reaction (PCR)-based methods. Two preliminary reports (Kato et al., 2001; Tapia et al., 2001) have demonstrated the usefulness of AFLP for the study of closely related, cultivated and noncultivated pineapple genotypes.

In this work, AFLP markers were used to assess the genetic diversity of the Mexican pineapple germplasm collection. The objectives were to determine the diversity of the collection which represents both commercial cultivars grown in Mexico and germplasm used in ongoing breeding programs. The results of the study are of importance in planning the acquisition of new material for the collection and in designing new breeding programs. The suitability of AFLP to study diversity in pineapple is also discussed.

Materials and Methods

Forty-six genotypes, including 39 genotypes of *A. comosus* var. *comosus*, two genotypes of *A. comosus* var. *ananassoides*, two genotypes of *A. comosus* var. *bracteatus*, and three other related species of the genus *Bromelia*, belonging to the Mexican National Pineapple Germplasm Collection at the INIFAP experimental station at Papaloapan, Veracruz, were analyzed (Table 1). The classification used in this article corresponds with that of Coppens d'Eeckenbrugge and Leal (2003), and cultivar names are the common names used locally. We have also indicated the species and the horticultural group and the previously accepted classification.

Leaf samples were obtained from plants growing in the field and transported in dry ice. DNA extraction was performed according to Dellaporta et al. (1993), with minor modifications. AFLP analysis was carried out according to Vos et al. (1995).

The oligonucleotide primers used for the preamplification step were:

EcoRI (EcoRI+A) 5'-AGACTGCGTACCAATTC/A-3',
MseI (MseI+A) 5'-GACGATGAGTCCTGAGTAA/A-3'

The preamplification 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 distances were calculated using the S-Plus 2000 for Windows software package (Mathsoft, Seattle, Wash.) using the simple matching coefficient (Skroch et al., 1992). 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. Confidence limits on relationships were determined by bootstrap analysis (B = 1500 replicates) (Felsenstein, 1985).

Results and Discussion

A total of 169 scorable AFLP markers were generated with four primer combinations (Table 2). The levels of polymorphism were as follows: 98.2% polymorphism considering both the genera *Ananas* and *Bromelia*, 73.3% among samples from the genus *Ananas*, and 63.9% considering only *A. comosus* var. *comosus*. In comparison, Duval et al. (2001), using RFLP markers found 94.4% polymorphism among 294 accessions covering the genus *Ananas* and 74.7% when 167 accessions of *A. comosus* var. *comosus* were analyzed. However, these results are not directly comparable to this work since here a much narrower collection of germplasm was analyzed. The percentages of polymorphism obtained with each AFLP primer combination are shown (Table 2).

With the exception of 'Hawaii IV' and 'Hawaii IX,' which have identical genotypes, all samples were differentiated by the AFLP technique (Fig. 1). The related species of the genus *Bromelia* 'Cardón Borrego' (*Bromelia penguin* L.), 'Che ornato' (*Bromelia* sp.), and 'Ornato epífita' (*Bromelia* sp.) were clearly separated from the genus *Ananas*, which forms a large distinct group. Within the genus *Ananas*, 'Ornamental Costa Rica' (*A. comosus* var. *ananassoides*) a noncultivated genotype was separated from the other genotypes. However, 'Tricolor Roja' and 'Tricolor Verde' (*Ananas comosus* var. *bracteatus*) grouped with the *A. comosus* var. *comosus* genotypes as did 'Wild Brazil' (*A. comosus* var. *ananassoides*). This result was unexpected since initial studies (Duval and Coppens d'Eeckenbrugge, 1993; Duval et al., 1996, 2003) did not indicate that *A. comosus*, *A. bracteatus* Baker, and *A. ananassoides* Baker L.B. Smith (under the previous classification) were so closely related. However, under the recently revised classification, *A. comosus*, *A. bracteatus*, and *A. ananas* (L.B. Smith) L.B. Smith are no longer regarded as distinct species but as botanical varieties of the single species, *A. comosus* (Coppens d'Eeckenbrugge and Leal, 2003). The results presented here therefore support this revised classification. The single exception which lies outside the group (Fig. 1) is the accession 'Ornamental Costa Rica' (*A. comosus* var. *ananassoides*) previously classified as *A. nanus* (L.B. Smith) L.B. Smith. This result suggests the need

Table 1. Pineapple cultivars and relatives analyzed in this study indicating old and new classifications, horticultural groups, and origin.

Sample no.	Previous classification	New classification	Cultivar	Horticultural group	Origin
1	<i>Bromelia pinguin</i>	-----	Cardón Borrego	None	Mexico
2	<i>Bromelia</i> sp.	-----	Bromelia Epífita	None	Mexico
3	<i>Bromelia</i> sp.	-----	Ché Ornato	None	Mexico
4	<i>Ananas nanus</i>	<i>A. comosus</i> var. <i>ananassoides</i>	Ornamental Costa Rica	None	Costa Rica
5	<i>A. bracteatus</i>	<i>A. comosus</i> var. <i>bracteatus</i>	Tricolor Roja	None	Ivory Coast
6	<i>A. bracteatus</i>	<i>A. comosus</i> var. <i>bracteatus</i>	Tricolor Verde	None	Ivory Coast
7	<i>A. ananassoides</i>	<i>A. comosus</i> var. <i>ananassoides</i>	Wild Brazil	None	Puerto Rico
8	<i>A. comosus</i>	<i>A. comosus</i> var. <i>comosus</i>	Pernambuco	Pernambuco	Puerto Rico
9	<i>A. comosus</i>	<i>A. comosus</i> var. <i>comosus</i>	Introduction 1	Maipure	Puerto Rico
10	<i>A. comosus</i>	<i>A. comosus</i> var. <i>comosus</i>	Manzana	Maipure	Colombia
11	<i>A.s comosus</i>	<i>A. comosus</i> var. <i>comosus</i>	Introduction 2	Spanish	Costa Rica
12	<i>A. comosus</i>	<i>A. comosus</i> var. <i>comosus</i>	Spanish Red	Spanish	Puerto Rico
13	<i>A. comosus</i>	<i>A. comosus</i> var. <i>comosus</i>	PR-1-56	Cayenne/Spanish	Puerto Rico
14	<i>A. comosus</i>	<i>A. comosus</i> var. <i>comosus</i>	PR-1-67	Cayenne/Spanish	Puerto Rico
15	<i>A. comosus</i>	<i>A. comosus</i> var. <i>comosus</i>	Cabezona Carranza	Spanish	Mexico
16	<i>A. comosus</i>	<i>A. comosus</i> var. <i>comosus</i>	Cabezona Tabasco	Spanish	Mexico
17	<i>A. comosus</i>	<i>A. comosus</i> var. <i>comosus</i>	Esmeralda	Smooth Cayenne	Mexico
18	<i>A. comosus</i>	<i>A. comosus</i> var. <i>comosus</i>	Criolla Quintana Roo	Spanish	Mexico
19	<i>A. comosus</i>	<i>A. comosus</i> var. <i>comosus</i>	Criolla Nayarit	Spanish	Mexico
20	<i>A. comosus</i>	<i>A. comosus</i> var. <i>comosus</i>	Criolla Guerrero	Spanish	Mexico
21	<i>A. comosus</i>	<i>A. comosus</i> var. <i>comosus</i>	Veracruz	Smooth Cayenne	Mexico
22	<i>A. comosus</i>	<i>A. comosus</i> var. <i>comosus</i>	Quintana Roo	Smooth Cayenne	Mexico
23	<i>A. comosus</i>	<i>A. comosus</i> var. <i>comosus</i>	Rayada Azueta	Smooth Cayenne	Mexico
24	<i>A. comosus</i>	<i>A. comosus</i> var. <i>comosus</i>	Rayada N3	Smooth Cayenne	Mexico
25	<i>A. comosus</i>	<i>A. comosus</i> var. <i>comosus</i>	Hojasverdes	Smooth Cayenne	Mexico
26	<i>A. comosus</i>	<i>A. comosus</i> var. <i>comosus</i>	Extravigor	Smooth Cayenne	Mexico
27	<i>A. comosus</i>	<i>A. comosus</i> var. <i>comosus</i>	Hawaii I	Smooth Cayenne	Hawaii
28	<i>A comosus</i>	<i>A. comosus</i> var. <i>comosus</i>	Hawaii II	Smooth Cayenne	Hawaii
29	<i>A. comosus</i>	<i>A. comosus</i> var. <i>comosus</i>	Hawaii III	Smooth Cayenne	Hawaii
30	<i>A. comosus</i>	<i>A. comosus</i> var. <i>comosus</i>	Hawaii IV	Smooth Cayenne	Hawaii
31	<i>A. comosus</i>	<i>A. comosus</i> var. <i>comosus</i>	Hawaii VI	Smooth Cayenne	Hawaii
32	<i>A. comosus</i>	<i>A. comosus</i> var. <i>comosus</i>	Hawaii VIII	Smooth Cayenne	Hawaii
33	<i>A. comosus</i>	<i>A. comosus</i> var. <i>comosus</i>	Hawaii IX	Smooth Cayenne	Hawaii
34	<i>A. comosus</i>	<i>A. comosus</i> var. <i>comosus</i>	Kona	Smooth Cayenne	Hawaii
35	<i>A. comosus</i>	<i>A. comosus</i> var. <i>comosus</i>	Libby	Smooth Cayenne	Puerto Rico
36	<i>A. comosus</i>	<i>A. comosus</i> var. <i>comosus</i>	Dorsey Edwards	Smooth Cayenne	Puerto Rico
37	<i>A. comosus</i>	<i>A. comosus</i> var. <i>comosus</i>	Blanca de Cuba	Pernambuco	Puerto Rico
38	<i>A comosus</i>	<i>A. comosus</i> var. <i>comosus</i>	Champaka I	Smooth Cayenne	Costa Rica
39	<i>A. comosus</i>	<i>A. comosus</i> var. <i>comosus</i>	Champaka II	Smooth Cayenne	Costa Rica
40	<i>A. comosus</i>	<i>A. comosus</i> var. <i>comosus</i>	Champaka III	Smooth Cayenne	Costa Rica
41	<i>A. comosus</i>	<i>A. comosus</i> var. <i>comosus</i>	Champaka IV	Smooth Cayenne	Costa Rica
42	<i>A. comosus</i>	<i>A. comosus</i> var. <i>comosus</i>	KU-2	Smooth Cayenne	Costa Rica
43	<i>A. comosus</i>	<i>A. comosus</i> var. <i>comosus</i>	MD-1	Smooth Cayenne	Costa Rica
44	<i>A. comosus</i>	<i>A. comosus</i> var. <i>comosus</i>	MD-2 INIFAP	Smooth Cayenne	Costa Rica
45	<i>A. comosus</i>	<i>A. comosus</i> var. <i>comosus</i>	MD-2 GOBVer	Smooth Cayenne	Costa Rica
46	<i>A. comosus</i>	<i>A. comosus</i> var. <i>comosus</i>	MD-2 Azueta	Smooth Cayenne	Costa Rica

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

Primer combination	Markers	Total	Level of polymorphism	Polymorphic	Level of polymorphism
		polymorphic markers		markers in <i>Ananas</i>	
EcoRI AAT/	(no.)	(no.)	(%)	(no.)	(%)
Mse-AAG	22	22	100	16	72.7
Mse AAC	44	44	100	30	68.2
Mse-AGT	39	36	92	26	66.6
Mse ATG	64	64	100	52	81.2

Fig. 1. Dendrogram showing the diversity of the Mexican pineapple germplasm collection. Accessions are presented with cultivar names. *Ananas comosus* botanical varieties are represented as 1) *A. comosus* var. *bracteatus*, 2) *A. comosus* var. *ananassoides*. Horticultural groups of *A. comosus* var. *comosus* are represented as follows: * = Smooth Cayenne, \$ = Spanish, + = Maipure, % = Pernambuco, C x S = Cayenne/Spanish hybrids, group of Smooth Cayenne cultivars = •••••, group of Spanish cultivars = —••—.

for a more detailed analysis of *A. comosus* var. *ananassoides* at the genotype level including many more accessions in order to clarify the classification of this group.

The greatest genetic distance (0.51) was found between members of the different genera *Ananas* and *Bromelia*. However, within each genus notable differences were also observed. A distance of 0.47 was found between *Bromelia pinguin* 'Cardón Borrego' and the other two *Bromelia* accessions and these other two accessions were separated by a distance of 0.34. The distance between *Ananas comosus* var. *ananassoides* 'Ornamental Costa Rica' and the other *Ananas* accessions was 0.32, whereas the other members of *Ananas* sp. were found to group at distances less than 0.27. Three small groups showed very high similarity with distances of less than 0.03, suggesting that these materials are essentially the same genotypes: 1) 'Cabezona Carranza'/'Cabezona Tabasco'; 2) 'Hawaii II'/'Hawaii IV'/'Hawaii IX'/'Kona'; 3) 'Criolla Guerrero'/'Criolla Nayarit'.

The bootstrap values shown on the dendrogram give an indication of how robust the different groupings on the dendrogram are. Higher numbers indicate nodes that are found in the majority of the bootstrap samples, whereas low numbers indicate that these

nodes are not so consistent. In terms of these values, the analysis presented here shows some interesting results. As mentioned above the samples from the related genus *Bromelia*, clearly form a separate group as do 'Ornamental Costa Rica' and 'Dorsey Edward', 'Hawaii I', and 'Libby' with bootstrap values of 100, 98, and 94 respectively. A fourth group with a high bootstrap number of 77 contains the majority of the samples, including *A. comosus* var. *bracteatus* and *A. comosus* var. *ananassoides* as well as most of the *A. comosus* var. *comosus* samples. Within this fourth group however many of the bootstrap values at the nodes are extremely low, suggesting that these nodes were not very consistent during the bootstrap analysis. This indicates that the samples within this group are genotypically quite similar and that the groups formed are distinguished by few AFLP bands.

In general, the results obtained from the AFLP analysis agree well with the morphological characterization of the germplasm collection. However some interesting differences were observed. The materials in the three clusters with distances <0.03 commented above were also classified as the same or closely related genotypes morphologically. This is also the case for the accessions 'Champaka I' and 'Champaka II'.

In a broad sense, all the Smooth Cayenne accessions are found within a single large group (••••• in Fig. 1), although Spanish, Pernambuco, and Maipure cultivars are also found dispersed within this group. The surprising exceptions to this arrangement are the accessions Smooth Cayenne: 'Dorsey Edwards', 'Hawaii I', and 'Libby', which are outwith the group. Another compact cluster consistent with the morphological classification is that denoted —••— on the dendrogram. This cluster contains all the samples classified as Spanish and within the group three improved cultivars classified as Smooth Cayenne: 'MD-2 INIFAP', 'MD-2 Azueta', and 'MD-2 GOBVer'. This grouping may suggest that these latter cultivars have been crossed with Spanish germplasm at some point during their development.

In contrast, the recognized hybrids from Smooth Cayenne x Spanish crosses 'PR-1-67', and 'PR-1-56' clearly group within the Smooth Cayenne cluster.

At the cultivar level, the most notable differences between the morphological classification and the molecular classification are the separation of members of the Pernambuco and Maipure horticultural groups (% and + respectively in Fig. 1), which rather than form groupings together are mixed within the Smooth Cayenne group. Duval and Coppens d'Eeckenbrugge (1993) and Duval et al. (1996) have suggested that some of the horticultural groups determined for pineapple may in fact associate cultivars with quite different genotypes that share a common outstanding morphological feature such as "piping" along the leaf edges as in the case of the Maipure group. This may explain the differences observed in this study for the Pernambuco and Maipure classes.

Most genotypes of the Mexican collection are derived from Smooth Cayenne clones (Table 1), with a few accessions derived from Spanish clones. This indicates that the genetic basis of the Mexican germplasm collection is very narrow. Other genotypes, although present in the collection, are not cultivated in Mexico and not widely used for breeding purposes. The information presented here could serve as the basis for the organization and development of the Mexican pineapple germplasm collection. For the collection to be more representative of the broad spectrum of pineapple germplasm, it should be enriched with more cultivated and non-cultivated genotypes. A core collection could be constructed taking into account the data presented here. For example 'Hawaii IV', 'Hawaii IX', 'Kona', and 'Hawaii II' are very similar genotypes; therefore in subsequent genotype analysis a single example of these accessions would be sufficient. This could also be done with 'Esmeralda' and 'Hawaii III' and with 'PR-I-67' and 'PR-I-58', etc. This core collection, although having fewer genotypes, would represent the genetic diversity of the complete collection and could be characterized extensively both at the morphological level and genetically in a more cost-effective manner. The potential of the AFLP technique to distinguish even very closely related accessions as shown here would also permit the study of variation within clones obtained from the same mother plant and from plants reproduced in tissue culture.

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