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Marker Assisted Characterization in *Tigridia pavonia* (L.f) DC

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1. Introduction

Conventional plant breeding programs largely depend on phenotypic selection, breeders' experience, and knowledge of plant genetics for traits of agronomic importance. A large amount of biological data is available from genetic studies of a crop of interest related to important target traits in crop breeding, which may in turn directly assist genotypic selection (Xu & Crouch, 2008). However, conventional breeding is time consuming and often testing procedures may be complex, unreliable or expensive due to the nature of the target traits (e.g. abiotic stresses) or the target environment (Choudhary et al., 2008).

Marker assisted characterization offers such a possibility by adopting a wide range of novel approaches to improving the selection strategies in horticultural plant breeding (Ibitoye & Akin-Idowu, 2010). The genetic markers involved in marker assisted characterization include morphological, biochemical and DNA fingerprints which can be employed in selection and identification of closely related genotypes.

Many variants of an enzyme, referred to as isozymes, can be resolved by electrophoresis and are very useful genetic markers. The alleles of most isozyme markers segregate in a codominant manner and rarely show epistatic interactions, which allows accumulation of many polymorphic isozyme loci in a single F_2 population and increases the efficiency of gene mapping. Once the map location of isozyme genes are known, they can be used efficiently as biochemical markers to tag other genes for morphological, physiological and phytopathological traits (Tanksley & Rick, 1980). However, only a relatively small number of protein variants may exist between the two parents and this limits the total number of protein loci that can actually be scored in a given mapping population (Young, 1999).

DNA markers have been used in the identification of varieties and characterization of genotypes because they offer a fast screening and a more precise discriminatory power (Vicente & Fulton, 2003).

With the discovery of restriction enzymes and the polymerase chain reaction (PCR), different markers systems such as Restriction Fragment Length Polymorphisms (RFLP), Random Amplified Polymorphic DNAs (RAPD), Amplified Fragment Length Polymorphisms (AFLP),

Simple Sequence Repeats or microsatellites (SSR), Single Nucleotide Polymorphism (SNP), Inter-Simple Sequence Repeats (ISSR) and others have been developed to visualize the composition of organisms at the DNA level and obtain their genetic fingerprints. Among these markers, RFLPs, AFLPs, SSRs and SNPs have been reported as highly polymorphic and reproducible. However RFLPs require large amount of DNA, are laborious intensive, time consuming and mostly require radioactively labeled probes, whereas AFLPs and SSRs are quite costly also require high resolution electrophoresis or automated sequences. On the other hand, although SNPs are less time consuming against rest of the markers and highly amenable to automation, initial cost involved is quite high (Jehan and Lakhanpaul, 2006). So, although marker informativeness is an important element when comparing different assay systems, other factors such as cost per assay, level of skills required, and reliability of assays should also be considered. PCR molecular markers like ISSR and RAPD would be an option because of the lower level of skill required, low costs per assays and the ready availability of primers can scan the entire genome and make more efficient the genotype characterization. Thus, due to their characteristics and efficiency for detecting polymorphism, the ISSR and RAPD markers have been successfully used to calculate the intra or inter-specific genetic diversity in different domesticated and wild species (Arzate-Fernández et al., 2005b; Escandón et al., 2005a; Tapia et al., 2005; Kumar et al., 2006; Luna-Paez et al., 2007; Marotti et al., 2007; Muthusamy et al., 2008; Ye et al., 2008).

The genus *Tigridia* comprises plants with highly colored flowers that exhibit great morphological variation, making many species potentially valuable as cultivated plants. *Tigridia pavonia* (L.f.) DC, also known as tiger flower or oceloxóchitl, is a species native to México which was used by the Aztecs for ornamental, nutritional and medicinal purposes (Hernández, 1959). Due to the great variability of the coloring of its flower and its ornamental potential, it is one of the principal phytogenetic resources of this country, considered the center of greatest genetic diversity of this species. In spite of fact that in Mexico, the potential for sustainable *T. pavonia* utilization is enormous, its remains largely unexploited due to the little information on the resource abundance and distribution, whereas in different countries of Europe, Asia and Australia this species is widely distributed and commercialized as a garden plant (Vázquez-García et al., 2001). Thus, genetic diversity studies are needed to provide information for its efficient conservation as well as for possible future use in breeding programs.

The widely distributed species generally present morphological and physiological variation, as well as in the genetic structure of their populations (Wen & Hsiao, 2001). Likewise, the dissemination in different regions could cause that the same variety or cultivar may be known under different names in different countries (and even within a given country) and different varieties sometimes may appear under the same name. Therefore, the study of phenotypic and genetic diversity to identify groups with similar genotypes is important for conserving, evaluating and exploiting genetic resources. Also, these studies are useful for determining the uniqueness and distinctness of the phenotypic and genetic constitution of genotypes with the purpose of protecting the breeder's intellectual property rights (Franco et al., 2001).

The focus of this chapter is in a more applied direction, encompassing the potential of the morphological, isozymes and DNA markers for characterization of nine *T. pavonia* varieties to their management into selection and breeding programme.

2. Materials

Nine botanical varieties of *Tigridia pavonia* (L.f.) DC, collected in three localities of the State of Mexico and registered in the National Seed Inspection and Certification Service (SNICS) were used in this study (Figure 1). The Sandra variety (TGD-008-030408) was collected in the municipality of Tenancingo, Carolina (TGD-002-030408), Trinidad (TGD-009-030408), Penelope (TGD-006-030408), Angeles (TGD-001-030408), Dulce (TGD-003-030408) and Mariana (TGD-005-030408), in the municipality of Temascaltepec and Samaria (TGD-007-030408) and Gloria (TGD-004-030408) in the municipality of Temoaya. All of the varieties were cultivated in a rustic greenhouse, at the Faculty of Agricultural Sciences (FCAgr) of the Autonomous University of State of Mexico (UAEM).

Fig.1. Nine botanical varieties of *Tigridia pavonia* (L. f.) DC used in the morphological and molecular characterization assay

3. Methodology

3.1 Morphological markers

To make the matrix of morphological data, 20 quantitative and one qualitative characters (Table 1) were evaluated among the nine *T. pavonia* varieties. Nine characters were previously published by Vázquez-García et al. (2001). Each character was evaluated in 14 individuals of each variety.

Character	Mean	Max	Min	Standar Deviation	CV (%)	*P*
1. Number of flowers per shoot	9.32	17.00	3.00	2.22	23.23	0.0753
2. Length of external tepal	8.65	11.70	7.00	0.88	7.87	<0.0001
3. Length of internal tepal	4.42	5.20	3.70	0.31	6.60	<0.0004
4. Type of internal tepal (oval formed, sharp pointed, lanceolated, rounded)	1.92	4.00	1.00	1.23	22.72	<0.0001
5. Length of staminal column	4.49	5.40	3.80	0.29	4.48	<0.0001
6. Distance from the base of the anther to the stigma	1.05	2.50	0.30	0.54	20.46	<0.0001
7. Length of anther	1.59	2.00	1.00	0.21	7.40	<0.0001
8. Length of the reproductive part	6.31	7.30	5.10	0.49	4.42	<0.0001
9. Number of fruits per shoot	4.84	13.00	1.00	2.32	34.44	<0.0001
10. Number of shoot per bulb	4.00	6.00	3.00	0.98	18.64	<0.0001
11. Length of shoot	60.03	96.50	38.60	18.68	7.03	<0.0001
12. Nodes per shoot	3.84	5.00	2.00	0.44	11.14	0.0240
13. Length of the internode	8.18	22.00	0	6.15	39.24	<0.0001
14. Number of branches per shoot	1.98	3.00	0.90	0.72	25.03	<0.0001
15. Length of the leaf	34.13	46.00	16.00	6.78	8.23	<0.0001
16. Width of the leaf	3.53	5.60	2.50	0.88	7.46	<0.0001
17. Length of the pseudopetiole	7.55	11.00	6.00	1.32	6.16	<0.0001
18. Floral scapus length	11.21	28.00	3.00	7.29	10.89	<0.0001
19. Floral scapus thickness	0.55	1.00	0.30	0.22	6.33	<0.0001
20. Length of the bract	11.29	24.50	8.00	3.88	16.60	<0.0001
21. Bract width	2.85	3.50	2.20	0.38	4.03	<0.0001

Table 1. Morphological characters and basic statistical data of nine varieties of *T. pavonia* (L. f.) DC.

3.2 Isozyme markers

For isozyme markers, two individuals of each *T. pavonia* variety were used. Leaf sections were taken from each individual eight to 12 weeks after budding initiated. All of the samples were processed in the laboratory of Plant Molecular Biology at the FCAgr of the UAEM.

Approximately 50 mg of fresh leaf tissue from each sample was homogenized using a plastic chopstick in 50 µL extraction buffer Tris-HCl 0.1M, 2% EDTA-2Na, 50.4% glycerol, 3.2% Tween-80, and 0.8% DTT, pH 7.5 (Arzate-Fernández et al., 2005a).

The samples were preserved at −20 °C until analysis. Two buffer systems of histidine-citric acid, pH 5.7 and pH 6.5 (H-AC) and Tris-citrate/Tris-histidine, pH 8.5 (T-C/T-H) were used following the procedures of Glaszmann et al. (1988) and Stuber et al. (1988).

The 18 isozyme systems evaluated were aspartate amino transferase (AAT; EC 2.6.1.1), acid phosphatase (ACP; EC 13.1.3.2), dehydrogenase alcohol (ADH; EC 1.1.1.1), aminopeptidase (AMP; EC 3.4.11.1), catalase (CAT; EC 1.11.1.6), endopeptidase (ENP; EC 3.4.23.6), esterase (EST; EC 3.1.1.1), formate dehydrogenase (FDH; EC 1.2.1.2), glutamate dehydrogenase (GDH; EC 1.4.1.2), glucose-6-phosphate dehydrogenase (G-6-PDH; EC 1.1.1.49), malic enzyme (MAL; EC 1.1.1.40), malate dehydrogenase (MDH; EC 1.1.1.37), phosphoglucose dehydrogenase (PGD; EC 1.1.1.44), phosphoglucose isomerase (PGI; EC 5.3.1 9), phosphoglucomutase (PGM; EC 2.7.5.1), phosphohexose isomerase (PHI; EC 5.3.1.8), peroxidase (POX; EC 1.11.1.7), and shikimate dehydrogenase (SKD; EC 1.1.1.25). The samples were loaded into a 12% hydrolyzed potato starch gel and electrophoresis was conducted at 4 °C for a mean duration of 3 h, 40-50 mA and 100-150 v. Each sample was run at least three times to verify reproducibility. Enzyme staining was performed according to the procedures described by Torres et al. (1978), Vallejos (1983), Glaszmann et al. (1988), Stuber et al. (1988) Wendel & Weeden (1990) and Ishikawa (1994).

3.3 DNA markers

Young leaves were collected of two individuals of each variety (in the same development stage), and stored at -20 °C prior to DNA extraction. The genomic DNA was extracted of approximately 100 mg of leaf tissue of each variety of *T. pavonia*. The extraction procedure was the CTAB as reported by Zhou et al. (1999). The DNA samples were stored to -20 °C prior to analysis. The polymerase chain reaction (PCR) was made in a final volume of 10 µL with: 1 µL of DNA (10 ng), 1 µL of 10X PCR buffer with ammonium (15 mM), 0.5 µL of $MgCl_2$ (15 mM), 1 µL of dNTPs (10 mM), 1 µL of the primer (20 mM) and 0.1 units of the enzyme Taq DNA polymerase.

3.3.1 ISSR markers

For ISSR markers, five primers of the anchored microsatellites type were used (3'-ASSR) (Yamagishi et al., 2002). In each primer, the anchor consisted of a triplicate of distinct sequence (Table 4). The amplification conditions for the primers 3'-ASSR02 and 3' -ASSR15 were those described by Arzate-Fernández et al. (2005b), and consisted of an initial cycle of 9 min at 94 °C, 1 min at 46 °C and 1 min at 72 °C and a final cycle of 9 min at 94 °C, 1 min at 46 °C and 10 min at 72 °C. For primers 3'-ASSR20, 3'-ASSR29 and 3'-ASSR35, the amplification cycles were those used by Yamagishi et al. (2002) and consisted of an initial cycle of 9 min at 94 °C, 1 min at 46 °C and 1 min at 72 °C and a final cycle of 10 min at 72 °C.

3.3.2 RAPD markers

For RAPD markers, five 10 base primers (Yamagishi, 1995), five 15 base primers (Yamagishi et al., 2002), and five 20 base primers (Debener & Mattiesch, 1998) were used (Table 5). The amplification conditions for RAPD primers of 10 base were the reported by Yamagishi (1995). The program for 15 base primers used in this study was followed according to Yamagishi et al. (2002) with minor modifications (40 cycles of 94 °C for 1 min, 53 °C for 3

min and 72 °C for 2 min). The PCR cycle conditions for 20 base primers were performed according to Debener & Mattiesch (1998).

The amplification of DNA fragments for ISSR and RAPD markers was made in a thermocycler (Mastercycler gradient, Eppendorf, Germany). The separation of the fragments was made in horizontal electrophoresis. A molecular marker of 100 to 3000 pb molecular weight was used. The running conditions for each sample were 100 V and 120 mA for 80 min, and the observation of the fragments was made in a transilluminator UVP.

3.4 Statistical analysis

For morphological markers, the 21 morphological characters were evaluated through an analysis of variance and a completely randomized design with 14 replicates, with the statistical program SAS version 8.0. With the data generated of the morphological characters, a binary matrix was made following the criteria described by Vicente & Fulton (2003); that is, the value of 1 was assigned if the character was present and 0 if it was absent.

For isozyme markers, a record of banding patterns was constructed and each band was assigned to a binary matrix as absent, 0, or present, 1.

For ISSR and RAPD markers, each band generated by each primer was considered as an independent locus calculated manually; that is, the value of 1 was assigned for the presence of a band and 0 for its absence. The total number fragments (FT), polymorphic fragments (FP), percentage of polymorphism (%P) and the Nei's genetic distance (G_D) were calculated using the program POPGENE version 1.32 (Yeh & Boyle, 1999). Also, capacity of each primer to differentiate the nine varieties under study was evaluated through the resolution power (*Rp*), according to Prevost & Wilkinson (1999).

In RAPD markers, a simple correlation analysis was performed to investigate the correlation between the length primer and the polymorphism generated by each primer group.

In all cases (morphological, isozyme and DNA markers), to determine genetic relationships and variety grouping, a dendrogram was constructed with the data using POPGENE with the UPGMA method (modified from the NEIGHBOR procedure of PHYLIP, version 3.5) (Felsenstein, 1990) based on the Nei (1972) matrix genetic distances. UPGMA is one of the simplest and most commonly used hierarchical clustering algorithms. It receives as input a set of elements and a dissimilarity matrix which contains pairwise distances between all elements, and returns a hierarchy of clusters on this set.

4. Results and discussion

4.1 Morphological markers

The values of the mean, maximum, minimum, standard deviation (SD) and coefficient of variation (CV) were calculated for each one of the 21 morphological characters evaluated in the nine varieties (Table 1). According to the values of the CV, the characters number of flowers per shoot, type of internal tepal, distance from the base of the anther to the stigma, number of fruits per shoot, length of the internode and number of branches per shoot showed a slightly high variation level. In contrast, the characters that presented low variation levels among the varieties were the length of the staminal column, length of the reproductive part and bract width.

The statistical analysis showed that the number of flowers per shoot and nodes per shoot were not significant. However, for the other 19 characters there were highly significant differences ($p \leq 0.0001$) (Table 1). These results show the high phenotypic diversity among the varieties analyzed, and can be used for the selection of parental lines within a plant breeding program.

The varieties formed two groups based on their morphological characteristics (Figure 2). Group I with average of $G_D = 0.42$ consisted of the varieties Carolina, Trinidad, Mariana, Angeles, Sandra, Penelope and Dulce, while group II included the varieties Gloria and Samaria, with an average of $G_D = 0.32$.

The morphological characters (18 of 21 equal) showed the close genetic relationship between the varieties Trinidad and Mariana ($G_D = 0.28$). In contrast, the least related varieties were Carolina and Samaria, as well as Penélope and Samaria ($G_D = 0.80$), with 4 and 5 of 21 similar morphological characters, respectively.

According to Vázquez-García et al. (2001), the varieties Gloria and Samaria can be easily distinguished by shoot length. In the present study, the results were similar and both varieties showed the highest values for length of shoot, length of internode, floral scapus length and length of the bract, as well as bract width.

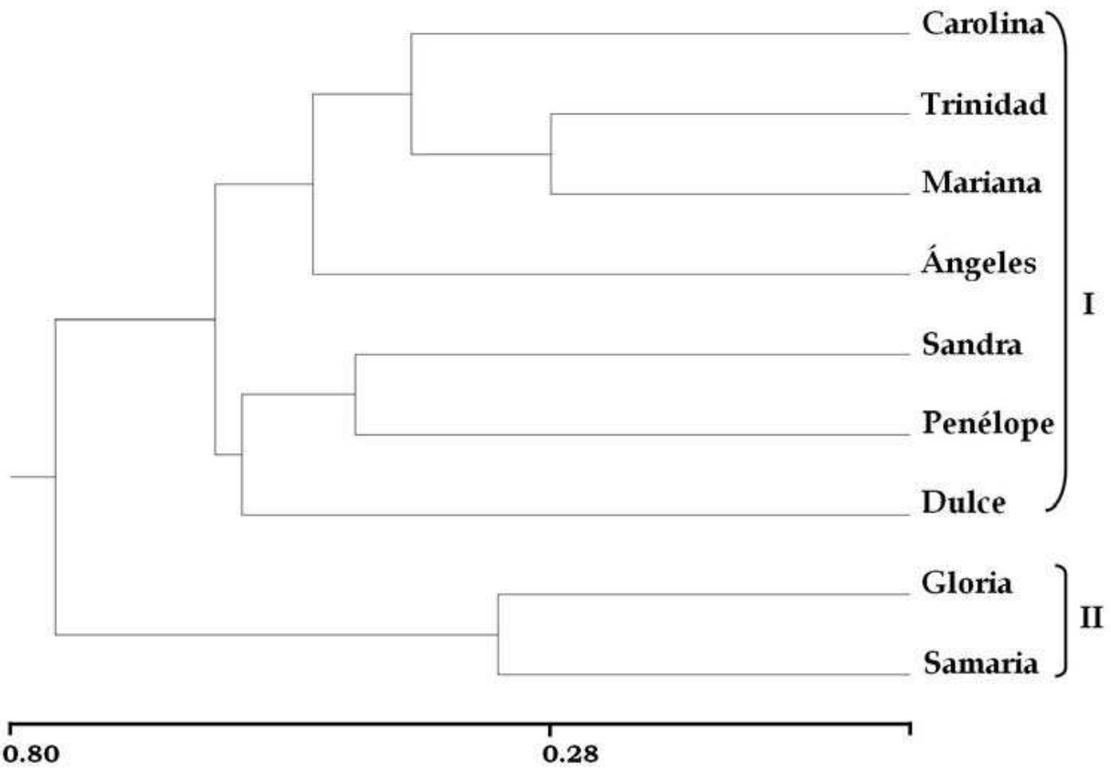

Fig. 2. Dendrogram of nine varieties of *T. pavonia* based on the genetic distance of Nei (1972), with the method UPGMA. The bar indicates the genetic distance among the varieties and the groups, calculated from morphological data.

4.2 Isozyme markers

The nine *T. pavonia* varieties evaluated were wild and free-pollinating. Therefore, they were considered to be heterozygotic; this agrees with the diverse isozyme banding patterns (IBPs) observed. Of the 18 isozymes tested, 12 could be stained, but only nine had sufficient resolution and clarity to be recorded and studied. Some bands were omitted because of their poor resolution. The isozymes ACP, MDH, PGI, PHI and PGM had better resolution when examined with the H-AC system, pH 5.7, and the isozymes AAT and PGD in the same system, but at pH 6.5. In contrast, the isozymes CAT and POX were better in the T-C/T-H system. Of the selected isozymes, nine zimograms in which 32 bands arranged in 20 IBP were identified: two for AAT, two for ACP, two for CAT, four for MDH, one for PGD, one for POX, three for PGI, three for PHI, and two for PGM (Figure 3).

In this study most of the varieties could be identified by the IBP obtained in each isozyme, as we describe in the following sections.

4.2.1 Aspartate amino transferase

AAT showed five active bands (Figure 3A) and varieties with four bands arranged in two banding patterns were detected. Pattern 1 was observed in the Angeles, Carolina, Dulce, Mariana, Penelope, and Sandra varieties, while the Gloria, Samaria, and Trinidad varieties exhibited pattern 2 (Table 2).

4.2.2 Acid phosphatase

ACP revealed four active bands (Figure 3B) and varieties with one or four bands arranged in two banding patterns were detected. The Angeles, Carolina, Dulce, Gloria, Mariana, Penelope, Sandra, and Trinidad varieties exhibited pattern 1 with four bands. Pattern 2 had a single band and was seen only in the Samaria variety (Table 2).

4.2.3 Catalase

CAT produced two active bands (Figure 3C) and varieties with one or two bands arranged in two banding patterns were detected. Pattern 1 with two bands was observed in Carolina, Dulce, Mariana, Penelope, and Samaria. Pattern 2 was observed in Angeles, Gloria, Sandra, and Trinidad (Table 2).

4.2.4 Malate dehydrogenase

MDH showed six active bands (Figure 3D) and varieties with three or four bands in four different arrangements were detected to produce four banding patterns. Patterns 1, 2, and 3 were composed of four bands. Pattern 1 was observed in Angeles, Carolina, Dulce, Mariana, Sandra, and Trinidad, while Penelope and Gloria exhibited patterns 2 and 3. Pattern 4 had three active bands and was observed in Samaria (Table 2).

4.2.5 Phosphoglucose dehydrogenase

PGD produced two active bands (Figure 3E) arranged in a single pattern, so that it was not possible to differentiate any of the varieties (Table 2).

4.2.6 Peroxidase

POX produced two active bands (Figure 3F) arranged in a single pattern, and thus it was not possible to differentiate varieties (Table 2).

4.2.7 Phosphoglucose isomerase

PGI produced four active bands (Figure 3G) and varieties with two, three or four bands arranged to produce three banding patterns were detected. Pattern 1 had four bands, and was observed in Angeles, Carolina, Dulce, Penelope, Samaria, Sandra, and Trinidad. Pattern 2 had three bands and was observed in Mariana. Pattern 3 had two bands and was observed in Gloria (Table 2).

4.2.8 Phosphohexose isomerase

PHI produced three active bands (Figure 3H) and varieties with two or three active bands in three arrangement producing three banding patterns were detected. Pattern 1 had three bands and was observed in ANG, CAR, DUL, PEN, SAN, and TRI. Patterns 2 and 3 had two bands each. Varieties GLO and SAM exhibited pattern 2 and variety MAR pattern 3 (Table 2).

4.2.9 Phosphoglucomutase

PGM produced four active bands (Figure 3I) and varieties with three or four bands arranged in two banding patterns were observed. Pattern 1 had four bands and was observed only in Gloria. Pattern 2 had three bands and was observed in Angeles, Carolina, Dulce, Mariana, Penelope, Samaria, Sandra, and Trinidad (Table 2).

As can be seen in Table 2, the nine isozymes produced sufficient polymorphic bands to distinguish most of the *T. pavonia* varieties evaluated. Of all the isozymes tested, MDH produced the highest number (4) of IBP and, therefore, the highest percentage of polymorphism (18.75%). Based on the observed IBP, this isozyme allowed to differentiate a larger number of varieties. In contrast, the isozymes PGD and POX produced only one IBP, and therefore neither isozyme was useful for differentiating the varieties used.

Twenty-nine bands derived from seven isozymes were only considered: five for AAT, four for ACP, two for CAT, six for MDH, four for PGI, three for PHI, and four for PGM (Figure 3). A dendrogram (Figure 4) was generated from the genetic relationship among these bands, clustering the varieties in two main groups. Group 1 comprises seven varieties: Angeles, Sandra, Carolina, Dulce, Mariana, Penelope, and Trinidad, with an average G_D of 0.074. Group II only includes the Gloria and Samaria varieties, with a G_D of 0.374.

Genetic analysis yielded an average genetic distance G_D of 0.194 among the nine *T. pavonia* varieties (Table 3). As observed in Table 3, the G_D between Angeles and Sandra varieties and between Carolina and Dulce was 0.0, revealing genetic similarity and, in spite of the number of isozymes tested, it was not possible to distinguish one variety from the other. In contrast, Vázquez-García et al. (2001) characterized these four varieties morphologically, differentiating them by flower color: white (Angeles), red (Sandra), pink (Carolina), and yellow (Dulce). The varieties with the highest G_D value (0.421) were Gloria-Mariana, Gloria-Penelope, and Mariana-Samaria.

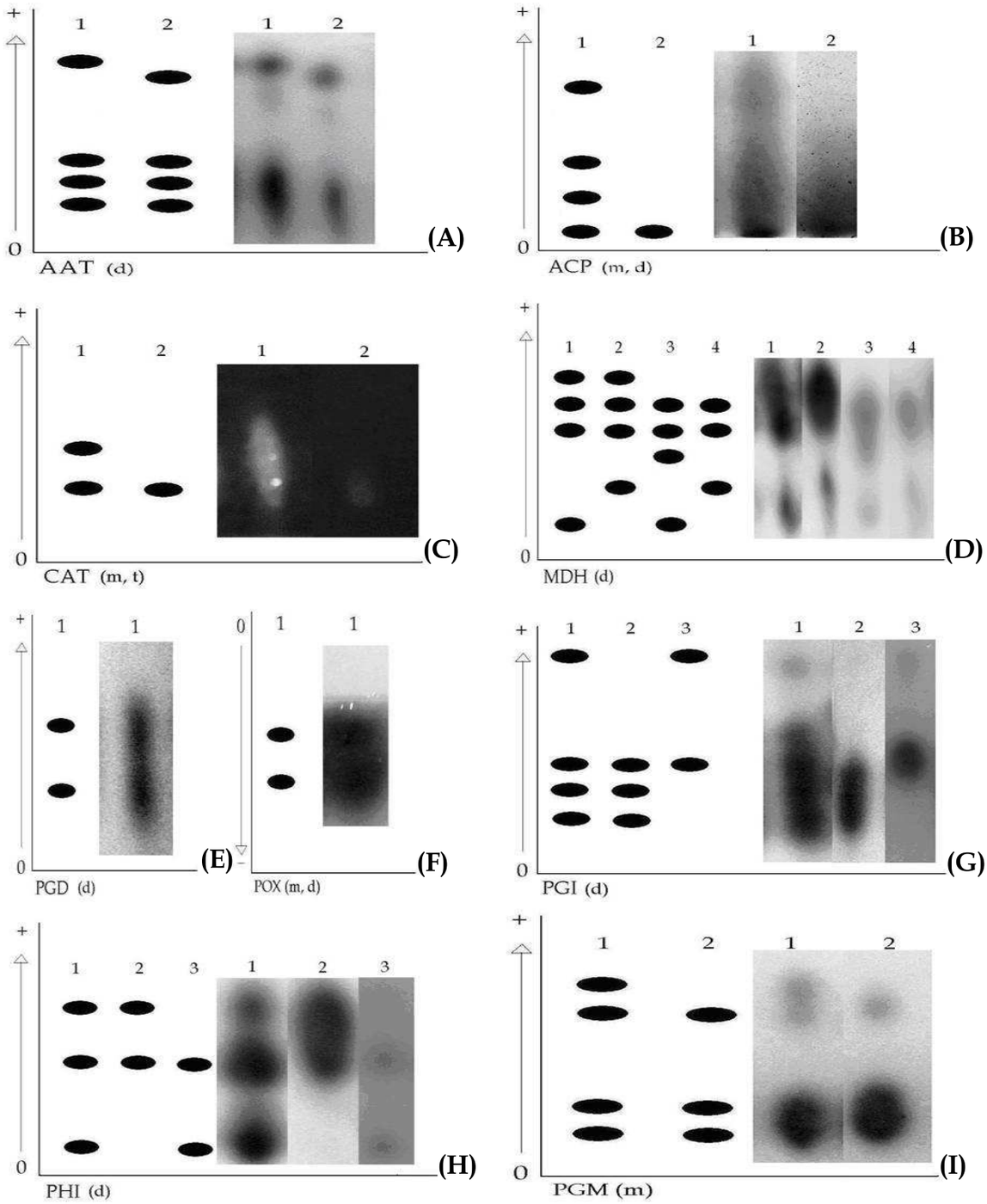

Fig. 3. Schematic representation of isozyme banding patterns (IBP) detected in nine *T. pavonia* (L.f.) DC. varieties, produced by A) AAT; B) ACP; C) CAT; D) MDH; E) PGD; F) POX; G) PGI; H) PHI; I) PGM. (m=monomer, d=dimer, t=trimer. "o" represents origin of electrophoresis.

Variety	Isozyme and pattern								
	AAT	ACP	CAT	MDH	PGD	POX	PGI	PHI	PGM
Ángeles[1]	1	1	2	1	1	1	1	1	2
Carolina[2]	1	1	1	1	1	1	1	1	2
Dulce[2]	1	1	1	1	1	1	1	1	2
Gloria	2	1	2	3	1	1	3	2	1
Mariana	1	1	1	1	1	1	2	3	2
Penélope	1	1	1	2	1	1	1	1	2
Samaria	2	2	1	4	1	§	1	2	2
Sandra	1	1	2	1	1	1	1	1	2
Trinidad	2	1	2	1	1	1	1	1	2

Table 2. Isozyme characterization of nine *T. pavonia* (L.f.) DC. varieties using nine isozyme systems. The number indicates the isozyme banding pattern (IBP) of each variety. AAT, aspartate amino transferase; ACP, acid phosphatase; CAT, catalase; MDH, malate dehydrogenase; PGD, phosphoglucose dehydrogenase; PGI, phosphoglucose isomerase; PGM, phosphoglucomutase; PHI, phoshohexose isomerase; POX, peroxidase. [1, 2] varieties with the same number can not be distinguished with the evaluated isozymes. § It was not possible determinate the IBP.

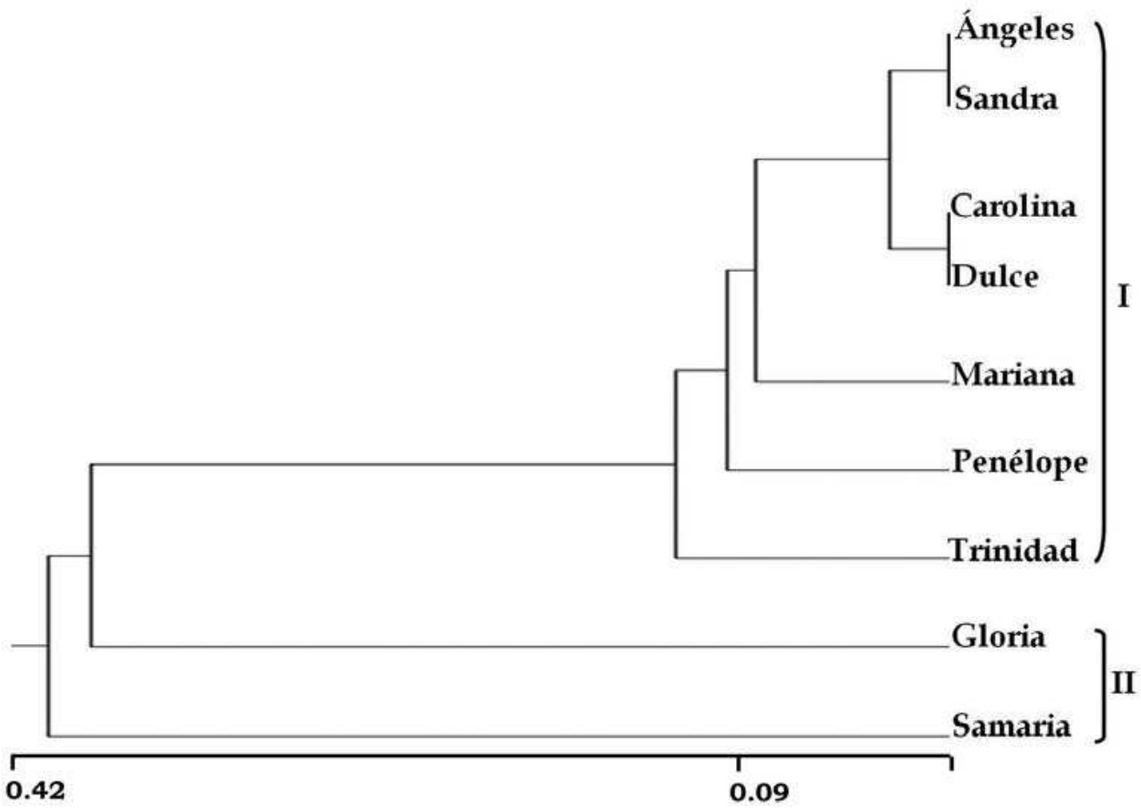

Fig. 4. Dendrogram of nine varieties of *Tigridia pavonia* based on Nei (1972) genetic distance, UPGMA method. The bar indicates the genetic distance among the varieties and the groups obtained with isozyme markers.

Variety	ANG	CAR	DUL	GLO	MAR	PEN	SAM	SAN
ANG								
CAR	0.031							
DUL	0.031	0.000						
GLO	0.287	0.330	0.330					
MAR	0.098	0.064	0.064	0.421				
PEN	0.098	0.064	0.064	0.421	0.133			
SAM	0.374	0.330	0.330	0.374	0.421	0.246		
SAN	0.000	0.031	0.031	0.287	0.098	0.098	0.374	
TRI	0.064	0.098	0.098	0.207	0.169	0.169	0.287	0.064

Table 3. Estimation of genetic distance (G_D), according to the formula of Nei (1972), for the nine varieties of *T. pavonia*.

4.3 DNA markers

4.3.1 ISSR markers

The total number of fragments reproducible with the five ASSR primers was 40, with an interval of 350 to 1900 pb in the size of the amplified fragments. Of the 40 fragments, 35 were polymorphic, with an average of seven per primer. The percentage of polymorphic fragments varied from 66.6 to 100%, with an average of 87.5% (Table 4). These results are similar with those reported by Yamagishi et al. (2002) and Arzate-Fernández et al. (2005b), who used these ASSR primers and proved their efficiency for detecting high polymorphism percentage in different species of the genus *Lilium.*

One aspect that favors the effectiveness of the ASSR primers as well as the type of the motive replicate in its sequence, is the sequence of its anchor. The CT motive sequences produce higher polymorphism with respect to the AT replicates (Pradeep et al., 2002; Hu et al., 2003), which despite being the most abundant in the plant genomes, have the disadvantage that the amplification of DNA fragments is low; this may be due to the semi-complementarity of the primer in the alignment stage of the PCR (Fang & Roose, 1997). In this study, the level of distinction among the variables depended on the sequence of the anchor of the primer. Thus, it was possible to obtain 100% polymorphism among the varieties of *T. pavonia* with primers ASSR02 and ASSR35 (Table 4).

Primer	Sequence (5'-3')	AF	PF	%P	*Rp*	FS (pb)
3´-ASSR02	CTCTCTCTCTCTCT ATC	9	9	100	3.12	500-1300
3´-ASSR15	CTCTCTCTCTCTCT ATG	8	6	85.7	2.2	350-1200
3´-ASSR20	CTCTCTCTCTCTCT GCA	7	6	85.7	2.2	600-1800
3´-ASSR29	CTCTCTCTCTCTCT GTA	6	4	66.6	1.8	550-1400
3´-ASSR35	CTCTCTCTCTCTCT TGA	10	10	100	5.54	350-1900
Total or mean		40	35	87.5	1.97	450

Table 4. Characteristics of the ISSR fragments amplified in nine varieties of *T. pavonia* (L.f.) DC.: total amplified fragments (AF), polymorphic fragments (PF), percentage of polymorphism (%P), resolving power (*Rp*) and size of the amplified fragments (FS) for each ASSR primer.

Prevost & Wilkinson (1999) described the resolution power (*Rp*) as a useful tool for evaluating the capacity of a primer in the distinction of various genotypes. In our results, the highest values of *Rp* were for the primers ASSR02 and ASSR35 (Table 4), with which the nine varieties were distinguished from each other. Escandón et al. (2005a) obtained similar results with just two ASSR primers and generated specific profiles for 18 of 21 collections of *Jacaranda mimosifolia*. The efficiency of the ASSR primers for discriminating genotypes in the inter-varietal level has also been reported in *Solanum tuberosum* (Prevost & Wilkinson, 1999), *Jacaranda mimosifolia* (Pérez de la Torre et al., 2003), *Nierembergia linaeriefolia* (Escandón et al., 2005b) and *Ficus carica* (Guasmi et al., 2006), among others.

The dendrogram generated with the ISSR data (Figure 5) formed two groups of varieties: group I included Carolina, Ángeles, Trinidad, Sandra, Dulce, Mariana and Penélope with an average G_D = 0.19 whereas the group II included Gloria and Samaria with a G_D = 0.39. The highest genetic association (G_D = 0.07) was found between the varieties Carolina and Ángeles, while the least related were Gloria and Dulce (G_D = 0.91).

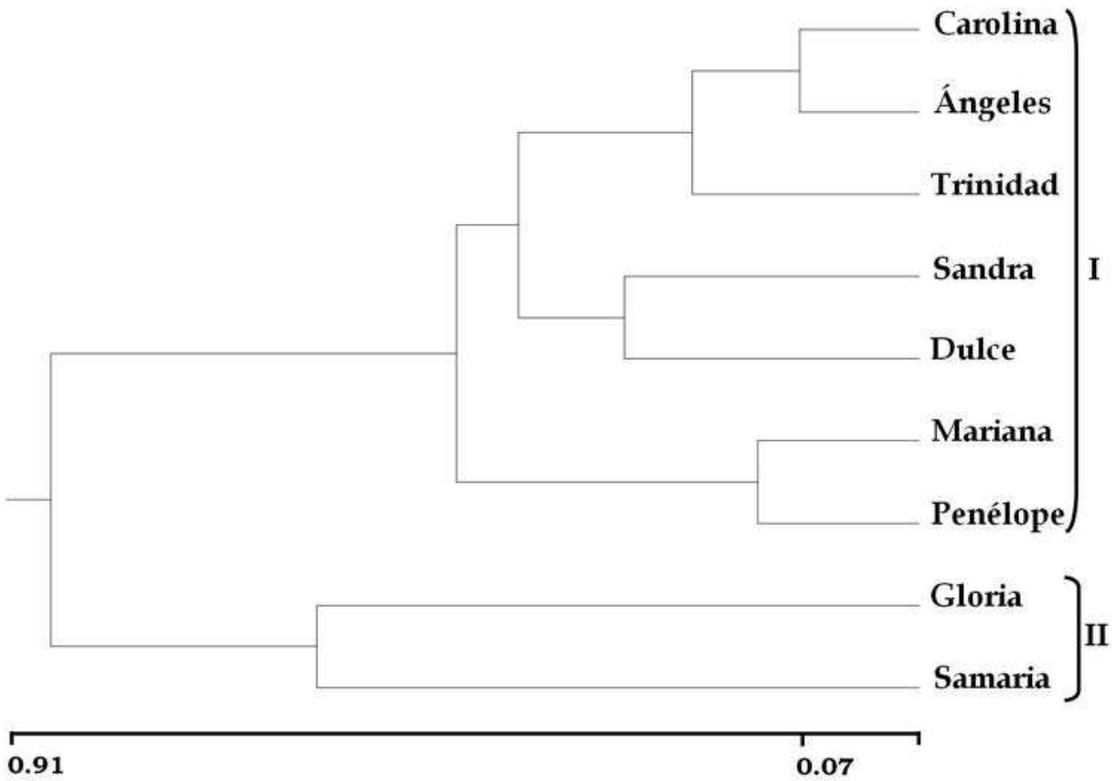

Fig. 5. Dendrogram of nine varieties of *T. pavonia* based on the genetic distance of Nei (1972), with the method UPGMA. The bar indicates the genetic distance among the varieties and the groups, calculated from ISSR data.

4.3.2 RAPD markers

In the present study, PCR amplification with 10, 15 and 20 length base RAPD primers led to reproducible fragment patterns for all varieties of *T. pavonia* evaluated. The majority of those RAPD fragments ranged from 250 to 2800 bp (Figure 6). For 10 base primers, the average of

total and the polymorphic fragments generated per primer were 8.8 and 8.2, respectively, whereas for 15 base primers, those values were 11 and 10.4, respectively, and for 20 base primers, 9.4 and 9, respectively (Table 5).

Generally, in RAPD analysis 10 base primers are preferred, nevertheless, because of the high annealing temperature applied in PCR reaction than their original, some 10 base primers could not hybridize with template DNA, generating only a few minor bands or none (Yamagishi et al., 2002), while the advantage of long primers is the smaller number of fragments containing repetitive DNA, thereby increasing the specificity and reproducibility of DNA fragments (Debener & Mattiesch, 1998). In the present study, the positive correlation ($r = 0.99$; $p \leq 0.05$) between primer length and the percentage of polymorphism generated per each primer was observed, indicating that the efficiency of primers to generate polymorphic fragments it increased by primer length. Thus, 15 or 20 base primers generated more DNA fragments (55), and a greater number of polymorphic fragments (52), in comparison with those obtained by the 10 base primers (44, and 41, respectively) (Table 5). The high efficiency of long primers to generate a large number of RAPD markers has been also reported in other species as *Lilium* spp. (Yamagishi et al., 2002), *Vitis vinifera* (Solouki et al., 2007) and *Matricaria chamomilla* (Solouki et al., 2008).

Primer	%GC	AF	PF	%P	*Rp*
Y24*	70	8	6	75	1.6
Y29*	70	10	10	100	5.1
Y37*	70	3	2	66.6	0.9
Y38*	70	15	15	100	7.5
Y41*	80	8	8	100	3.3
Average	72	8.8	8.2	93.1	3.6
P619**	66	13	13	100	5.8
P625**	66	8	6	75	2.6
P628**	60	4	3	75	1.3
P635**	73	17	17	100	8.9
P647**	60	13	13	100	6.4
Average	65	11	10.4	94.5	5
P495***	60	11	10	90.9	3.9
P496***	60	6	5	83.3	2.6
P497***	60	8	8	100	3.8
P498***	60	12	12	100	4.9
P500***	60	10	10	100	3.8
Average	60	9.4	9	95.7	3.8

Table 5. Percentage of GC (%GC), total amplified fragments (AF), polymorphic fragments (PF), percentage of polymorphism (%P) and resolving power (*Rp*) for each RAPD primer used. *10 base (Yamagishi, 1995). **15 base (Yamagishi et al., 2002). ***20 base (Debener & Mattiesch 1998).

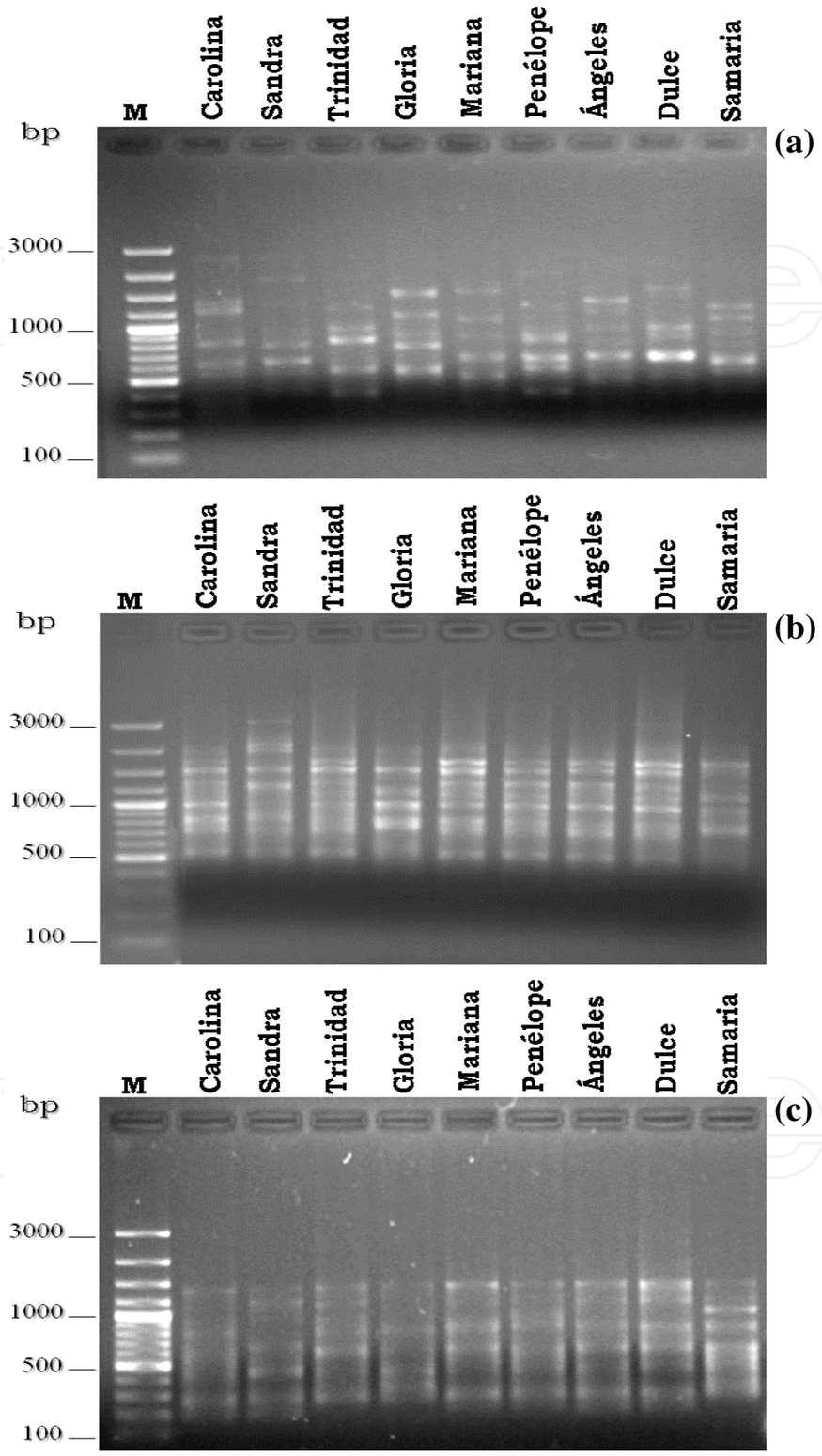

Fig. 6. RAPD profiles of nine varieties of *T. pavonia* generated by the 10 base primer Y38 (a), 15 base primer P647 (b) and 20 base primer P497 (c).

Although it is no clear exactly why the long primers produced more polymorphic bands, it has been reported that the GC content may be a factor that determine the efficiency of a primer (Solouki et al., 2007), since GC content is associated with annealing temperature and related to generation of more DNA fragments. Thus, it has been observed that with long primers with a lower GC content, the efficiency for amplifying polymorphic bands is higher, in comparison with short primers with a higher GC content (Ye et al., 1996; Solouki et al., 2007). According to this, it is possible that the GC content has favored the major efficiency of the 15 (65 % GC) and 20 (60 % GC) base primers used in the present study, because more DNA fragments were amplified and also a greater percentage of polymorphism, in comparison with those amplified with 10 base primers with 72 % GC content (Table 5). Our results are closely similar with those reported by Solouki et al., (2008) where it were obtained more DNA fragments and 100 % of polymorphism, using a long primer with low GC content.

The high efficiency of long primers in the genetic differentiation of *T. pavonia* varieties was also confirmed with the measurement of resolving power. The highest values of *Rp* belonged to the 15 and 20 base primers (5 and 3.8, respectively) (Table 5), indicating a better distinction of the varieties. So, these results also confirm the utility of the *Rp* as measure of capacity of a primer to discriminate among closely related individuals as was pointed out by Prevost &Wilkinson (1999) and Escandón et al. (2007).

The dendrograms based on UPGMA analysis of the 10, 15, 20 and the pooled (10, 15 and 20) RAPD data showed the genetic differentiation of the nine varieties of *T. pavonia* (Figure 7). In the dendrogram generated with decamers, the G_D among the varieties ranged from 0.20 to 0.69, with an average of 0.24. For the 15 base dendrogram, the G_D ranged from 0.13 to 0.78, with an average of 0.32, while the dendrogram of the 20 base showed a range of G_D of 0.11 to 0.67, with an average of 0.28, and finally the dendrogram with pooled data showed a range of G_D of 0.20 to 0.68, with an average of 0.24.

All four dendrograms clearly grouped the varieties in two major clusters. In the dendrograms of 10 and 15 bases, and in the pooled, the cluster I consisted of the varieties Carolina, Dulce, Trinidad, Penelope, Angeles, Mariana, and Sandra, whereas the cluster II grouped the varieties Gloria and Samaria. The close relationship observed in these dendrograms, among the varieties collected in Tenancingo and Temascaltepec municipalities, might be the result of having some common morphological characters such as: number of shoots by bulb, number of nodes by shoot, length of leaf, and number flowers by shoot. The separation of Gloria and Samaria collected in Temoaya, could be due to the highest values for length of shoot, length of internode, and length of floral escape, besides that the flowering of these varieties is delayed in comparison with the rest of them, as it was reported in our previous study (Piña-Escutia et al., 2010).

On the other hand, the dendrogram developed with 20 base primers also grouped the cluster I similar to the dendrograms generated with other primers sets, except for the variety Sandra, which was grouped with the varieties Gloria and Samaria in the cluster II. Thus, this dendrogram showed a best differentiation of the varieties evaluated, confirming the utility of long primers in generation high polymorphism and genetic discrimination of plant species, which was also reported by Solouki et al. (2007), and Solouki et al. (2008).

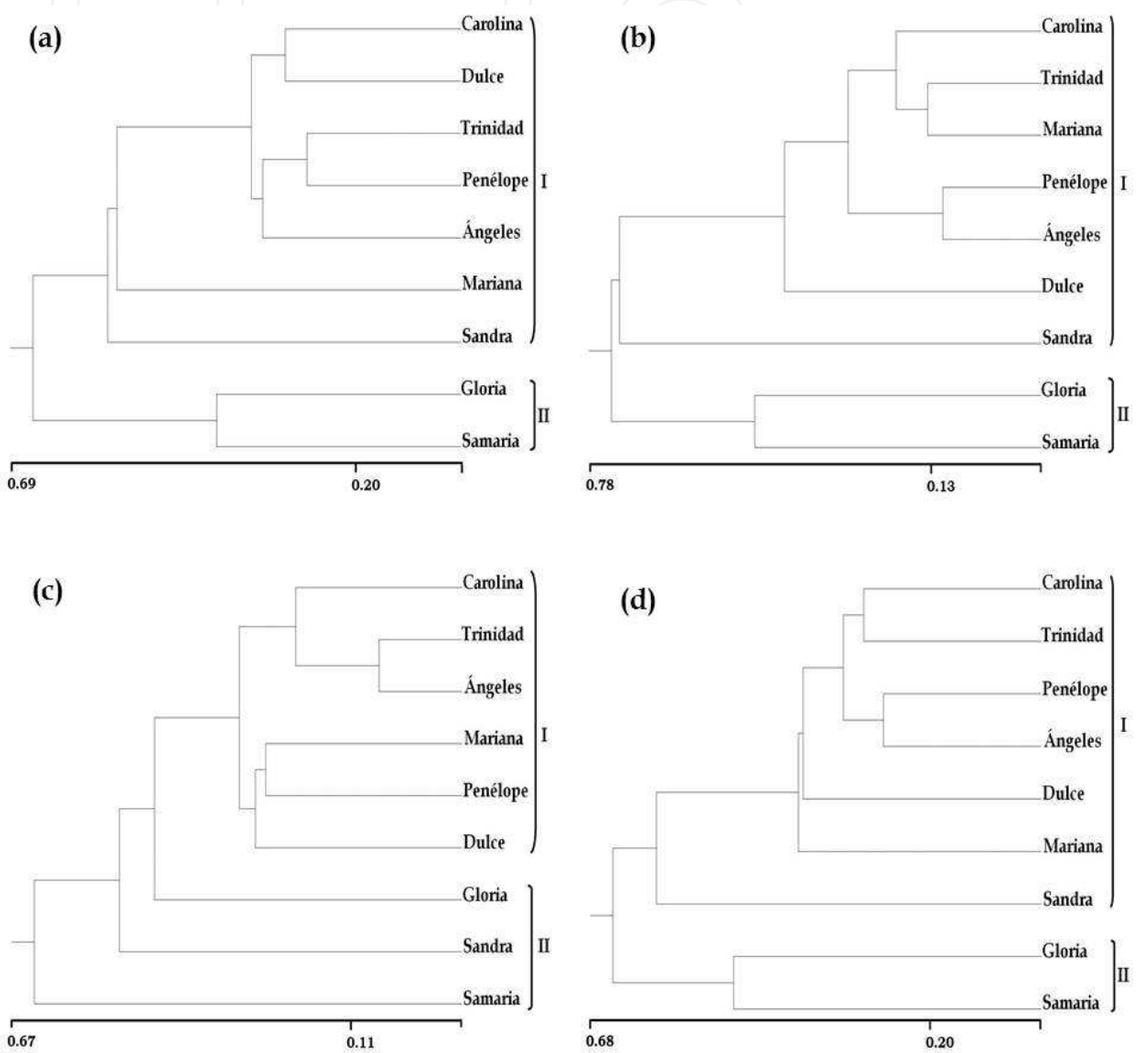

Fig. 7. Dendogram generated using UPGMA analysis, showing relationships between nine varieties of *T. pavonia*, based on RAPD data of 10 (a), 15 (b), 20 (c) and the pooled (10, 15 and 20) base of length primer (d). The bar indicates the genetic distance among the varieties and the groups.

5. General conclusions and perspectives

The assessment of genetic variability of Mexican native species is fundamental for the conservation, and plant variety protection. In this review we have conducting the morphological analysis in combination with the molecular assays to distinguish *T. pavonia* varieties. The analyses of the morphological and DNA markers (ISSR and RAPD) seem to be more efficient than the isozymes, because whereas the former showed polymorphism among the nine varieties evaluated and permit the distinction among them, the biochemical characterization revealed that the varieties Angeles and Sandra as well as Carolina and Dulce were the same, indicating the low discrimination power of isozymes which can be due to inadequate genome coverage. So, our results indicate that the marker assisted characterization can be applied to complement existing conventional breeding programmes and, despite its application in genetic improvement of horticultural crops is limited, their use as balance between molecular and non-molecular methods can offers opportunities of improved phenotypic selection in the future.

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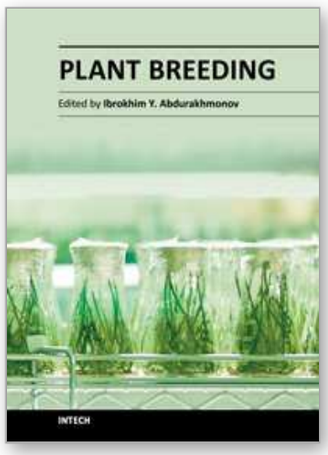

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Modern plant breeding is considered a discipline originating from the science of genetics. It is a complex subject, involving the use of many interdisciplinary modern sciences and technologies that became art, science and business. Revolutionary developments in plant genetics and genomics and coupling plant "omics" achievements with advances on computer science and informatics, as well as laboratory robotics further resulted in unprecedented developments in modern plant breeding, enriching the traditional breeding practices with precise, fast, efficient and cost-effective breeding tools and approaches. The objective of this Plant Breeding book is to present some of the recent advances of 21st century plant breeding, exemplifying novel views, approaches, research efforts, achievements, challenges and perspectives in breeding of some crop species. The book chapters have presented the latest advances and comprehensive information on selected topics that will enhance the reader's knowledge of contemporary plant breeding.

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