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Molecular differentiation of Xoconostle (*Opuntia* spp.) using AFLP molecular markers

Diferenciación molecular de Xoconostle (*Opuntia* spp.) usando marcadores moleculares AFLP

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Abstract. The technologies based on molecular markers of DNA are the most advanced and possibly the most effective and accurate means to identify genetic variation. The objective of this study was to use molecular genetic markers namely AFLP as a tool for the molecular differentiation of 36 accessions of xoconostle collected in the Centro Regional Universitario Centro Norte of the Universidad Autónoma de Chapingo (CRUCEN-UACh.). DNA extraction was performed by the method of cellular ruptor using FastDNA® Kit, and quantification of purity and yield of DNA was carried out through the Nanodrop 2000. It was observed that the DNA concentrations ranged from 108.4 ng/µL to 38.5 ng/µL. Two hundred and ninety five molecular markers were generated with a molecular weight ranging from 50 to 800 bp. The average diversity index (ID) of the 36 accessions was 0.8124. A binary matrix was generated which was analyzed through clusters by the unweighted pair clustering method (UPGMA). In the dendogram two groups of 5 and 31 accessions were generated and the last group was divided into two subgroups consisting of 8 and 23 accessions, respectively. It was observed that there were differences between the accessions of xoconostle, presenting a high degree of similarity because its grouping was realized at short distances and in a staggered way. The accessions Orito Xoconostle 14, Orito Xoconostle 16 had a zero euclidean distance, being considered duplicate, so that only one of them should be established inside the germplasm bank.

Keywords: *Xoconostle*; AFLP; Molecular markers; Genetic diversity.

Resumen. Las tecnologías basadas en marcadores moleculares de ADN son los más avanzados y posiblemente los medios más efectivos y precisos para identificar la variación genética. El objetivo de este estudio fue utilizar marcadores genéticos moleculares AFLP como una herramienta para la diferenciación molecular de 36 accesiones de xoconostle colectadas en el Centro Regional Universitario Centro Norte de la Universidad Autónoma de Chapingo (CRUCEN-UACh). La extracción de ADN se realizó mediante el método de ruptor celular utilizando FastDNA® Kit, y la cuantificación de la pureza y el rendimiento de ADN se llevó a cabo a través de la Nanodrop 2000. Se observó que las concentraciones de ADN osciló entre 108,4 ng/µL a 38,5 ng/µL. Se generaron 295 marcadores moleculares con un peso molecular de 50 a 800 pb. El índice de diversidad promedio (ID) de las 36 accesiones fue 0,8124. Se generó una matriz binaria que fue analizada a través de conglomerados por el método de agrupación de pares no ponderada (UPGMA). En el dendograma se generaron dos grupos de 5 y 31 accesiones y el último grupo se dividió en dos subgrupos integrados por 8 y 23 accesiones respectivamente. Se observó que hubo diferencias entre las accesiones de xoconostle, presentando un alto grado de similitud debido a que su agrupación se realizó a distancias cortas y de manera escalonada. Las accesiones Orito Xoconostle 14, Orito Xoconostle 16 tuvieron una distancia euclidiana cero siendo consideradas duplicadas, por lo que sólo se debe establecer una de ellas en el banco de germoplasma.

Palabras clave: *Xoconostle*; AFLP; Marcadores moleculares; Diversidad genética.

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INTRODUCTION

Among the natural riches of Mexico is the xoconostle (from Nahuatl: "xoco" meaning acid and "noxtle" meaning tuna) that modern taxonomists have decided to name as *Opuntia matudae* and even *Opunia xoconostle* better known as acidic fruit (Bravo & Sanchez, 1991). Xoconostles have a wide inner edible wall and a thin outer wall that is not easily removed, and seeds are arranged in the center of the fruit with dry funiculus, whose species are used in human food and traditional medicine (Scheinvar et al., 2009). This variety of fruit is important because the xoconostle has been a relevant factor in the economic support of the Mexican peasant. The peel is rich in fiber, minerals, soluble phenols and betalains, it presents higher antioxidant capacity than strawberry, raspberry, red plum, grapefruit, pear and apple (García et al., 2005; Scheinvar et al., 2009; Guzmán et al., 2010).

Xoconostles are distributed throughout the country, but the greatest concentration of xoconostles is found in the arid and semi-arid zones of the Chihuahua desert and in the semi-arid zone of Tehuacán-Cuicatlán (Scheinvar et al., 2010). This genetic wealth must be considered as part of the country heritage, so attention should be paid to its conservation, improvement and use, making it an active element in the country development (de Luna et al., 2016).

DNA markers have been used to characterize the genetic diversity of various plant species including the cactus (García et al., 2009). The Amplified Fragment Length Polymorphism (AFLP) was originally designed by Vos et al. (1995). It is a highly sensitive method for the generation of genetic fingerprints that can be used in a wide variety of fields. This is the result of the molecular genetic variations that exist between genetically nearby species, varieties or cultivars. These variations in the DNA sequence are exploited by AFLPs in such a way that genetic fingerprints are generated, which can be produced routinely and consistently (Cenis, 2000). It has been reported that AFLPs are efficient to distinguish differences between accessions by detecting up to 69% of genetic diversity, with polymorphism ranges from 0.3 to 0.93 (Pecina, 2012). The analysis of AFLP markers to estimate genetic diversity has been used to complement agronomic data when information is limited or non-existent (Jiang, 2010).

The objective of the present study was to use molecular genetic markers namely AFLP to make the molecular differentiation of 36 accessions of xoconostle, estimating the degree of similarity between them and initiating the generation of the genetic fingerprinting of each accession.

MATERIALS AND METHODS

Plant material. The material analyzed consisted of 36 xoconostle accessions (Table 1), collected in several localities of the State of Zacatecas. They were deposited in the Centro Regional Universitario Centro Norte of the Universidad Autónoma de Chapingo (CRUCEN-UACh.), in the state of Zacatecas, within a locality "Huertas El Orito" (22° 44′ 47.28″ N, 102° 36′ 26.90″ W) for its establishment at the bank of germplasm of that Institution. The samples were taken to the Biotechnology Laboratory of the Facultad de Agronomía of the UANL for analyses.

Table 1. Plant material collected in the Centro Regional Universitario Centro Norte.
Tabla 1. Material vegetal colectado en el Centro Regional Universitario Centro Norte.

Accesion	Common name
1	Orito Xoconostle 11
2	Orito Xoconostle 12
3	Orito Xoconostle 13
4	Orito Xoconostle 14
5	Orito Xoconostle 15
6	Orito Xoconostle 16
7	Orito Xoconostle 17
8	Orito Xoconostle 18
9	Orito Xoconostle 19
10	Orito Xoconostle 20
11	Orito Xoconostle 21
12	Orito Xoconostle 22
13	Orito Xoconostle 23
14	Orito Xoconostle 24
15	Cambray
16	Blanco arroyo hondo
17	Cuaresmeño blanco zac
18	El Chocho
19	Apastillado
20	Cueron
21	Rojo sainero
22	Café
23	Chavenito
24	Cenizo
25	Guerito
26	El Ranchito
27	Xoconostle Rosita
28	Xoconostle Manso 7637
29	Xoconostle de Cerro Blanco
30	Xoconostle Cuaresmeño de pulpa blanca
31	Xoconostle de Cerro Rojo
32	Xoconostle Cuaresmeño (fruta 9)
33	Xoconostle Cueresmeño
34	Xoconostle del Borrego
35	Xoconostle Apan
36	7592

Extraction of DNA. It was performed by the cell-ruptor method with the FastDNA® kit (Qbiogene, Inc. C.A.). Quantification of the DNA was performed by Nanodrop 2000 and the quality was determined on a 1% agarose gel (120 mL of .5X TBE and 1.2 g of agarose) stained with 2 µL of SYBR GREEN using a 100 bp marker of the BIOLINE brand; DNA separation was performed by electrophoresis at 120V for one hour.

Generation of AFLP in Xoconostle. The generation of AFLP markers was performed according to the protocol of Vos et al. (1995) modified by Simpson & Gil (2004), using the commercial kit IRDye ™ Flourescent AFLP® Kit for Large Plant Genome Analysis of LI-COR® (LI-COR Biosciences, Lincoln, NE, USA).

Digestion of genomic DNA using restriction enzymes. DNA digestion was performed as specified in LI-COR® Biosciencies IRDye™ Fluorescent AFLP kit. A 12.5 µL reaction volume was handled with the following components in the reaction mixture: 5X buffer, 100 ng of DNA, EcoR1/Mse1 enzyme mixture and deionized water. The incubation time was 2 hours at 37 °C with a final enzyme inactivation step at 70 °C for 15 minutes. Digestion was corroborated on a 1% agarose gel.

Connection of adapters. The total volume of the reaction mixture was 25 µL. The components of the mixture were: adapter solution, 12 µL; T4 DNA ligase, 0.5 µL, and sterile deionized water to complete the reaction volume. The reaction was incubated at 20 °C for two hours. Subsequently, a 1:10 TE dilution of the digested and bound DNA mixture was performed.

Pre-amplification. In the case of preamplification, a reaction volume of 25.5 µL was handled, the components of the reaction mixture were: digested and ligated DNA, 2.5 µL; AFLP preamp mix, 20 µL; Buffer solution 10 X (provided with Taq polymerase), 2.5 µL, and Taq DNA polymerase (5 U/µL), 0.5 µL. The reaction mixture was subjected to amplification in a thermocycler under the 20 cycle thermal program at: 94 °C for 30 seconds, 56 °C for one minute and 72 °C for one minute. At the end of the program, the samples were maintained at 4 °C. Pre-amplification was corroborated on a 1% agarose gel. A 1:40 dilution in TE was performed with the reaction mixture.

Selective amplification. Selective amplification was performed in the "duplex" mode, a MseI primer with two EcoRI primers labeled with IRDye at 700 and 800. The reaction volume was 11 µL. The components of the mixture were: preamplified and diluted DNA, 2 µL; Working solution of Taq DNA polymerase, 6 µL; MseI primer, 2 µL; EcoRI primer labeled at IRDye 700, 0.5 µL, and EcoRI primer labeled at 800 IRDye, 0.5 µL. Because the volume for sample handling for preparation of the working solution of Taq DNA polymerase was too small, 200 µL were prepared which is recommended and sufficient to process 33 samples. The mixture and proportion of the reagents were: sterile deionized water, 158 µL; Buffer solution 10 X, 40 µL, and Taq DNA polymerase, 2 µL. The amplification was performed in thermocycler under the thermal program of: one cycle of 94 °C for 30 seconds, 65 °C for 30 seconds and 72 °C for one minute. Twelve consecutive cycles where the alignment temperature (65 °C) decreases

Table 2. Combinations of primers for the generation of selective amplification.
Tabla 2. Combinaciones de iniciadores para la generación de la amplificación selectiva.

Combination	primer Mse1	primer EcoR1 IRDye 700	primer Eco R1 IRDye 800
1	M-CAA	E-AAC	E-ACT
2	M-CTA	E-ACA	E-AGC
3	M-CAG	E-AAG	E-ACG
4	M-CTG	E-ACC	E-AGC
5	M-CTT	E-ACA	E-AGG
6	M-CAA	E-AAG	E-ACG
7	M-CAA	E-ACA	E-AGC
8	M-CAA	E-ACC	E-AGG
9	M-CAC	E-AAC	E-ACG
10	M-CAC	E-AAG	E-ACT
11	M-CAC	E-ACA	E-AGC
12	M-CAC	E-ACC	E-AGG
13	M-CAG	E-AAC	E-ACT
14	M-CAG	E-ACA	E-AGC
15	M-CAG	E-ACC	E-AGG
16	M-CAT	E-AAC	E-ACG
17	M-CAT	E-AAG	E-ACT
18	M-CAT	E-ACA	E-AGC
19	M-CAT	E-ACC	E-AGG
20	M-CTA	E-AAC	E-ACG
21	M-CTA	E-AAG	E-ACT
22	M-CTA	E-ACC	E-AGG
23	M-CTC	E-AAC	E-ACG
24	M-CTC	E-AAG	E-ACT
25	M-CTC	E-ACA	E-AGC
26	M-CTC	E-ACC	E-AGG
27	M-CTG	E-AAC	E-ACG
28	M-CTG	E-AAG	E-ACT
29	M-CTG	E-ACA	E-AGG
30	M-CTT	E-AAC	E-ACG
31	M-CTT	E-AAG	E-ACT
32	M-CTT	E-ACC	E-AGC

by 0.7 ˚C for each cycle (the denaturation temperature of 94 ˚C and extension of 72 ˚C were maintained); and 23 cycles of 94 ˚C for 30 seconds, 56 ˚C for 30 seconds and 72 ˚C for one minute. After the program was completed, the samples were stored at 4 ˚C. The 64 primer combinations of the kit (Table 2) were tested in 32 duplex reactions.

Electrophoresis. Separation and analysis of the amplified fragments were performed on 6.5% polyacrylamide gel using the LI-COR IR^2 4200 sequencer (LI-COR Biosciences, Lincoln, NE) with the SAGAMX program. The data collection conditions were 16 bits, voltage 1500, power 40 W, current at 40 mA, temperature at 45 ˚C and a scanner speed of 4.

Analysis of molecular data. To estimate the similarity of the xoconostle we used cluster analysis or "agglomerative hierarchical cluster analysis" (Luna et al., 2007), because this analysis is so robust for the reconstruction of hierarchical phylogenetic relationships as cladistic methods (James & McCulloch, 1990). Well defined amplified DNA fragments (bands) obtained with the primers were analyzed to generate a binary data matrix (0 and 1) where "0" represented the absence of the band and "1" represented the presence of the band. With the binary data matrix the genetic distances matrix was constructed by comparing each pair of genotypes using the method of Nei & Li (1979). For the formation of the clusters he used the unweighted arithmetic mean grouping (UPGMA) method which maximizes the variation among the groups and minimizes it within them (Hair et al., 2014).

RESULTS

DNA concentrations obtained an average yield of 74.04 ng/μL. The highest value was 108.4 ng/μL in the Orito Xoconostle 24 accession, and the lowest value was 38.5 ng/μL in the Orito Xoconostle 13 accession.

In the AFLP generation the 64 primer combinations were used (Table 2), obtaining fragments whose weight ranged from 50 to 800 bp, in all amplification products (Fig. 1 and 2).

The fragments produced were potentially polymorphic, with a greater number of bands observed when the IRDye 700 labeled primers were used. The highest amplification was obtained with the M-CAC/E-AAG combination that was amplified in 77% of the samples. This combination was reported by Espinoza (2014) in which the fragments generated were used to construct a dendrogram, and obtain the respective genetic relation. Forty percent of the samples were the least amplified with the M-CAG/E-ACG combination. Two hundred and fifty nine molecular markers were generated with an average diversity index (ID) of 0.8124.

The UPGMA analysis (Fig. 3) evidenced the degree of genetic differentiation and separated the accessions into two groups formed at a euclidean distance of 10. The first group consisted of five accessions (Cuaresmeño pulpa blanca, Xoconostle Cerro Rojo, Xoconostle Cuaresmeño (fruta 9), Xoconostle Cerro Blanco, Xoconostle Manso) and the second group consisted of 31 accessions. The latter was subdivided into two subgroups composed of 8 accessions grouped to an Euclidean distance of 8 (Cuaresmeño Blanco Zacatecas, Cueron, El Chocho, Blanco Arroyo Hondo, Cambray, Apastillado, Orito Xoconostle 27, Orito Xoconostle 22), and 23 accessions grouped to an Euclidean distance of 7 (Xoconostle 21,

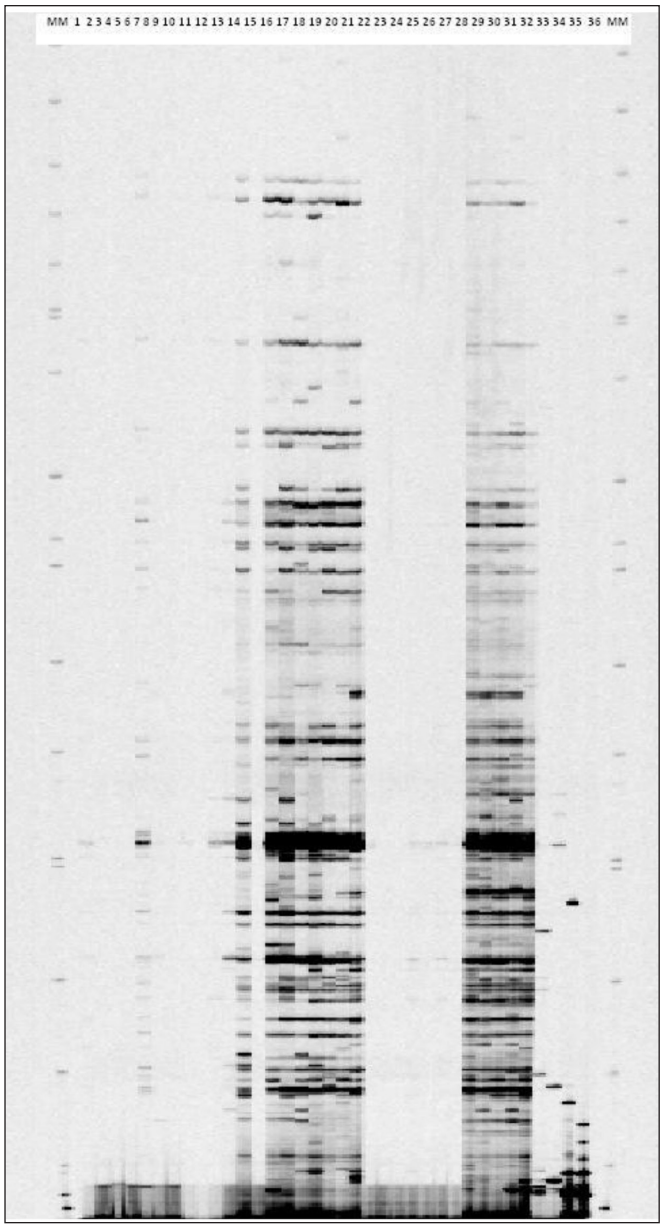

Fig. 1. Amplification products of the 36 accessions using AFLP with combination of initiator M-CAC-E-AAG marked with IRDye 700.

Fig. 1. Productos de amplificación de las 36 accesiones por medio de la técnica de AFLP con la combinación de iniciador M-CAC-E-AAG marcado con IRDye 700.

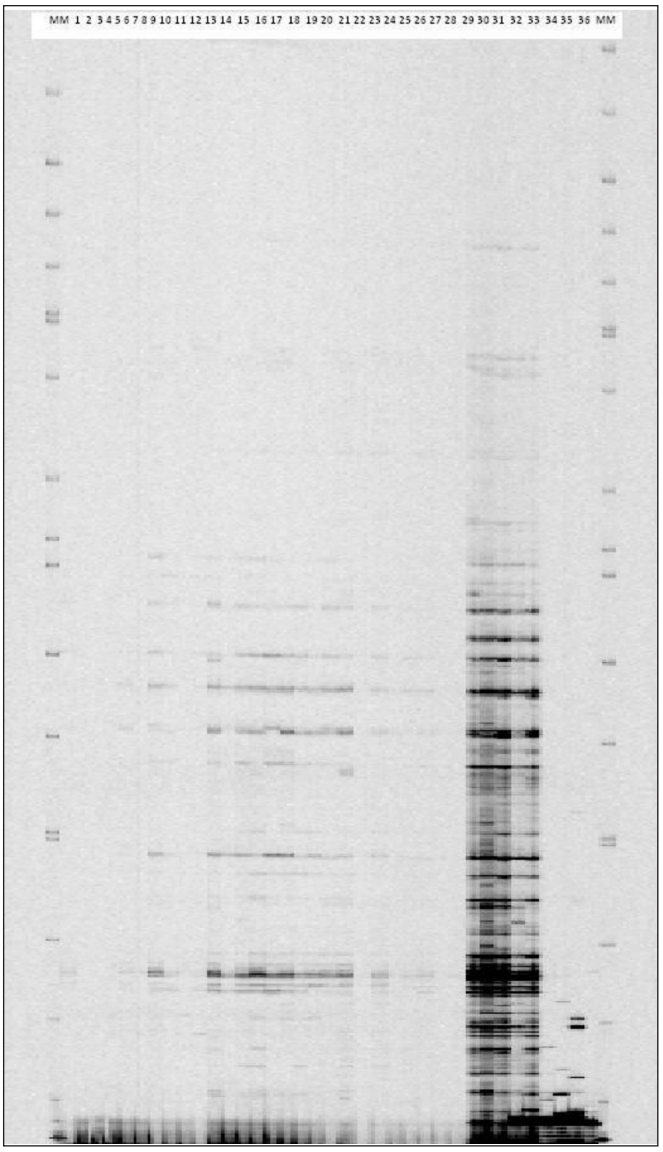

Fig. 2. Amplification products of the 36 accessions using AFLP with combination of initiator M-CAC-E-ACT marked with IRDye 800.

Fig. 2. Productos de amplificación de las 36 accesiones por medio de la técnica de AFLP con la combinación de iniciador M-CAC-E-ACT marcado con IRDye 800.

Orito Xoconostle 15, Orito Xoconostle 23, Orito Xoconostle 17, Xoconostle Cuaresmeno, Guerito, Chavenito, Café, Rojo Sainero, Xoconostle Apan, Orito Xoconostle 20, Orito Xoconostle 18, Cenizo, Orito Xoconostle 12, 7592, Xoconostle del Borrego, Xoconostle Rosita, Orito Xoconostle 19, Orito Xoconostle 16, Orito Xoconostle 14, Orito Xoconostle 13, El Ranchito, Orito Xoconostle 11). The accessions Orito Xoconostle 14 and Orito Xoconostle 16 presented an Euclidean distance of zero.

DISCUSSION

DNA concentrations were found to be higher in young plant tissue (108.4 ng/µl) compared to old plant tissue (38.5 ng/µL).

Molecular markers were defined from well defined bands and with the same intensity, generating two hundred and ninety five molecular markers with all combinations of primers of the analyzed material.

When analyzing the dendrogram generated from the UPGMA analysis, it is not possible to identify groups with a common molecular marker. This is because the bands that were obtained were polymorphic, in addition to that there was no phenotypic evaluation of the accessions studied. It is possible to distinguish different types of xoconostle because the differences in their strings of DNA were very notorious. There was a high degree of kinship in some accessions (Orito Xoconostle 13, Orito Xoconostle 19) because they were grouped at a short euclidean distance (1), until all the accessions were grouped in a staggered form at a distance of 13.

The average diversity index (ID) of the 36 accessions of xoconostle was 0.8124. This value is close to that reported by Escalante (2012) in cactus, indicating that the collected material has a high genetic variability.

In the dendrogram the accessions Orito Xoconostle 14, Orito Xoconostle 16 presented an Euclidean distance of zero. Because of this, they were considered like duplicated. As a result, only one of them must be established within the bank.

CONCLUSION

The use of molecular markers AFLP is an efficient tool to differentiate the accessions of xoconostle. Two hundred and fifty nine molecular markers of the AFLP type were generated, which served to more accurately estimate the genetic diversity among the xoconostle accessions.

It was possible to differentiate the xoconostle accessions in spite of a high degree of kinship, because the grouping was realized at short distances and in a staggered form.

The accessions Orito Xoconostle 14, Orito Xoconostle 16 presented an Euclidean distance of zero, being considered duplicates and only one of them must be established in the bank of germplasm.

Disclosure policy. The authors declare that there is no conflict of interests regarding the publication of this paper.

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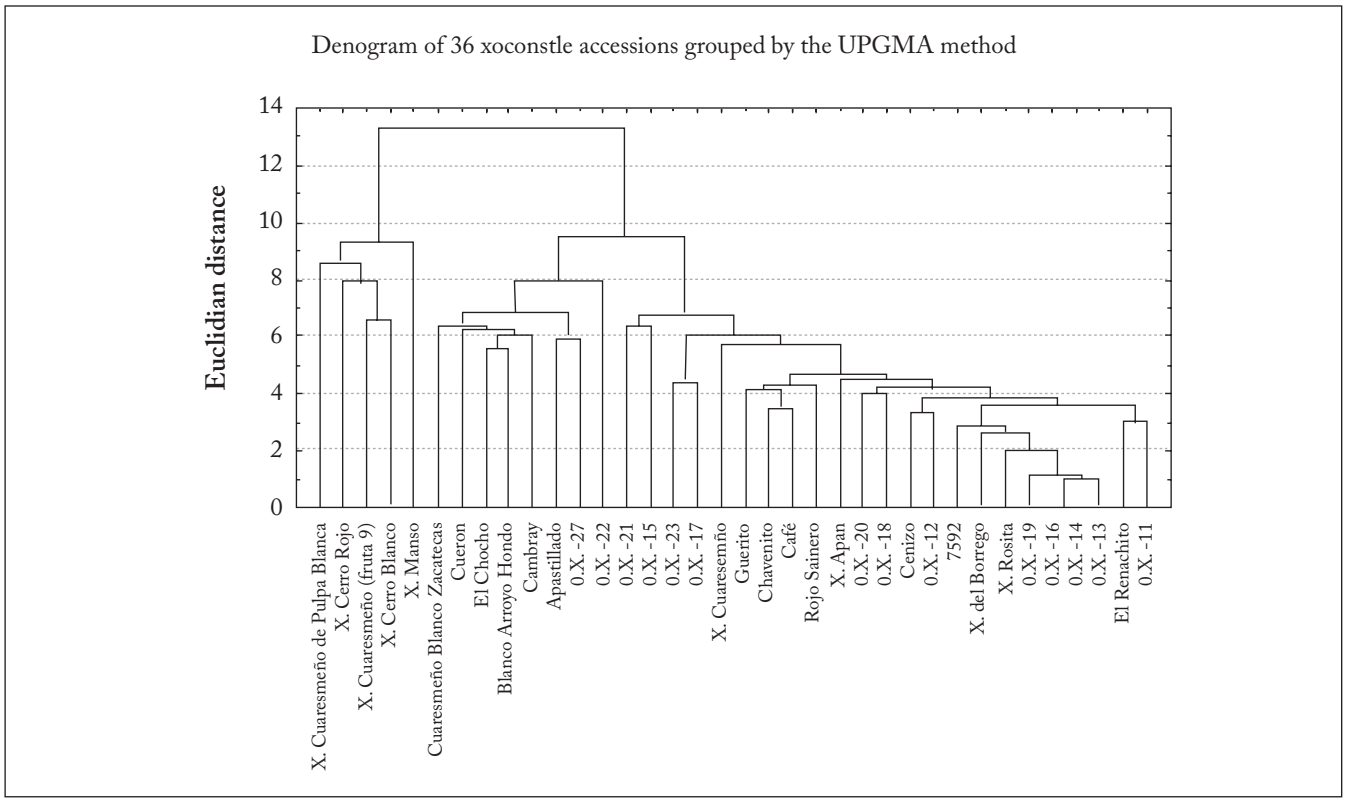

Fig. 3. Dendrogram generated with 36 accessions from 259 AFLP markers by estimating genetic similarity coefficient of Nei and Li (1979), using the method of grouping the non-weighted arithmetic mean (UPGMA).
Fig. 3. Dendograma generado con las 36 accesiones a partir de 259 marcadores AFLP estimando similitud genética según el coeficiente de Nei y Li (1979), utilizando como método de agrupamiento el de la media aritmética no ponderada (UPGMA).

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