Electronic Journal of Biotechnology 17 (2014) 65–71

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

Efficiency of RAPD, ISSR, AFLP and ISTR markers for the detection of polymorphisms and genetic relationships in camote de cerro (*Dioscorea* spp.)

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ARTICLE INFO

Article history:
Received 5 August 2013
Accepted 7 January 2014
Available online 30 January 2014

Keywords:
Dioscoreaceae
Genetic diversity
Molecular markers
Tuber

ABSTRACT

Background: At present, species known as camote de cerro (*Dioscorea* spp.) are found only in the wilderness in Mexico, but their populations are extremely depleted because they are indiscriminately collected, it is urgent to evaluate the conservation status of these plants in order to design conservation genetics programs. In this study, genetic diversity parameters along with cluster analysis based on Jaccard's coefficient were estimated with the objective to assess the efficiency of Random Amplified Polymorphic DNA (RAPD), Inter Simple Sequence Repeat (ISSR), Amplified Fragment Length Polymorphism (AFLP) and Inverse Sequence Tagged Repeat (ISTR) molecular DNA markers in the *Dioscorea* genus.
Results: The polymorphic information contents were quite similar for all markers (≈0.48). Genetic variation of *Dioscorea* spp., in terms of average heterozygosity was lower with ISTR (0.36), and higher when other markers were used (RAPD = 0.43; ISSR = 0.45 and AFLP = 0.47).
Conclusion: This indicates an important level of genetic differences despite the fact that the plant is asexually propagated. Based on the diversity statistics, any marker tested in present work can be recommended for use in large-scale genetic studies of populations. However, the low correlations among different molecular marker systems show the importance of the complementarity of the information that is generated by different markers for genetic studies involving estimation of polymorphism and relationships.

1. Introduction

The genus *Dioscorea* (family Dioscoreaceae) comprises multiple species that grow in Africa, Asia, South America and the Caribbean Islands [1]. Some of these species are polyploid, producing tubers, which allow their vegetative propagation. The genus *Dioscorea* has been reported as producing diosgenin, a secondary metabolite which is very important in pharmaceutical industry because it is used as raw material for the semi synthesis of steroidal drugs and also as a complement to traditional medicine in the treatment of various diseases [2]. The tuber is used as food because of its contents of carbohydrates, vitamins (especially vitamin C), minerals and proteins [3,4]. This genus includes 600 species, of which 65 to 70 were reported for Mexico [5]. In western Mexico this plant, commonly known as camote de cerro, cannot be found cultivated but occurs in wild form [6]. It is distributed in the mountainous areas with evergreen and subperennifoliar forests. From ancient times these tubers were harvested, cooked and consumed as food by people of the region. Because this plant is collected only, the extensive acceptance of its tuber is threatening the genetic diversity of many local populations. Therefore, studies designed to the estimation of the genetic diversity of camote de cerro, can be very important in supporting breeding and conservation strategies of this genetic resource, in assessing its potential as field crop and to determine the origin and relationships among different forms and species. There are many molecular techniques that can help to generate information and assess the polymorphism among individuals and populations. In those cases in which institutions of developing countries do not have access to next generation sequencing technologies, the most popular molecular markers can be quickly and easily utilized to begin to assess genetic diversity of plant genetic resources. Molecular tools have been proved

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Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

Production and hosting by Elsevier

http://dx.doi.org/10.1016/j.ejbt.2014.01.002

useful for the correct identification of taxa, species and specific genotypes [7,8,9]. DNA fingerprinting techniques have been utilized to characterize the diversity of genetic collections. Molecular markers can detect specific locations at DNA level that differ among cultivars or improved species, and they can be selected for many purposes and technical facilities. Examples of markers are: RAPD (Random Amplified Polymorphic DNA), ISSR (Inter Simple Sequence Repeat); AFLP (Amplified Fragment Length Polymorphism) and ISTR (Inverse Sequence Tagged Repeat). Even if the markers are randomly determined, they have different properties, and require different DNA quantities and qualities. The four molecular markers listed above have the ability to detect different parts of the genome, they have a dominant inheritance and used together they can be more informative. RAPD is used to amplify a specific sequence of the genome. Williams et al. [10] utilizes short synthetic oligonucleotides (10 bases long) of random sequences as primers to amplify nanogram amounts of total genomic DNA under low annealing temperatures by PCR. The ISSR [11] is a type of genetic marker that allows us to get the levels of variation in microsatellite regions that are scattered in various genomes, particularly nuclear. These regions consist of tandem repeats of simple patterns as (CT)n or (AC)n repeating sequences located between nuclear eukaryotic genome. Repeated motifs, also called SSRs (simple sequence repeats) may be penta-, tetra-, tri-and dinucleotides. The biallelic markers are dominant AFLP [12]. They can detect single nucleotide simultaneous variations in unknown regions of the genome in which a mutation can be found frequently in functional genes indeterminate. ISTR is a retrotransposon based marker which has the ability to characterize wild species and genetic relationships at an individual level [13,14]. It profits from the abundant repeats that are characteristic of plants with large genomes [15,16]. The acquisition of knowledge about the polymorphisms that can be detected in the wild *Dioscorea* genus with molecular markers is essential for the implementation of conservation programs, as well as for domestication and breeding.

The objectives of this study were to evaluate the value of RAPD, ISSR, AFLP and ISTR marker systems for their ability to distinguish *Dioscorea* populations and their efficiency to estimate genetic diversity parameters, including number of fragments, unique profiles and polymorphic levels per assay unit.

2. Materials and methods

2.1. Plant material

Fresh leaf tissue was collected from 24 asexually propagated wild individuals from nine locations in various regions of state of Jalisco in Mexico (Fig. 1). Tubers of each individual were planted in a nursery shade mesh at Centro Universitario de Ciencias Biológicas y Agropecuarias (CUCBA), Universidad de Guadalajara, Jalisco, Mexico (20°45′N, 103°31′ W; 1650 msnm). Materials were placed in plastic containers 60 × 40 × 27 cm with substrate to promote budding. The plants were maintained with fertilizer formula 20–10–20 until the branches were sufficiently developed to collect leaves for DNA extraction. Names and codes for vegetal materials are shown in Table 1.

2.2. DNA extraction

DNA was isolated from fresh leaves. Two protocols, reported by Keb-Llanes et al. [17] and Cota-Sánchez et al. [18], respectively, were assessed in order to determine the ability to eliminate the excess content of carbohydrates, phenolic compounds, and proteins present in the leaves, which may cause inhibition on *Taq* polymerase action during PCR [3]. The DNA extracted was assessed for quality, using electrophoretic and spectrophotometric methods, and stored at -20°C until processing. The yield and quality obtained measuring OD_{260} and OD_{280} (OD = Optical Density) with both methods were compared by Analysis of Variance (ANOVA).

2.3. Analysis of molecular markers

In this study, the informativeness and efficiency of the molecular markers RAPD, ISSR, AFLP and ISTR were compared. All markers used are based on random sequences and involve different levels of technical difficulty. Different numbers of primer sequences were used, as reported in Table 2.

Conditions for PCR were specific for each marker. RAPD and ISSR analyses were carried out in a 25 μL reaction container for RAPD: 2 ng

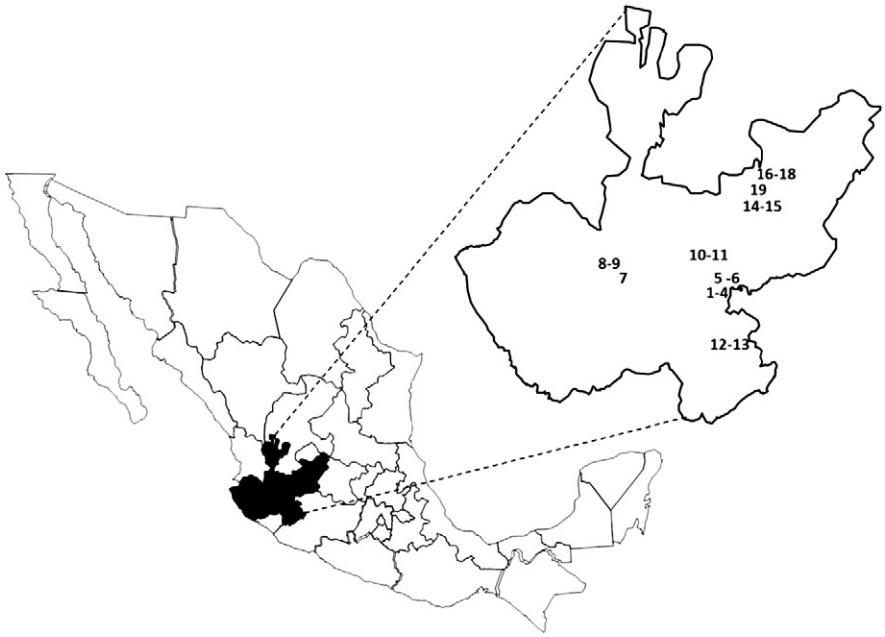

Fig. 1. Locations of the State of Jalisco, Mexico, where the number of 1 to 4 belong to Chapala, 5–6 San Antonio, 7 Cocula, 8–9 Ahualulco, 10–11 Ixtlahuacan de los Membrillos, 12–13 La Manzanilla de la Paz, 14–15 Acatic, 16–18 Ixtlahuacan del Rio and 19 San Gabriel.

Table 1
Dioscorea spp. accessions used in this study.

No.	Name of the locality	Code	Scientific name	Region of Jalisco
1	Chapala	Ch3	*Dioscorea sparsiflora* Helms	Marsh
2	Chapala	Ch4	*Dioscorea sparsiflora* Helms	Marsh
3	Chapala	Ch6	*Dioscorea sparsiflora* Helms	Marsh
4	Chapala	Ch7	*Dioscorea sparsiflora* Helms	Marsh
5	San Antonio	SA3	*Dioscorea sparsiflora* Helms	Marsh
6	San Antonio	SA5	*Dioscorea sparsiflora* Helms	Marsh
7	Cocula	CO1	*Dioscorea sparsiflora* Helms	Center
8	Ahualulco	Ah1	*Dioscorea sparsiflora* Helms	Valleys
9	Ahualulco	Ah3	*Dioscorea sparsiflora* Helms	Valleys
10	Ixtlahuacan de los Membrillos	Ce1	*Dioscorea sparsiflora* Helms	Center
11	Ixtlahuacan de los Membrillos	Ce2	*Dioscorea sparsiflora* Helms	Center
12	La Manzanilla de la Paz	LaM1	*Dioscorea sparsiflora* Helms	Southeast
13	La Manzanilla de la Paz	LaM2	*Dioscorea sparsiflora* Helms	Southeast
14	Acatic	A2	*Dioscorea remotiflora* Kunth	Highland
15	Acatic	A3	*Dioscorea remotiflora* Kunth	Highland
16	Ixtlahuacan del Río	IR2	*Dioscorea sparsiflora* Helms	Center
17	Ixtlahuacan del Río	IR3	*Dioscorea sparsiflora* Helms	Center
18	Ixtlahuacan del Río	IR6	*Dioscorea sparsiflora* Helms	Center
19	San Gabriel	Te1	*Dioscorea sparsiflora* Helms	South
20	San Gabriel	Te2	*Dioscorea sparsiflora* Helms	South
21	San Gabriel	Te3	*Dioscorea sparsiflora* Helms	South
22	San Gabriel	Te4	*Dioscorea sparsiflora* Helms	South
23	San Gabriel	Te5	*Dioscorea sparsiflora* Helms	South
24	San Gabriel	Te6	*Dioscorea sparsiflora* Helms	South

DNA, 1.75 mM $MgCl_2$; 1× buffer PCR; 0.2 mM dNTPs, 1.5 μM primers; 0.1 U *Taq* polymerase, and for ISSR: 2.5 ng DNA, 2.5 mM $MgCl_2$; 0.8 μM primers; 1× buffer PCR; 0.25 mM dNTPs; 0.05 U *Taq* polymerase. ISTR analysis was performed in 20 μL with 4 ng of DNA; 0.025 U of *Taq* DNA polimerase, 3 mM $MgCl_2$, 0.3 μM of each primer and 1× buffer. Amplification for every marker was performed with thermal cycler (TECHNE) using specific cycling parameters in each case. RAPD and ISSR amplifications were carried out under the conditions reported by Sarwat et al. [7]. ISTR analysis was carried out according to the conditions described by Torres-Morán et al. [8]. Amplicons generated by RAPD and ISSR were separated by electrophoresis in 1.5% agarose, and ethidium bromide stains were used for visualizing bands.

AFLP markers were analyzed according to the conditions reported by Das et al. [19] and Sarwat et al. [7,20,21]. These markers required a highly purified genomic DNA (300 ng), which was restricted simultaneously with 2.5 units of *Eco*RI and *Mse*I, each in 5× reaction buffer (50 mM Tris–HCl, pH 7.5, 50 mM Mg-acetate, 250 mM K-acetate) in a 25 μL reaction volume, followed by subsequent ligation of *Mse*I and *Eco*RI adapters. The adapter-ligated DNA was diluted and preamplified using adapter-specific primers with one additional selective nucleotide at the 3′ end (*Mse*I adapter specific primer + C; *Eco*RI adapter-specific primer + A) (Table 2). Preamplification was carried out for 20 cycles, each consisting of incubation at 94°C for 30 s, 56°C for 60 s, and 72°C for 60 s. The preamplified DNA was diluted 50-fold with sterile water and utilized as a template for selective amplification using *Eco*RI and *Mse*I primers with three selective nucleotides at the 3′ end (*Eco*RI + 3 N and *Mse*I + 3 N). The cycling parameters for PCR consisted of denaturation at 94°C for 30 s, annealing at 68°C for 30 s and 72°C for 30 s, followed by a touchdown cycling protocol in which the annealing temperature was reduced by 1°C in each cycle. This procedure was continued until the annealing temperature reached 56°C. Finally, amplification was carried out for 23 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 60 s. The amplified products were separated using polyacrylamide gels according to Sambrook and Russell [22]. After electrophoresis, the gels were lifted onto a chromatographic paper, covered with plastic wrap, and dried under a heated vacuum, and the bands were visualized by exposure to Kodak X-Omat film for different periods ranging between overnight and several days.

Table 2
Primer sequences used in this study.

Molecular marker	Primer	Sequence
RAPD	1	GGTGCGGGAA
	2	GTTTCGCTCC
	3	GTAGACCCGT
	4	AAGAGCCCGT
	5	AACGCGCAAC
	6	ACGTGGAATG
ISSR	855	(AC)8(CT)C
	864	(ATG)6
	890	ACGACTACG(GT)7
	812	(GA)8
	888	BDB(CA)7
	891	HVH(TG)7
ISTR	F31/B31	GTCGACATGCCATCTTTC-FATTCCCATCTGCACCATT-R
	F91/B1	ATATGGACTTAAGCAAGCCA-FATCAGGAAGGTCTGTAAAGC-R
	F61/B8	ATATATGGACTTAAGCAAGCA-FATACCTTTCAGGGGGATG-R
AFLP	AAC + CTA	GATGAGTCCTGAGTAACAGGATGAGTCCTGAGTCGTACC
	AAC + CAA	GATGAGTCCTGAGTAACAGGACTGCGTACCAATTCACT

F: forward; R: reverse.

2.4. Data analysis

Parameters for calculating the marker efficiency and genetic characteristics were used. polymorphic information content (PIC) was calculated using the formula of Roldán-Ruiz et al. [23], $PIC = 2f_i(1 - f_i)$ where f_i is the frequency of the amplified allele and $1 - f_i$ is the frequency of null allele. Heterozygosity per locus was calculated according the formula: $He = 1 - p^2 - q^2$, where $p^2 = f_i$. Average heterozygosity per marker was calculated based on: $H_{av} = \sum(H_e / L)$, where L = total of detected bands. Multiplex ratio was calculated as $MR = L/T$, where T = total number of primer combinations. The Marker Index (MI) was obtained by multiplying the average heterozygosity by Multiplex Ratio: $MI = H_{av}$ *XMR*. A binary matrix of presence/absence was obtained from gels for each marker. Jaccard's similarity coefficient was calculated for use in clustering analysis by Unweighted Pair-group Method with Arithmetic Average (UPGMA). All analyses were performed using the software

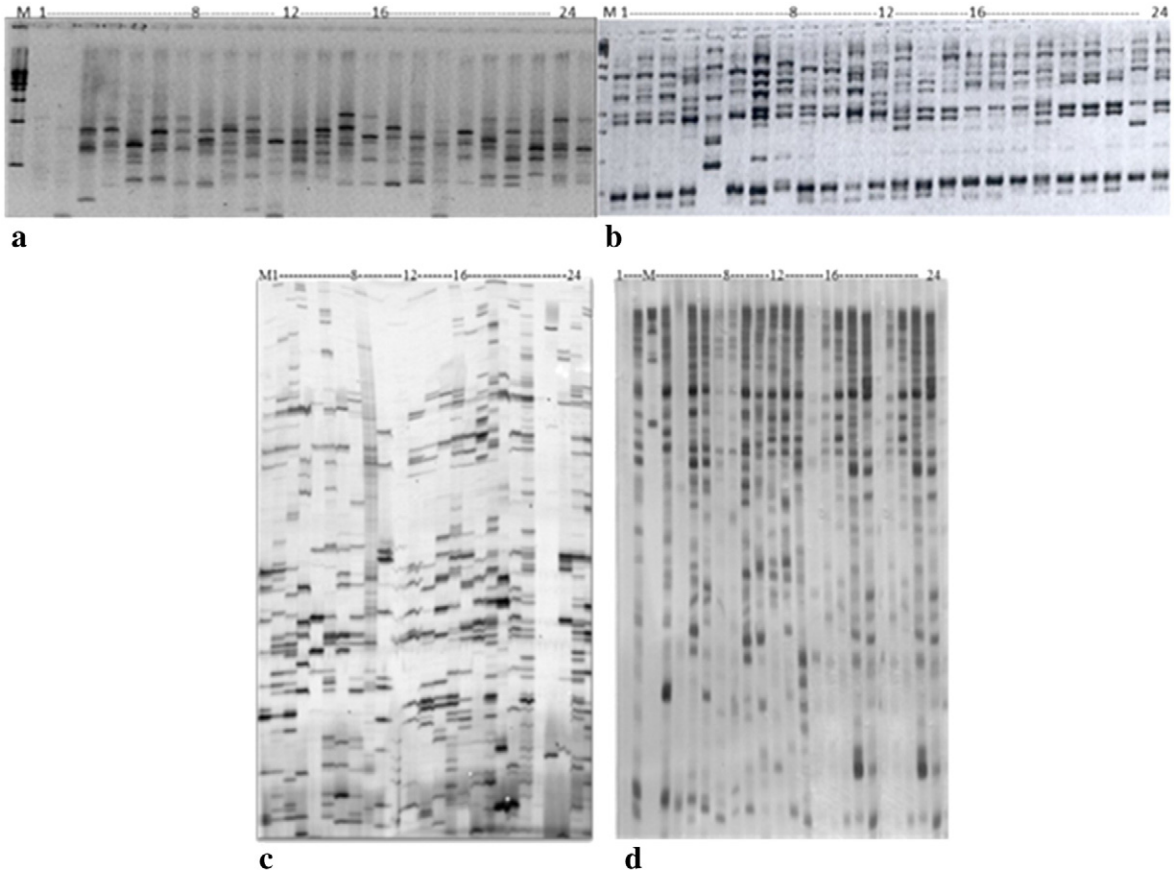

Fig. 2. Banding patterns in gels with four molecular marker systems showing genetic polymorphism in *Dioscorea* spp. (a) RAPD, (b) ISSR, (c) AFLP and (d) ISTR profile. M: Molecular weight reference, localities 1–8 Chapala, San Antonio, Ahualulco, 9–12 Ahualulco, Ixtlahuacan de los Membrillos, La Manzanilla de la Paz, 13–16 La Manzanilla de la Paz, Acatic, Ixtlahuacan del Rio, 17–24 Ixtlahuacan del Rio, San Gabriel.

NTSYS version 2.21 [24]. The results are represented as dendrograms. The degree of confidence at the nodes of the dendrogram was evaluated with Bootstrap with 1000 replicates, using FreeTree and TreeView software [25]. The correspondence between the RAPD, ISSR, AFLP and ISTR similarity coefficient matrices was tested based on Mantel test. Mantel test evaluates correlation between distance matrices; the statistical significance of this association is measured by a randomization procedure in which the order of the elements in one of these matrices is randomly permutated several times and a Z-value is calculated for each permutation [26]. The correspondence tests among different markers were performed based on 10,000 randomizations by using the MXCOMP module of NTSYS version 2.21 software [24].

3. Results

3.1. Isolation DNA

Differences among the DNA yields from the two extraction methods were highly significant ($P < 0.0043$ for DO_{260}, and $P < 0.0020$ for DO_{280}), and the quality obtained with Keb-Llanes's protocol was higher than that obtained with a Cota protocol. During the experiment, samples for all individuals for molecular markers tests were extracted by Keb-Llanes's method.

3.2. Efficiency of molecular markers

In order to assess efficiency of the marker systems in *Dioscorea* spp. with regard to detection of polymorphism, several diversity statistics were calculated. A different degree of DNA banding pattern polymorphism was detected for each marker. Differences were found between the efficiency parameters and the detected loci (Fig. 2). A comparison between marker systems with regard to the statistics of diversity was carried out and is shown in Table 3.

A range of bands between 92 and 302 was produced, in which RAPD showed the highest level. The PIC was equivalent for all the markers. All four molecular markers used were informative, with PIC from 0.4999 in ISSR to 0.4398 in RAPD. The highest expected heterozygosity (H_{av}) was estimated for AFLP and the lowest for ISTR; similar results were obtained for MI, where the highest Marker Index was observed for AFLP (25.38) and the lowest for ISTR (11.03). Despite the differences in some of the diversity statistics, these results show that any of these markers can be used to estimate the level of polymorphism in accessions of *Dioscorea* spp.

Table 3
Relative efficiency of molecular markers in determining polymorphism in *Dioscorea* spp.

Parameters for marker efficiency	RAPD	ISSR	AFLP	ISTR
Number of individuals	24	24	24	24
Total number of bands (L)	302	155	108	92
Polymorphic bands (p)	158	127	88	79
Total number assays/primer combinations (T)	6	6	2	3
Multiplex ratio (MR) (L/T)	50.33	25.83	54	30.66
Polymorphism percentage (%p)	58.27	81.93	81.48	85.86
Polymorphic information content (PIC)	0. 44	0.49	0.48	0.47
Average heterozygosity (H_{av})	0.43	0.45	0.47	0.36
Marker index (MI) = $H_{av} \times$ MR	21.6	11.62	25.38	11.03

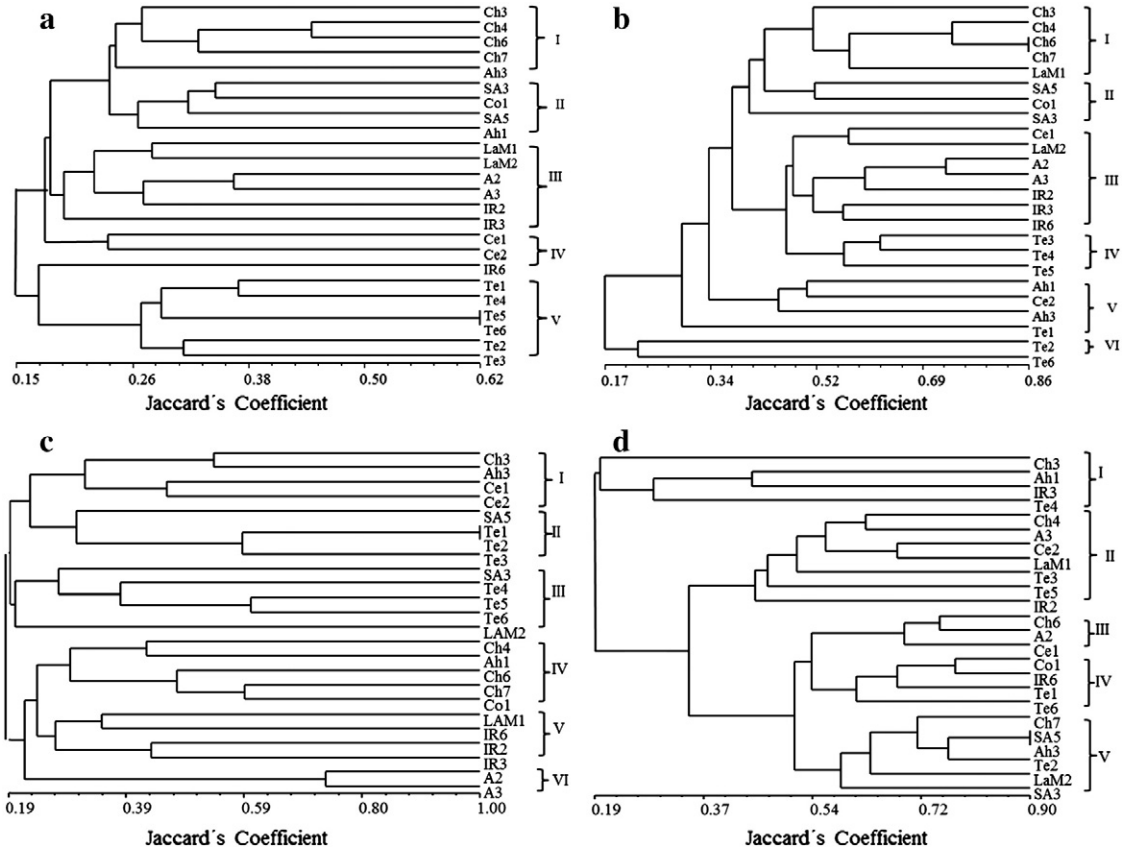

Fig. 3. Comparison by similarity of relationships between *Dioscorea* spp. genotypes as revealed by (a) RAPD, (b) ISSR, (c) AFLP and (d) ISTR marker systems.

3.3. Genetic relationships

RAPD, ISSR, AFLP and ISTR banding profiles of each sample were checked manually for the presence/absence of bands. These data were used for calculating the similarity among individuals. An individual analysis was carried out for each marker. A dendrogram based on a UPGMA algorithm for each marker shows a correspondence between similarity among individuals and geographical regions. Each marker detected a similarity of 0.15 to 1.00 between individuals (Jaccard's coefficient). AFLP and ISSR markers detected six groups in which clusters were composed by individuals from the same region, while RAPD and ISTR detected five groups. According to the results, individuals

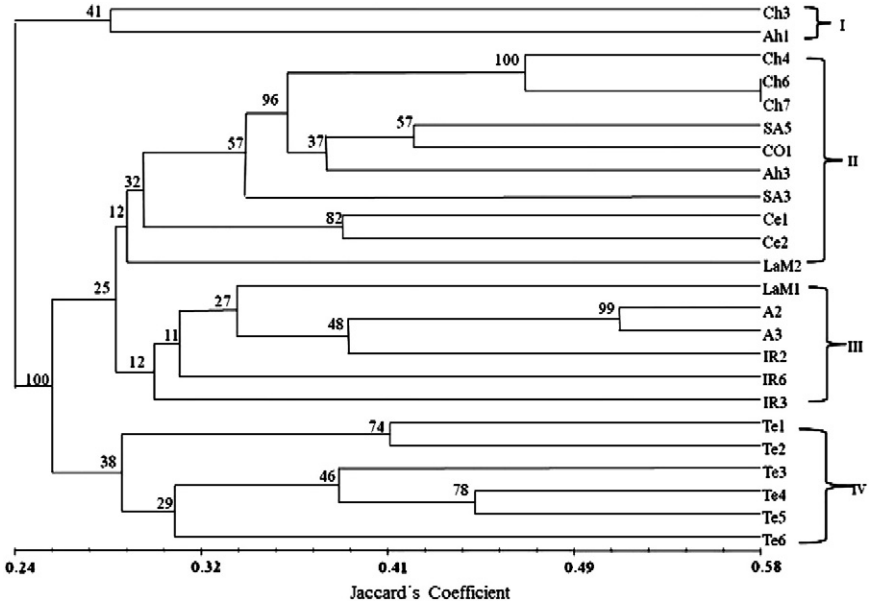

Fig. 4. Clustering analyses and bootstrap for 24 individuals of *Dioscorea* spp. based on molecular markers.

Table 4
Correlation among similarity matrices derived from RAPD, ISSR, AFLP and ISTR markers.

	RAPD	ISSR	AFLP	ISTR	ALL[a]
RAPD	1				
ISSR	0.24575	1			
AFLP	0.31720	0.12418	1		
ISTR	0.14620	−0.062288	0.10157	1	
Pooled[a]	0.66821	0.57112	0.59068	0.48288	1

[a] Combined analysis of all the markers.

from Acatic (A2 and A3) showed high similarity, as did those from Tepozal (Te) and Ixtlahuacan del Río (IR) when analyzed with AFLP, ISSR and RAPD. The ISTR marker showed a different clustering tendency due to its retrotransposon based primer sequences.

A global analysis was carried out using the total bands produced for all the markers (Fig. 3). Four groups were detected in these analyses. It is worth noting that two individuals (Ch3 and Ah1) were placed in group I. These plants were collected from different regions (Marsh and Valleys), and the similarity between them is low (0.2384, Jaccard's coefficient), compared to the maximum similarity found in other materials (Ch6 and Ch7), which is 0.58. Three groups were formed with individuals with common geographical origin (II, III and IV) and average similarities of ≈0.322. The bootstrap value is shown in Fig. 4. The only two materials belonging to *Dioscorea remotiflora* were clustered into the third group (III), but based on a similarity of 0.34165 with LaM2 and 0.3196 with respect to the IR2, IR6 and IR3.

3.4. Comparison of different marker systems

In order to determine the correlation among similarity matrices of the marker systems, a Mantel test was carried out to obtain the degree of relationship among them; as reference, a similarity matrix including the information of all markers was used. The similarity matrix was based on Jaccard coefficient. The results for the correlation coefficients *r* among similarity matrices are presented in Table 4. Values of *r* were non-significant and ranged from −0.06288 between ISSR and ISTR to 0.3172 between AFLP and RAPD. Correlations among the pooled matrix and individual marker systems were higher, ranging from 0.48288 (AFLP) to 0.66821 (RAPD). Based on the results of the Mantel test (Table 4) and the marker index that involves average heterozygosity and multiplex ratio (Table 3), the most efficient marker system detecting genetic differences among *Dioscorea* spp. accessions were RAPD and AFLP.

4. Discussion

Among the marker systems evaluated, RAPD and AFLP were found to be more efficient in the estimation of molecular diversity of different accessions of *Dioscorea* spp.; it was evident from large values of polymorphism percent, PIC, Multiplex ratio and average number of polymorphic bands per primer. Ferrao et al. [27] indicated that the efficiency of a molecular marker is a balance between the level of polymorphism it can detect (information content) and its capacity to identify multiple polymorphisms; deciding which technique is the most appropriate depends on several factors, including the objective of the research, genetic structure and the resources available. However, the robustness, the informativeness and the polymorphism level should be the primary criteria for choosing a method. According to Mignouna et al. [28], AFLPs have better discriminatory power for polyploid species; however we have found that the ISTR marker is also very efficient in the detection of differences for these species. Considering percent of polymorphism, ISTR was found to be more efficient than ISSR, AFLP and RAPD, having identified 86% polymorphic DNA markers vs 82% and 81% detected with ISSR and AFLP, respectively. Such high levels of polymorphism (>80%) were also found in ten populations of *Podophyllum hexandrum* in two different types of habitats [29] using RAPD, ISSR and AFLP. The highest average heterozygosity (H_{av}) was identified using AFLP (0.47), while the lowest (0.36) was found with ISTR. This is due to the total number of bands, according to Demey et al. [30]. Within and among accessions, major diversity was found within individuals collected on Southeast, Highland and South regions of Jalisco.

The clustering patterns based on the different marker systems were primarily related to the geographical distribution of the accessions. These results support the fact that region specific variations exist, which can be explained by a long-term adaptation process to soil and climate, multiple generations of selection and overexploitation [6]. In the regions where the samples were collected, only two species of *Dioscorea* genus were found (*Dioscorea sparsiflora* and *D. remotiflora*). These are not clearly separated in the clustering analysis, sometimes these have been reported as the same species *D. remotiflora* var. *sparsiflora* (Hemsl.) [31]. Differences within individuals in a population of *Dioscorea* spp. were previously reported [32,33]. The maintenance of genetic diversity within a plant species has been shown to be influenced by its biological characteristics, and *Dioscorea* spp. has been proven to include plants with different sex and different ploidy levels [34], mating system and geographical distribution [35].

The four marker systems used were an accruable tool to detect relationships among materials of *Dioscorea* spp., to identify association between genetic similarity and geographic distribution, and to estimate genetic diversity. However more efficiency was found in RAPD and AFLP. The low correlations among different molecular marker systems show the importance of using different markers for estimating diversity and genetic similarity; therefore, it is important to stress that the complementarity of the information that is generated by different markers is valuable for genetic studies involving both, diversity and relationships. Using a combined data set with at least two of the markers would be advisable [36].

The generation of genetic information between *Dioscorea* species observed in this study should greatly facilitate strategies for breeding and conservation programs. Similarly, the high level of genetic diversity observed within populations was encouraging, as it should provide a broad genetic base to maintain the potential of these species to respond to new selection pressures imposed by environmental changes. The results indicate an important level of genetic differences despite the fact that the plant is predominantly asexually propagated. Any marker tested in this work can be recommended for use in a large-scale of genetic studies of populations of this genus carried out in order to develop conservation strategies.

Conflict of interest

Authors declare that there is no conflict of interest.

Acknowledgments

We thank the National Council for Science and Technology (CONACyT) for the funding awarded to Ing. Ana Paulina Velasco Ramírez, fellow number 272575, for the development of this project.

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