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Molecular characterization of Colombian introductions of squash *Cucurbita moschata*

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ABSTRACT

The molecular characterization was carried out by means of the amplified fragment length polymorphism (AFLP) of 121 introductions of *C. moschata*, of the germplasm bank of the Vegetables Research Program at the Universidad Nacional de Colombia campus Palmira, originating from eight Colombian departments. The AFLP data were evaluated using multiple correspondence analysis (MCA), genetic distance, genetic analysis with the TFPGA program and the UPGMA cluster analysis. The genetic diversity of these introductions was high and was accordingly to morpho-agronomic diversity, previously studied. The F_{ST} values indicated that exists genetic structure among most of the introductions. Most of the genetic variation among introductions was attributed to variations between individuals within each location.

Key words: Squash, *Cucurbita moschata*, genetic diversity, genetic structure, molecular markers AFLP.

INTRODUCTION

Squash, *Cucurbita moschata* (Duchesne ex Lam.) Duchesne ex Poir., is one of five domesticated species of the genus *Cucurbita* in independent or simultaneous events in Mesoamerica and northern South America (Vallejo & Estrada, 2004; Ferriol *et al*., 2004; Sanjur *et al*., 2002; Decker–Walters & Walters, 2000; Wessel–Beaver, 2000; Robinson & Decker– Walters, 1997; Nee, 1990; Esquinas–Alcázar & Gulick, 1983; Whitaker & Davis, 1962).

Molecular markers have been used to study the genetic diversity of *C. moschata*. Jeon *et al.* (1994), used RAPD markers to discriminate between cultivars of *C. moschata* and *C. pepo* x *C. moschata*. Youn *et al*. (1998), used them to investigate genetic relations between local varieties of native Korean squash. Gwanama *et al*. (2000), analyzed genetic diversity in traditional varieties grown in South-Central Africa and classified them to assist in parental selection to improve fruit characteristics. Baranek *et al*. (2000), used them to reveal genetic diversity within species and between the species of *C. pepo*, *C. moschata*, and *C. maxima*. The only work so far reported, which had achieved AFLP (Vos *et al*., 1995) in *C. moschata* was carried out by Ferriol *et al*. (2004), to determine molecular diversity of squash germplasm collection in Spain.

The aim of this research was to determine the genetic diversity of Colombian introductions of the squash germplasm bank from the Universidad Nacional de Colombia campus Palmira, Colombia by means of characterization with AFLP markers.

MATERIALS AND METHODS

In the Experimental Center of the Universidad Nacional de Colombia, campus Palmira (CEUNP), we planted, in seedlings with peat, 121 introductions collected in eight Colombian departments (Montes *et al*., 2004) and the cv. UNAPAL Bolo Verde of *C. moschata*. Molecular characterization was carried out in the Alexander von Humboldt Institute for research in Biological Resources, located in the International Center for Tropical Agriculture (CIAT), with leaves of 13-day-old seedlings.

For DNA extraction we used the DNeasy Plant Mini Kit protocol of QIAGEN® (2004), with some modifications (Rueda *et al*., 2006) and adapted to *C. moschata*. DNA was displayed on 0.8% agarose gel dyed with ethidium bromide by means of an ultraviolet light trans-illuminator. DNA was quantified using a Hoefer DyNA Quant 200, HiTech-Trader ™ fluorimeter. A previous DNA cut was carried out with the restriction enzyme EcoRI (Invitrogen ™) by means of digestion at 37˚C for six hours, in a PCR PTC-100 Peltier Thermal Cycler machine by MJ Research ™.

For molecular characterization we used the kit of the system of analysis I of AFLP of Invitrogen ™ (2003) and half of the amounts of the protocol. The AFLP fragments were separated by electrophoresis on 6% polyacrylamide gels dyed with silver nitrate (Bassam, 1991). We used molecular weight markers: DNA Ladder 10 bp (330-10 bp) and DNA Ladder 25 bp (500-25 bp) of Invitrogen ™.

AFLP fragments of 70-550 bp of the combination of the "primer" EcoRI-AGC/MseI-CTC were counted manually as follows: present (1) and absent (0), using a white light trans-illuminator. With these data we establish a 0 and 1 matrix.

To carry out a multiple correspondence analysis (ACM) and from genetic distance of the 121 introductions, we used a matrix with all of the monomorphic and polymorphic bands. The statistical package used was NTSYS-pc version 2.0 (Rohlf, 1997).

For genetic analysis we used a binary matrix only with the polymorphic bands; the origin department of the introductions was taken into account. The statistical analyses were carried out with this matrix. The TFPGA software, version 1.3 of 2000 was used (Miller, 1997).

The percentage of polymorphic loci (P) was estimated, with the average heterozygosity; the unbiased Nei formula (1978), was used. To estimate population differentiation among locations, the statistical F_{ST} was calculated. To interpret the F_{ST} value the Wright classification (1978), was used.

The exact test of population differentiation of Raymond & Rousset (1995), was carried out. A matrix of Euclidian distances was developed starting from the matrix of binary data of the introductions. The software AMOVA-PREP version 1.01 (Miller, 1998) was used. Distance matrixes were used to carry out the molecular variance analysis (AMOVA) by means of the WINAMOVA 1.55 software (Excoffier *et al*., 1992). The Bartlett test was also used throughout WINAMOVA 1.55 software (Excoffier *et al*., 1992).

Distance calculations were based on the measurement of the unbiased minimum genetic distance accordingly to Nei (1978). A dendrogram was also carried out by means of the UPGMA grouping method (Sneath & Sokal,

1973). The unbiased minimum distance of Nei (1978), was used. Calculations and dendrograms were established with the TFPGA software (Miller, 1997).

RESULTS AND DISCUSSION

Extraction of DNA and AFLP

With the extraction method, we obtained a great amount of pure, non-degraded DNA ($\overline{X}$: 230 ng/ul), of good quality. We determined 15 minutes, is the adequate timing for lysis of macerated plant tissue with Buffer AP1 at 65˚C. The concentration was higher than obtained by Restrepo (2005), in *C. moschata* (200 ng/ul), which was very close to the range of concentrations (10 ng/ul-300 ng/ul) obtained by Montes (2003), to the concentration (240 ng/ul) obtained by Brown *et al.* (1998), and to the concentration (250 ng/ul) obtained by Chen & Ronald (1999).

DNA performed a complete digestion with EcoRI in all samples, thus confirming the good quality of DNA and the efficacy of this method of DNA extraction. This DNA performed ideal qualities to be used in the AFLP technique. In the 121 introductions plus the cv. UNAPAL Bolo Verde, 117 bands were performed, from which 91 were polymorphic (77.8%) (Figure 1). Each introduction performed different bands that indicated molecular genetic variation among themselves.

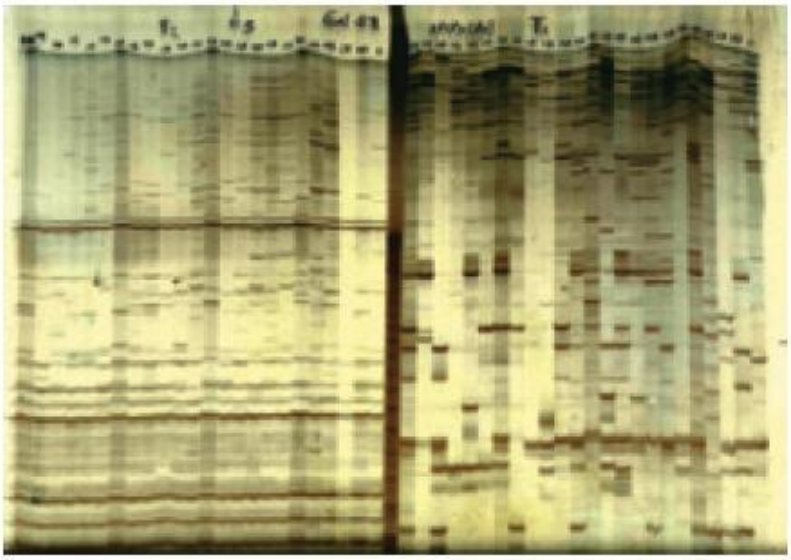

Figure 1. AFLP molecular markers of the combinations of primers T2 (EcoRI-AGC / MseI-CTC) and T1 (EcoRI-ACG / MseI-CAT) of introductions of *C. moschata*, separated by electrophoresis on 6% polyacrylamide gel, dyed with silver.

Genetic diversity of introductions originating from eight Colombian departments

The number of polymorphic loci in the introductions of Colombian *C. moschata* (89.01%) was higher than obtained by Ferriol *et al.* (2004), (85.90%) and the values per location fluctuated between 43.96 and 87.91%, respectively (Table 1). The higher percentages of polymorphic loci were performed in the introductions coming from Valle, Cauca, and Caldas departments. The lowest percentages of polymorphic loci were performed in the

introductions from Risaralda, Quindío and Bolívar departments and were related with small sample size (Risaralda, n = 4; Quindío, n = 8; and Bolivar, n = 6).

Table 1. Polymorphic loci and heterozygosity of *C. moschata* in eight Colombian departments, according to the square root method of recessive genotype.

Departments	% of polymorphic loci (*)	Estimated average heterozygosity (**) Square root of q^2
Valle (n=24)	87.91	0.2510
Cauca (n=18)	81.32	0.2339
Quindío (n=8)	54.94	0.1778
Risaralda (n=4)	43.95	0.1609
Caldas (n=9)	79.12	0.2093
Magdalena (n=16)	54.94	0.1702
Atlántico (n=37)	71.43	0.2029
Bolívar (n=6)	50.55	0.2036
Total introductions: (n=122)	89.01	0.2529

* A 99% criterion was used to define the % of polymorphic loci.
** Assuming Hardy-Weinberg equilibrium.

The value of unbiased average heterozygosity or genetic diversity from all Colombian introductions of *C. moschata* was high (0.2529), compared to that obtained by Ferriol *et al*. (2004), (0.17), in accessions collected in different regions of Spain, 12 accessions from South and Central America (Ecuador, Cuba, Perú, Guatemala, and Dominican Republic), two accessions from Morocco, and the commercial squash cv. Butternut from USA. The genetic diversity obtained is in agree with the high morpho-agronomic diversity recorded in this same collection by Montes *et al*. (2004). Values fluctuated between 0.1609 (Risaralda) and 0.2510 (Valle) (Table 1). Valle and Cauca departments exhibited the highest heterozygosity levels, possibly due to the closeness and to anthropogenic exchanges, while Risaralda department presented the lowest heterozygosity (0.1609). The average heterozygosity value for all the introductions, was relatively higher than the average heterozygosity for each department, which could indicate a degree of significant genetic differentiation among departments.

The genetic diversity found in the Colombian collection of *C. moschata* agrees with previous studies carried out by Ferriol *et al*. (2004), Wessel–Beaver (2000), and Whitaker & Davis (1962). It also agrees with the identification of areas of high diversity of *C. moschata* in Colombia, where traditional varieties exhibit common primitive traits (Zhiteneva, 1930; Nee, 1990; Wessel–Beaver, 2000; Sanjur *et al*., 2002).

Genetic differentiation between introductions

The value of average F_{ST} (0.1357 ± 0.0136, with a confidence interval of 95% between 0.1112 and 0.1614) indicated moderate genetic differentiation (Wright, 1978) among introductions coming from the eight Colombian departments. The average F_{ST} between all pairs of departments was 0.1355, which fluctuated from -0.0027 for the linked departments Valle-Cauca, with small genetic differentiation and geographic neighbors, and 0.3116 for the linked departments Quindío (center)-Magdalena (Atlantic Coast) with high genetic differentiation and geographically distant (Table 2).

Table 2. F_{ST} by means of the method of distance between introductions of *C. moschata* coming from eight Colombian departments.

Departments	Valle	Cauca	Quindío	Risaralda	Caldas	Magdalena	Atlántico	Bolívar
Valle	*****							
Cauca	-0.0027	*****						
Quindío	0.1059	0.1196	*****					
Risaralda	0.0434	0.0708	0.0452	*****				
Caldas	0.0618	0.0762	0.0134	0.0003	*****			
Magdalena	0.1545	0.1757	0.3116	0.2269	0.2460	*****		
Atlántico	0.0976	0.1260	0.1981	0.1107	0.1706	0.0543	*****	
Bolívar	0.0701	0.1003	0.2207	0.0732	0.1519	0.1469	0.0465	*****

Most F_{ST} values between linked departments were significantly high, excepting the linked departments Valle-Cauca, Valle-Risaralda, Quindío-Risaralda, Quindío-Caldas, Risaralda-Caldas, and Atlántico-Bolivar, respectively, whose F_{ST} values indicated a small genetic differentiation. The small differentiation was due to the fact they share morpho-agronomic traits such as shape and fruit size, shape and seed size, and flowering time. This is according to the five groups comprised in the morphologic characterization (Montes *et al*., 2004), in which the groups of the Andean introductions from Valle, Cauca, Quindío, Risaralda, and Caldas departments and those of the Atlantic Coast (Atlántico, Bolivar, and Magdalena departments) were different. In addition, the six pairs or linked departments are very geographically closed and with possible anthropogenic effect.

F_{ST} values indicated a moderate genetic differentiation from the linked departments Valle-Quindío, Valle-Caldas, Valle-Atlántico, Valle-Bolivar, Cauca-Quindío, Cauca-Risaralda, Cauca-Caldas, Cauca-Atlántico, Cauca-Bolivar, Risaralda–Atlántico, Risaralda–Bolívar, Magdalena–Atlántico and Magdalena–Bolívar, respectively. Montes *et al.* (2004), also found morpho-agronomic traits, which were common in some linked departments where was distanced each other (Valle–Caldas, Cauca–Quindío, Cauca– Caldas) and in others distanced (Valle–Atlántico, Cauca–Atlántico, Risaralda–Bolívar), with possible anthropogenic effect.

The F_{ST} indicated great genetic differentiation between the linked departments Valle–Magdalena, Cauca–Magdalena, Quindío–Atlántico, Quindío–Bolívar, Risaralda–Magdalena, Caldas–Magdalena, Caldas–Atlántico, and Caldas–Bolívar, respectively. These linked departments conform groups, which are different from the morpho-agronomic traits (Montes *et al*., 2004). They are departments distant from one another, one located in the Colombian southwest and the other one in the northwest. The F_{ST} of Quindío-Magdalena indicates a high genetic differentiation between these two departments and is correlated with the great geographic distance existing between Quindío in the southwest and Magdalena in the Colombian northwest. Values indicate that there is a genetic structure between the introductions of the eight Colombian departments and it is not a continuous structure or panmictic unity.

The exact test of differentiation between introductions (Table 3) confirmed significant genetic differentiation between departments of the Atlantic Coast and those of the Andean region. These results agree with the diversity index (62%) estimated in the investigation of Montes *et al.* (2004).

Table 3. Differentiation test between introductions of *C. moschata* coming from eight Colombian departments.

Departments	Valle	Cauca	Quindío	Risaralda	Caldas	Magdalena	Atlántico	Bolívar
Valle	*****	1.0000	0.4702	1.0000	0.6346	0.0000*	0.0000*	0.9978
Cauca	92.2306	*****	0.6885	1.0000	0.5635	0.0000*	0.0000*	0.9896
Quindío	182.7625	172.1378	*****	1.0000	1.0000	0.0000*	0.0000*	0.6535
Risaralda	88.9584	107.1105	71.7906	*****	1.0000	0.9163	0.9994	1.0000
Caldas	174.8626	178.3111	75.3443	55.4763	*****	0.0000*	0.0000*	0.8525
Magdalena	289.5888	289.7867	315.1221	156.3110	324.1026	*****	0.9964	0.9671
Atlántico	330.0943	342.7471	291.0938	126.4934	328.3445	134.8445	*****	1.0000
Bolívar	132.3211	140.7959	173.9252	75.0473	162.1226	148.5233	111.0275	*****

* P<0.05, there are differences between departments.
** Chi-square values at the bottom of the diagonal.
*** Probability values at the top of the diagonal.

The analysis of molecular variance (AMOVA) (Table 4) showed significant differences between departments (P<0.001). 88.76% of the total genetic variation was due to the existence between introductions within each department.

Table 4. Analysis of Molecular Variance for the introductions of *C. moschata* coming from eight Colombian departments.

Source of variation	D. F.	Sum of Squares	Mean Squares	V (Variance)	(%) of Variation	Probability
Between departments	7	207.3196	29.6170	1.3378	11.24	<0.001
Within departments	114	1204.4099	10.5650	10.5650	88.76	
Valle		272.5000				
Cauca		191.7778				
Quindío		74.1250				
Risaralda		32.0000				
Caldas		106.8899				
Magdalena		118.8750				
Atlántico		353.2432				
Bolívar		55.0000				
Total	121	1411.7295				

Genetic variation between departments was highly significant (P<0.001) and the total genetic structure, Φ_{ST}: 11.24, differed significantly from cero. The percentage of variation between departments is assimilated as the value of Φ_{ST} in all cases. Between the value of total Φ_{ST} and that of F_{ST} no significant difference was found that confirms that there exists a genetic variation between the introductions coming from the eight departments, although most of it was found within each department.

The heterogeneity of the variance between departments estimated by means of the Bartlett test of homogeneity of variances (Table 5) was of Bp = 0.95653, with a level of significance of P < 0.001, that indicates significant levels of variability between introductions coming from the different departments.

Table 5. Bartlett test of variance homogeneity for introductions of *C. moschata* coming from eight Colombian departments. Bartlett statistics (*) and probability (**).

Departments	Valle	Cauca	Quindío	Risaralda	Caldas	Magdalena	Atlántico	Bolívar
Valle	*****	0.6703	0.0000	0.2478	0.0000	0.0000	0.0000	0.0000
Cauca	0.9213	*****	0.0000	0.1209	0.0000	0.0000	0.0000	0.0000
Quindío	2.2465	2.2712	*****	0.0060	0.3307	0.0000	0.0000	0.0000
Risaralda	1.1624	1.3007	1.0486	*****	0.2228	0.0000	0.0450	0.2787
Caldas	0.1719	1.9032	1.1019	0.9018	*****	0.0000	0.0000	0.0000
Magdalena	4.8340	4.6517	5.0980	2.5006	4.8299	*****	0.0000	0.0000
Atlántico	4.1855	4.3269	3.8789	1.6765	3.9694	2.4069	*****	0.0829
Bolívar	1.5752	1.7912	2.4239	1.1208	2.0006	2.3975	1.4100	*****

* Bartlett statistics, values at the bottom of the diagonal.
** Probability values at the top of the diagonal.

The linked departments which did not present differences between their variances were: Valle–Cauca, Risaralda–Valle, Risaralda–Cauca, Risaralda–Quindío, Caldas–Quindío, Caldas–Risaralda, Bolívar–Risaralda, and Bolívar–Atlántico, respectively. The majority of these linked departments presented little genetic differentiation and are located geographically close, except for the linked departments Bolivar-Risaralda.

The values of genetic distance, with an average of 0.0360 (Table 6), fluctuated between 0.0040 for the linked departments Valle-Cauca and 0.0884 for Quindío-Magdalena. The genetic distance analysis agreed with the results provided by the differentiation tests between introductions of *C. moschata* coming from eight Colombian departments, which confirms that there exists a genetic structure.

The tree of genetic distance (Figure 2) presented seven groupings between introductions of *C. moschata*. Between the departments of Valle and Cauca, and Quindío and Caldas, it presented distance values close to cero, which indicates that they are related or that there is a high similarity. Between the groups of the Atlantic Coast and the Andean region one highlights the great genetic differentiation and geographic distance except for Risaralda. Although they are grouped with departments of the Atlantic Coast, they do not share morphological traits (Montes *et al*., 2004). This is probably the anthropogenic effect.

Analysis of multiple correspondence and genetic distance of all of the introductions of *C. moschata*

On carrying out the multiple correspondence analysis we did not observe a tendency to conform groups between the 121 introductions of *C. moschata*. However, the classification analysis was carried out (Figure 3). In the dendrogram one observes the tendency to form three great groups (Table 7). Group I with introductions originating in the Andean region, with a similarity index of Nei and Li (1979) of approximately 0.68. Group II with introductions coming from the Andean region and the Atlantic Coast, mostly from the latter, with a similarity index of 0.71. And Group III with other introductions coming from the Andean region with a similarity index between 0.48 and 0.68. Atlantic Coast introductions were grouped into Group II because they have close genetic distances, share the same northwestern geographic Colombian region, and share morphological traits. In this group some Andean introductions were placed with those of the Atlantic Coast,

possibly due to the anthropogenic effect or because they share qualitative and quantitative morphological traits (Montes *et al*., 2004).

Table 6. Nei's unbiased genetic distance between introductions of *C. moschata* coming from 8 Colombian departments.

Departments	Valle	Cauca	Quindío	Risaralda	Caldas	Magdalena	Atlántico	Bolívar
Valle	*****							
Cauca	0.0040	*****						
Quindío	0.0277	0.0327	*****					
Risaralda	0.0356	0.0417	0.0418	*****				
Caldas	0.0220	0.0301	0.0052	0.0339	*****			
Magdalena	0.0518	0.0556	0.0884	0.0483	0.0741	*****		
Atlántico	0.0265	0.0323	0.0506	0.0323	0.0449	0.0138	*****	
Bolívar	0.0360	0.0431	0.0749	0.0325	0.0616	0.0262	0.0143	*****

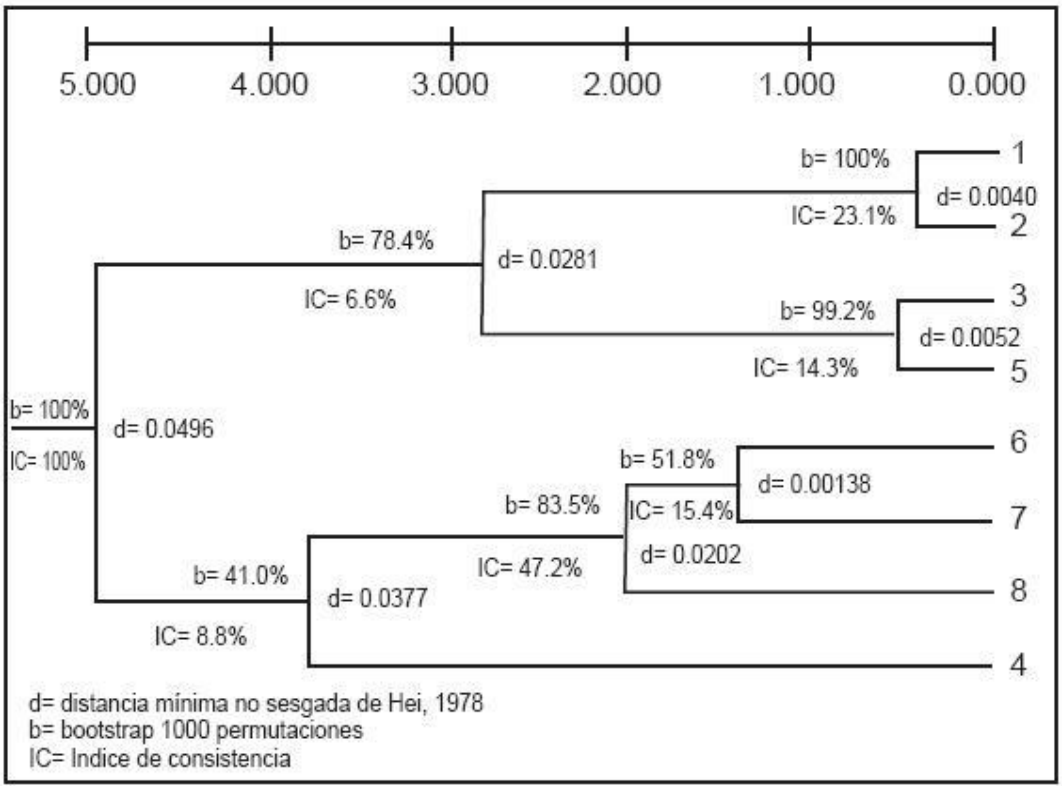

Figure 2. Nei's unbiased tree of distance, based on the UPGMA method for *C. moschata* introductions coming from 8 Colombian departments: Valle (1), Cauca (2), Quindío (3), Risaralda (4), Caldas (5), Magdalena (6), Atlántico (7), and Bolivar (8) (d= minimum unbiased distance of Nei, 1978; b= bootstrap 1000 permutations; IC=CI= Consistency Index).

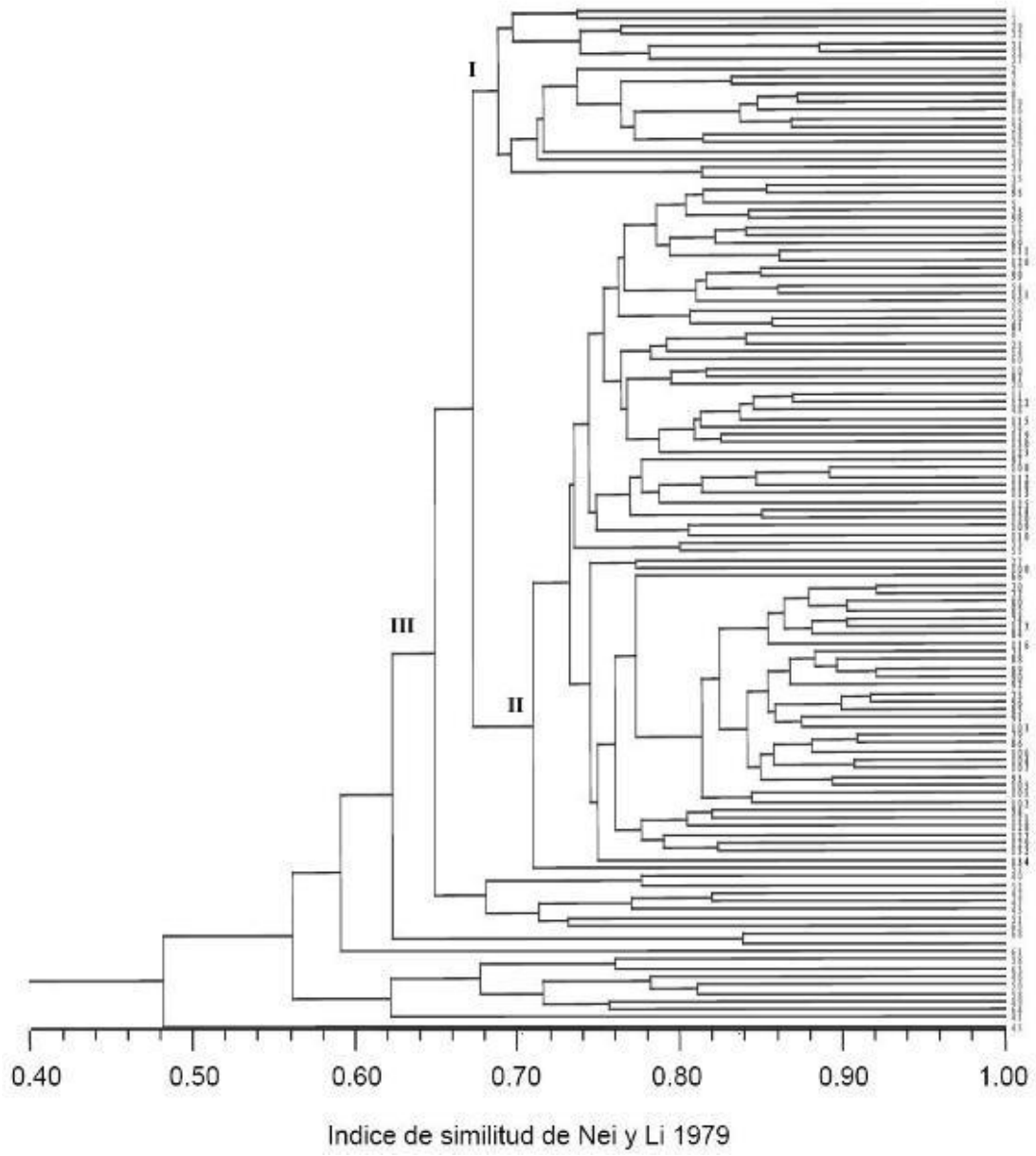

Figure 3. Dendrogram generated with Nei and Li's genetic similarity indexes, estimated in the analysis of AFLP fragments, for 121 Colombian introductions of *C. moschata*.

Table 7. Groups of introductions of *C. moschata* formed by means of classification analysis.

Groups	Regions	Introductions
I	Andean	1,3,29,32,31,33,37,2,7,9,8,19,16,15,24,18,26,17,36,21,35.
II	Andean Atlantic Coast	4,5,12,25,22,54,28,56,59,6,23,14,60,10,20,11,48,34,53,55,27,134. 95,73,98,69,111,126,99,131,81,87,122,115,119,130,123,97,108,112,118,113, 125,114,120,109,110,100,66,70,72,80,82,74,117,84,116,71,88,89,90,92,75,96 ,85,91,103,79,86,106,104,107,93,105,101,102,94,121,128,127,129,132.
III	Andean	57,40,52,42,47,43,51,65,68,135,61,38,63,46,50,58,49,64,41,45.

CONCLUSIONS

The molecular genetic diversity of the 121 introductions of *C. moschata* coming from eight Colombian departments was high (0.2529) and was in accordance with the great morpho-agronomic diversity previously recorded.

The mean heterozygosity of all of the introductions (25.3%) was higher than the mean of heterozygosity for each group of introductions coming from the eight departments. The F_{ST} values indicate that there is a genetic structure between most of the introductions coming from the eight Colombian departments and not a continuous structure or panmictic unity.

There was significant genetic variation of 11.24% between the introductions coming from the eight departments. Most of the total variation was due to variation between individuals of *C. moschata* within each province (88.76%).

In the classification analysis, the 121 introductions were grouped in three great groups: two with introductions from the Andean region and another one mixed with Andean and Atlantic Coast introductions, but mostly from the Atlantic Coast.

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