Research article

Intra and interspecific genetic diversity of yam (*Dioscorea* spp.) from the Colombian Caribbean region by AFLP markers

Diversidad genética intra e inter-específica de ñame (*Dioscorea* spp.) de la región Caribe de Colombia mediante marcadores AFLP

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Abstract

Knowing the genetic variability of yams, *Dioscorea* spp., supports the development of breeding and conservation strategies of this genetic resource. The aim of this study was to carry out the molecular characterization of 20 accessions of *Dioscorea* spp. using the AFLP molecular technique to determine the distribution of their intra and interspecific genetic variation. Using multiple correspondence analysis and level of reliability of the genetic groups by resampling, the results showed high genetic variability among the accessions studied giving as a result four genetic groups: *D. alata* L., *D. rotundata* Poir., *D. esculenta* (Lour.) Burkill and *D. trifida* L.f., which confirmed a correspondence between the morphological and molecular characterization. The average similarity values ranged from 41.81% in *D. alata* and *D. rotundata*, and 33.51% in *D. trifida* and *D. esculenta*. These data are consistent with previous morphological characterizations and systematics of the species in relation to their botanical sections. The analysis also revealed the heterogeneous composition of *D. alata* in the Colombian Caribbean region; these studies will help to define an adequate strategy for conservation to support future efforts in breeding programs.

Key words: Agronomical characters, Colombian Caribbean region, *Dioscorea* spp., genetic marker, molecular characterization, plant genetic resources.

Resumen

Conocer la variabilidad genética del ñame, *Dioscorea* spp., permite apoyar estrategias de mejoramiento y conservación de este recurso fitogenético. El objetivo de este estudio fue la caracterización molecular de 20 accesiones de *Dioscorea* spp. mediante la técnica molecular de AFLP para determinar cómo se distribuye la variabilidad genética de manera intra e inter-específica. Los datos fueron analizados mediante los métodos de agrupación de correspondencia múltiple y análisis de similaridad de Dice, estableciendo los niveles de confiabilidad de los grupos genéticos mediante remuestreos. En términos de diversidad interespecífica, los valores promedios de similitud variaron entre 41.81% entre *D. alata* L. y *D. rotundata* Poir., y 33.51% entre *D. trifida* L.f. y *D. esculenta* (Lour.) Burkill, lo que sugiere alta diversidad genética entre las accesiones estudiadas, que formaron cuatro grupos genéticos: *D. alata*, *D. rotundata*, *D. esculenta* y *D. trifida*, confirmando correspondencia entre la caracterización morfológica, clasificación botánica y la caracterización molecular. En términos de diversidad intraespecífica para la especie *D. alata*, el análisis también reveló una composición heterogénea en la región Caribe colombiana. Estos estudios ayudarán a definir una estrategia adecuada para fines de conservación y apoyar los esfuerzos futuros en los programas de mejoramiento genético.

Palabras clave: Características agronómicas, caracterización molecular, *Dioscorea* spp., marcadores genéticos, recursos fitogenéticos, región Caribe de Colombia.

Introduction

In several places in the tropics, the yam, *Dioscorea* spp., is a food of high importance, mainly in Western Africa, some parts of Sub-Eastern Asia, India, some areas of Brazil and other countries in tropical America (Tamiru, 2006; Lebot *et al.*, 1998). Yam has a high genetic diversity, both at the intra and interspecific levels (Martin and Rhodes 1977; Okoli, 1991). Wild yam diversity is enhanced by crop domestication in numerous countries (Mignouna y Dansi, 2003). However, the genetic diversity levels of several *Dioscorea* species and their genetic relationships have not been completely studied. The attempts to characterize yam by morphological characters (Hamon and Toure, 1990b;), isoenzymes (Hamon and Toure, 1990a; Dansi *et al.*, 2000) and molecular markers have not yield conclusive results, because of the high variability of this crop (Tamiru *et al.*, 2007).

Some of the most important morphological variations could be the result of differences in only few genes (Bradley *et al.*, 1997). These observations have been supported by studies that showed high genetic diversity events, in both, wild and domesticated yam, at the South of Ethiopia and in cultivated species like *D. alata* L., *D. bulbifera* L., *D cayenensis* L. and *D. rotundata* Poir. in Western Africa (Tamiru *et al.,* 2007). However, genetic diversity studies on this specie done by Bustamante *et al.* (2003) showed that *Dioscorea* genus presents high similarity between characterized Colombian accessions. For taxonomic, phylogenetic and genetic diversity studies in yam there have been used molecular markers like Amplified Fragment Length Polymorphism (AFLP) (Vos *et al.,* 1995), (Ramser *et al.*, 1997; Mignouna *et al.*, 1998; Dansi *et al.*, 2000). This has allowed the detection of differences between varieties that have been considered similar based on morphological and isoenzyme markers, demonstrating its utility as a tool to discriminate *Dioscorea* spp. Introductions (Dansi *et al.*, 2000) and, allowing the detection of duplicates in germplasm collections (Mignouna *et al.,* 2003).

The main objective of this work was to analyze yam intra and interspecific genetic diversity of the yams in the collection of Universidad de Córdoba, Colombia, using AFLP molecular markers.

Materials and Methods

Plant Material. For this study it was collected leaf material of 20 accessions from the yam collection of the Universidad de Córdoba (Table 1). These accessions were coming from different regions of Cordoba, Sucre and Bolivar (Colombia). Leaf material was kept with 50 g of silica-gel on plastic bags which can keep until 5 g of tissue in good conditions. Later, tissue was grinded on liquid nitrogen and kept at -196 °C.

DNA Extraction. Molecular techniques were done in the Molecular Biology Lab of the Institute for Research on Biological Resources Alexander von Humboldt. For DNA extraction, grinded leaf tissue of the different accessions was kept on liquid nitrogen. DNA extraction was done with the Qiagen® commercial kit with some modifications as the incubation temperature was reduced to 60 °C and centrifugation time was 5 minutes. DNA quality was quantified by electrophoresis on 1% agarose gel dyed with ethidium bromide and visualized with UV light. Quantification was done using a DNA Lambda 20 bp weight marker.

AFLP markers and PCR amplification. Isolated DNA from each accession was digested with EcoRI and MseI restriction enzymes, subsequently two adapters to the generated DNA fragments were added and ligated using T4 DNA ligase enzyme from Invitrogen®. Amplification was done by PCR (Polymerase Chain Reaction) following the instructions of the Invitrogen® commercial kit. Complementary primers to the adapters sequence were used and a preamplification +1/+1 was done with additional nucleotides. Amplifica-

Table 1. Yam (*Dioscorea* spp.) accession list of this study

Specie	Code	Common name	Department
D. alata L.	9404- 002	Ñame pepita	Córdoba
D. alata L.	9503- 005	Osito	Sucre
D. alata L.	9503- 008	Ñame peludo	Córdoba
D. alata L.	9504- 009	Mampuján	Córdoba
D. alata L.	9506- 022	Pico de botella	Córdoba
D. alata L.	9506- 027	Diamantes	Córdoba
D. alata L.	9603- 037	Mampuján	Córdoba
D. alata L.	9605- 054	Ñame seda	Magdalena
D. alata L.	9605- 062	Manteco	Córdoba
D. alata L.	9811- 094	Mampuján	Córdoba
D. alata L.	9811- 098	Mampuján	Córdoba
D. alata L.	0106- 100	Ecuatoriano	Córdoba
D. alata L.	0504- 130	Te encontré	Bolívar
D. alata L.	0504- 140	Coco	Córdoba
D. esculenta (Lour.) Burkill	0403- 104	Ñame familia	Córdoba
D. esculenta (Lour.) Burkill	0504- 139	Ñame familia	Córdoba
D. rotundata Poir.	9811- 076	Ñame espino	Córdoba
D. rotundata Poir.	0403- 129	Espino venezolano	Bolívar
D. trifida L.f.	0403- 102	Ñampin	Córdoba
D. trifida L.f.	0403- 105	Ñampin	Córdoba

tion was done on a PTC-100^{tm} MJ Research Inc. thermocycler using the fragments from each digestion. Fragments were amplified with +3/+3 nucleotide primers in different combinations, this allowed the determination of the combinations that generate more number of polymorphic bands.

To study the genetic variability of the yam species (*D. alata* L., *D. rotundata* Poir., *D. esculenta* (Lour.) Burkill y *D. trifida* L.f.) the amplification reaction was performed using different combinations of primers pairs. It was found that the primers combination in the treatments E-ACA/M-CAT, E-AAC/M-CAC, E-AAG/M-CTC, showed higher polymorphism, therefore these ones were used for that study. For *D. alata* the study was done with a combination of highly polymorphic primers E-ACA/M-CAT, in 14 accessions.

PCR program for the beginning of the +3/+3 cycle was as follows: one cycle at 94 °C for 30 s, 65 °C for 30 s., and 72 °C for 60 s. Annealing temperature was reduced 0.7 °C in each cycle for 12 cycles and 23 cycles were performed for a total of 2 h, 2 min in his way: 94 ºC for 30 s, 56 ºC for 30 s y 72 ºC for 60 s, finally, the amplified product was kept at 4 °C. Amplified reactions were observed on a 6% polyacrylamide gel dyed with silver, and the loci between 40 and 330 bp distribution was captured.

Results analysis. Due to the dominant nature of AFLP markers, binary matrices were coded of each level by bands presence (1) or absence (0) from the amplification of each digested fragment. With the presence/absence matrix it was calculated the Dice's similarity index adapted by Nei and Li (1979) for molecular data. This index makes an average of the similarity values for each pair of individuals by the following equation:

$Sij = 2a/(2a + b + c)$, where, SiJ = similarity between the i and j individuals;

a = number of shared loci by i and j;

b = number of loci present in i but absent in j; and

c = number of loci present in j but absent in i.

Similarity matrices and dendograms were built with the NTSYS-PC, version 2.02i software (Rohlf, 1998) with the UPGMA and SAHN grouping methods, respectively. Additionally, the relations among individuals by multiple correspondence analyses (ACM) with all the population was done to get a graphical representation of the distance between accessions. To estimate the reliability of the genetic groups assigned in the dendograms, a confirmation of the conglomerates analyses and diversity groups was done by resampling (1000 permutations) with the WinBoot software (Nelson, 1996).

Results and discussion

AFLP Polymorphisms

Primers combinations used in this study showed highly polymorphic patterns among and within species, in that way different loci patterns were observed ensuring a good discrimination power to identify different genetic groups among *Dioscorea* spp. species. DNA amplified fragments with AFLP oscillated on a 40 to 330 bp range. E-ACA/M-CAT y E-AAC/M-CAC primers combination showed the highest polymorphism percentage, the total loci number obtained in this study was 206 fragments (Table 2), which was extended to 74 loci for the E-ACA/M-CAT primers combination, and to 64 loci for E-AAG/M-CTC primers combination, with an average of 68.66 loci per primer pair. E-AAC/M-CAC primers combination (Figure 1) showed higher polymorphism with 68 loci, from which 54 showed polymorphism (94.11%). Using E-ACA/M-CAT primers combination it was obtained 91.89% polymorphism with 74 loci in total and 68 showing polymorphism (91.89%). These results are similar to the ones found by Tamiru *et al.* (2007) in yam when they quantified polymorphisms above 90% and highlighted a high number of polymorphic bands when using primers combinations.

Inter and intraspecific genetic diversity in *Dioscorea* spp.

In this work stands out the importance of *Dioscorea* spp. variation in the Colombian Caribbean region. The molecular characterization by AFLP had a high sensibility allowing the separation of yam accessions in four groups according to the specie: *D. alata*, *D. rotundata*, *D. esculenta* and *D. trifida*. The comparison of genetic similarities among the four species are presented in Table 3. Average values were between 41.81% between *D. alata* and *D. rotundata*, and 33.51% between *D. trifida* and *D. esculenta.* These results were consistent with the species classification based on their botanical selections. *D. alata* and *D. rotundata* belong to the Enanthiophillum section of the *Dioscorea* spp. genus, which are originated in Southeast Asia and Western Africa; while *D. trifida* belongs to the Macrogynodium section originated in tropical America and *D. esculenta* in the Combilium

Table 2. Polymorphism in evaluated *Dioscorea* spp. accessions with different AFLP primers combinations.

Primers combinations	Loci number			Polymorphism %[2]
	Total	P[1]	M[1]	
E-ACA/M-CAT	74	68	6	91.89
E-AAC/M-CAC	68	64	4	94.11
E-AAG/M-CTC	64	51	13	79.68
Total	206	183	23	
Average	68.66	61	7.66	88.56†

[1]P = Polymorphic, M = Monomorphic.
[2]Determined based on the polymorphic loci number out of the total amplified loci for a primers combination in all the varieties.
†Average polymorphism.

section which is originated in Southeast Asia. Genetic similarity comparison between species pairs indicates that *D. alata* is genetically closer to es genéticamente *D. rotundata* (41.81%) than to *D. esculenta* (32.21%), while *D. trifida* is genetically more distant from*D. alata* (29.95%) (Table 3). Conglomerate analysis supports these relationships (Figure 1) with the bootstrap values on the distance matrix -100% for the relation between *D. alata* vs. *D. rotundata*, 49.8% for the relation of *D. esculenta* with both species, and 97.9% for *D. trifida*—. Variability levels among taxonomical groups have been studied by Malapa *et al.* (2005) who found genetic variability among *Dioscorea* spp. taxonomical groups; they discriminate six species in the Enantiophyllum section by using AFLP markers (Vos *et al.*, 1995). These results were expected due to the high variability in the *Dioscorea* genus, as it was demonstrated by Sonibare *et al.* (2010) when they studied *Dioscorea* samples collected in Eastern and Central Africa. Hildebrand *et al.* (2002) found high diversity in native varieties of yam in Southeast Ethiopia separating 23 native yum types.

The multiple correspondence analysis (ACM) estimated the variation among individuals in three dimensions (x, y, z axis) (Figure 2) and shows similar information as the one in the dendogram splitting four groups. The results from this study confirmed and are consistent with the ones obtained by Durango and Padilla (1998) in previous morphological characterizations of the same genotypes. In group 1 we found 14 accessions (9506-27 to 9605-54) with a trend to group in the center of the coordinate formed by *D. alata*, with an average similarity value of 89.51% (Table 3). These are characterized by a squared stem without thorns, dextral rolling and four wings or creases, acute apex leaves, some accessions produce brown cylindrical, spherical, deltoid and irregular subterranean tubers. These results support the findings of Egesi *et al.* (2003) who found differences in color, taste, consistency, floury and viscosity in tubers from 40 *D. alata* accessions. These tubers have good acceptance at the local markets, but some genotypes are susceptible to anthracnose caused by the fungi *Colletotrichum gloeosporioides* (Campo *et al.*, 2009).

Group 2 consisted on *D. rotundata* specie (Figure 2), includes two accessions (9811-076 y 0504-129) that share an average similarity value of 79.12% (Table 3). They are characterized by a slim rounded stem, with thorns and dextral rolling, leaves are wider in the apical part and thorns are absent in petioles, inflorescence is a simple spike. 9811-076 genotype showed cylindrical subterranean tubers with brown epidermis and white flesh. 0405-129 genotype produced tubers with good organoleptic characteristics for a good acceptance in the local and international markets, but it is susceptible to anthracnose (Campo *et al.*, 2009).

Group 3, consisted on *D. esculenta* specie (Figure 2), including two accessions (0403-104 y 0504-139) which share an average similarity index of 82.22% (Table 3). This group has a lower dispersion due to a higher genetic closeness among both accessions. They are characterized by a brown rounded stem with thorns, and sinistral rolling, leaves are wide and petioles have thorns, produces small light brown subterranean tubers and there is no knowing of flowers neither of aerial tubers.

The fourth conglomerate is composed of *D. trifida* (Group 4) including accessions (0403-105 y 0403-102) that share an average similarity index of 55.56% (Table 3). The accessions of this group are dispersed along the coordinates (x, y, z axis) (Figure 2) with some separation degree among the accessions of the same species. . These accessions are cha-

Table 3. Average of genetic similarities (%) among and within *Dioscorea* spp. species.

Specie	*D. alata*	*D. rotundata*	*D. esculenta*	*D. trifida*
D. alata	82.51			
D. rotundata	41.81	79.12		
D. esculenta	32.21	37.11	82.22	
D. trifida	29.95	25.50	33.51	55.56

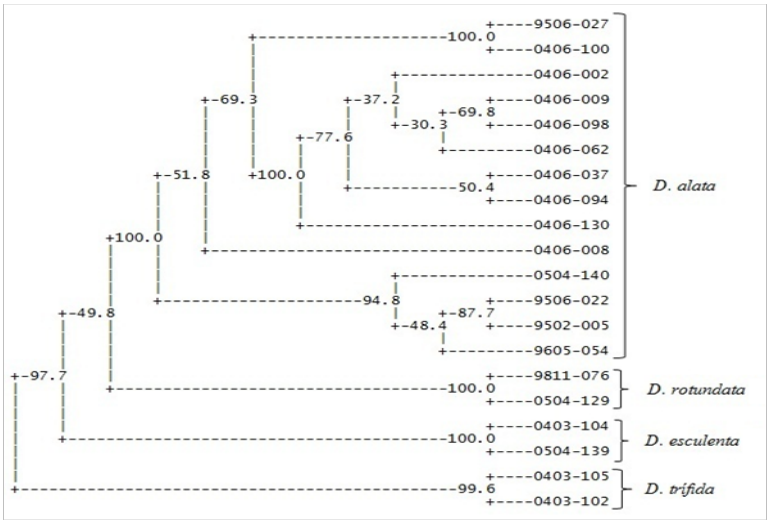

Figure 1. Similarity dendogram by Dice's coefficient, using UPGMA grouping method based on 206 AFLP markers generated by four combinations of initiators. Values in percentage on each branch correspond to a 1000 replicates analysis.

racterized by a green stem with two brown creases, sinistral rolling, rustic and five lobe leaves. These species have hermaphrodite flowers, tubers are brown and oval forming a bunch connected to roots hanging from the stem.

The results of this study are similar to the ones of Malapa *et al.* (2005) who found consistency between species systematics and botanical sections by AFLP. In the same way Tamiru *et al.* (2007) evaluated genetic diversity and structure in yam from Ethiopia comparing it to Easthern Africa species, and found high genetic variability in the evaluated accessions. Similar results were obtained by Mignouna *et al.* (2002) using enzymatic markers to confirm the genetic variability in *Dioscorea cayenensis* Lam. and *Dioscorea rotundata* genotypes. In this study the high variability in the *Dioscorea* spp. genus in the yam collection of the Universidad de Cordoba was proven, additional to the molecular results that were similar to the previous morphological characterizations and botanical classification. However, these results differ from the ones reported by Bustamante *et al.* (2001) who, using DNA fingerprinting, found similarity between the accessions from the Universidad de Córdoba yam collection and some genotypes from the IITA (International Institute of Tropical Agriculture, Ibadan, Nigeria).

Dioscorea alata L. genetic diversity

Studies in other species allowed the genetic variability estimation of commercially grown yam in different regions (Arnau *et al.*, 2009; Tostain *et al.*, 2006). This suggests that it is possible to use molecular markers with high polymorphism in the generated bands in order to differentiate genotypes within the same species of de *D. alata* (Malapa *et al.*, 2005). In this study, the data similarity analysis through AFLP was done (Figure 3) to evaluate the relationship among *D. alata* accessions. This grouping showed four main groups with some genetic variability degree. Group 1 con sisted on 9506-027, 0406-100, 0406-002, 0406-009, 0406-098, 0406-062, 0406-094 genotypes, collected on Cordoba, Colombia, and 0406-130 collected on Bolívar, Colombia A first subgroup is separated from the other accessions, it consists of 9506- 027 and 0406-100 genotypes, they have male flowers, stem with green creases, subterranean tubers with white flesh; this group has a similarity value of 0.976. A second subgroup (Figure 3)

formed by 9404-002, 9504-009, 9603-037, 9605-062 and 0406-094 genotypes has a squared stem with small purple creases; this similarity value of 0.972, has female flowers, genotype is totally wild. It has acute apex leaves, flowers are simple, yellow and in bunch. Tubers are irregular, hairy and with several roots. These results correspond to the ones of Malapa *et al.* (2005), who demonstrated that this is a highly heterogeneous specie, with high variability within the species, these was evident in the morphology of the studied accessions that vary in phenotypic characteristics such as color, shape and size of leaves, stem and tubers. 9404-002, 9504-009 and 9603-037 genotypes are resistant to anthracnose, which is agronomically important since those accessions could be involved in future breeding programs to obtain *C. gloeosporioides* resistant yam crops. This resistance was evaluated by Campo *et al.* (2009) with antracnose incidence and severity assays. In the Colombian Caribean region yam is known by common names, 9404-002 ('ñame pepita'), 9504-009, 9603-037, and 0406-094 ('mampuján'), 9605-062 ('ñame manteco'), and 0406-130 ('te encontré'). Results showed that 9504-009 and 9603-037 accessions are the same genotype, because they have a similarity coefficient of 1 and share morphological characteristics such a: green and purple coloration in the stem, simple leaves with a heart shape and acute apex, simple and compound yellow flowers; large tubers with hairs and several roots; and resistance to anthracnose

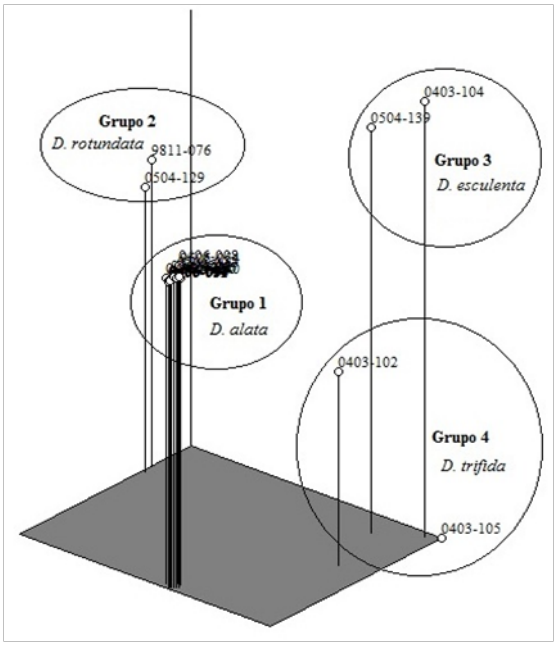

Figure 2. Spatial representation of the genetic structure of 20 *Dioscorea* spp. accessions using AFLP markers, done by multiple correspondence analysis (ACM).

Group 2 consisted on 0406-008 (*Ñame peludo*) accession, collected in Cordoba, Colombia, has a similarity coefficient of 0.82. Its main characteristic is a squared stem in green and brown color, small creases, indeterminate growth, with 4-6 m length and dextral rolling. Leaves are sagittate with not profound lobes and an acute apex. Flowers are feminine, tubers are branched, hairy and with several dark brown roots, flesh is white, tubers are

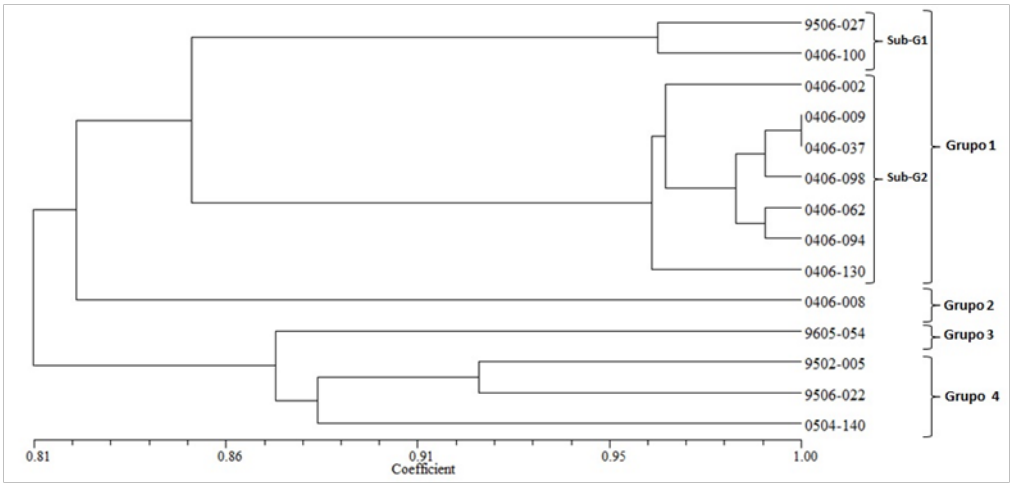

Figure 3. Result of the grouping analysis of the evaluated yam (*Dioscorea alata* L.) accessions, done by Dice's similarity coefficient.

irregular in shape. There are aerial rounded tubers.

Group 3, with the 0406-054 ('ñame seda') accession, collected in Magdalena, Colombia, has a similarity index of 0.871. Its main characteristics are absence of aerial tubers and secondary roots in the tubers, precociousness, and thorns at the base of young stems.

Group 4 consisted on the 9502-005 accession collected in Sucre and, 9506-022 and 0504-140 collected in Cordoba, Colombia, with a similarity coefficient of 0.92. They form aerial tubers, have tubers with secondary roots and are resistant to *C. gloeosporioides* fungi (Campo *et al.*, 2009).

In previous studies that included phenotypic criteria (Lebot *et al.,* 1998; Malapa, 2000), it was demonstrated the wide morphological variability of *D. alata* which has diverse tubers. This variation, could be important as variability resource for breeding programs (Malapa *et al.,* 2005), but must be considered from an individual point of view in order to preserve agronomical and crop production characteristics.

The high interchange degree of seeds coming from Cordoba, Sucre, Bolivar and Magdalena farmers, indicate that clones have been widely distributed in these areas, however, this affirmation should be taken with precaution since, there are not towns largely enough that include different geographical transepts of the Colombian Caribbean region that allow a detailed study of such variability.

Conclusions

- AFLP technique allowed the detection of genetic variability in the twenty evaluated yam accessions, in order to differentiate species using morphological patterns of organization well defined by four genetic groups belonging to the *D. alata, D. rotundata, D. esculenta* and *D. trifida* species.
- Genetic groups share characteristics like presence or absence of stem creases and thorns, leaf and tuber shape and size, and there are accessions grouped according to agronomical characteristics such as anthracnose resistance. These characters are more related to typical qualitative and quantitative characteristics of each genotype and not to the geographical localization from which the accession was collected. The genetic variability found in this study is useful for the preservation of this genetic resource and to increase the collection.
- The molecular characterization allowed the detection of genetic variability among *D. alata* accessions, where a high morphological divergence is appreciated, i.e. dioecism, tuber shape and size. This information could be of high use in the development of strategies for future breeding programs.

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