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Genetic diversity of *Cosmos* species revealed by RAPD and ISSR markers

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ABSTRACT. The genus *Cosmos* is native of America and is constituted by 34 species; 28 of them are endemic of Mexico. The cosmos are used as a nematicide, antimalarial, and antioxidative agent. The aim of this study was to estimate the genetic diversity among 7 cosmos species based on random amplified polymorphic DNA (RAPD) and inter-simple sequences repeats (ISSR) markers. With RAPD markers, the obtained polymorphism was 91.7 % and the genetic diversity was 0.33, whereas these values were 65.6%, and 0.22 from ISSR markers, respectively, indicating the presence of high genetic diversity among the *Cosmos* species that were analyzed. The unweighted pair group method with arithmetic mean dendrograms that were obtained with both markers were notably similar, revealing 2 clusters and indicating a clear genetic differentiation among the *Cosmos* species that were assessed. The first cluster comprised the species *Cosmos sulphureus*, *Cosmos pacificus*, and *Cosmos diversifolius*, while the second cluster included the species *Cosmos purpureus*, *Cosmos crithmifolius*, *Cosmos bipinnatus*, and *Cosmos parviflorus*. Besides this, the *Cosmos* species were

clustered according to their collection sites. The Mantel test corroborates the correlation between the genetic distance and the geographic altitude of each *Cosmos* species. The results suggest that it is necessary to preserve the *Cosmos* species in their natural habitat in addition to the germoplasm collection for *ex situ* conservation.

Key words: *Cosmos*; Genetic diversity; Inter-simple sequence repeat (ISSR); Random amplified polymorphic DNA (RAPD); DNA profiles

INTRODUCTION

The genus *Cosmos* belongs to the family Asteraceae, is native of America, and is constituted by 34 species, including 28 that are endemic of Mexico. The genus comprises plants with late-flowering annual or tuberous perennial behavior. These plants can attain a height of 0.5 to 3 m and have an open and sprawling habit, require little care, and tolerate dry and infertile soil. The cosmos have been reported to act as a nematicide (Tsay et al., 2004). Likewise, their flower extracts can be used as natural dye (Kale et al., 2006), antimalarial (Botsaris, 2007), antioxidative, and antigenotoxic agents (Jang et al., 2008). The main importance of cosmos is the great color and morphological variation of flowers, making many species potentially valuable as ornamental plants. Thus, some species such as *Cosmos sulphureus*, *Cosmos bipinnatus*, and *Cosmos diversifolius* are commercialized as garden plants in different countries of Europe and Asia. Moreover, new varieties have been developed in those places (Oku et al., 2008). In contrast, *Cosmos* species remain largely unexploited in Mexico because of the small amount of information on the resource abundance and distribution. Therefore, genetic diversity studies are needed to provide information for its efficient conservation as well as for possible future use in breeding programs.

Genetic diversity of a species is related to geographic distribution, mode of reproduction, breeding system, and seed dispersal mechanism. Therefore, successful management and preservation of populations of rare, threatened, or endangered species depend on a complete understanding of the species, including levels and structure of genetic variation (Wallace, 2002 cited by Arzate-Fernández et al., 2005). Genetic diversity has been traditionally assessed by morphological markers. However, these markers are time consuming and often the testing procedures are complex or unreliable.

Molecular markers offer fast screening and a wide range of novel approaches to improve the selection strategies in horticultural plant breeding (Ibitoye and Akin-Idowu, 2010). Different marker systems such as restriction fragment length polymorphisms (RFLPs), amplified fragment length polymorphisms (AFLPs), simple sequence repeats (SSRs), and single nucleotide polymorphisms (SNPs) have been reported as highly polymorphic and reproducible. However, RFLPs require a large amount of DNA, are labor-intensive, time-consuming, and mostly require radioactively labeled probes, whereas AFLPs and SSRs are quite costly and also require high-resolution electrophoresis or automated sequences. On the other hand, although SNPs are less time-consuming that the rest of the markers and highly amenable to automation, the initial cost that is involved is quite high (Jehan and Lakhanpaul, 2006). Polymerase chain reaction (PCR) molecular markers like inter-simple sequence repeats (ISSRs) and random amplified polymorphic DNA (RAPD) would be an option because of the lower level of skill required, low cost per assay, and the ready availability of primers allow the scanning of the entire genome and efficient genotype characterization. Thus,

because of their characteristics and efficiency for detecting polymorphisms, the ISSR and RAPD markers have been successfully used to calculate the intra or inter-specific genetic diversity in different domestic and wild species (Escandón et al., 2005; Muthusamy et al., 2008; Li et al., 2011).

To date, there have been no reports on the genetic diversity of *Cosmos* species. In this study, RAPD and ISSR markers were used to investigate the genetic diversity and differentiation of 7 *Cosmos* species. The objectives were to: 1) estimate the genetic diversity of the species, 2) assess the genetic relationships between different *Cosmos* species, and 3) to determine if the genetic variation among the species is related to an altitudinal gradient.

MATERIAL AND METHODS

Plant material

Seven *Cosmos* species from different altitudes were collected in 6 municipalities of Estado de México, México (Figure 1; Table 1).

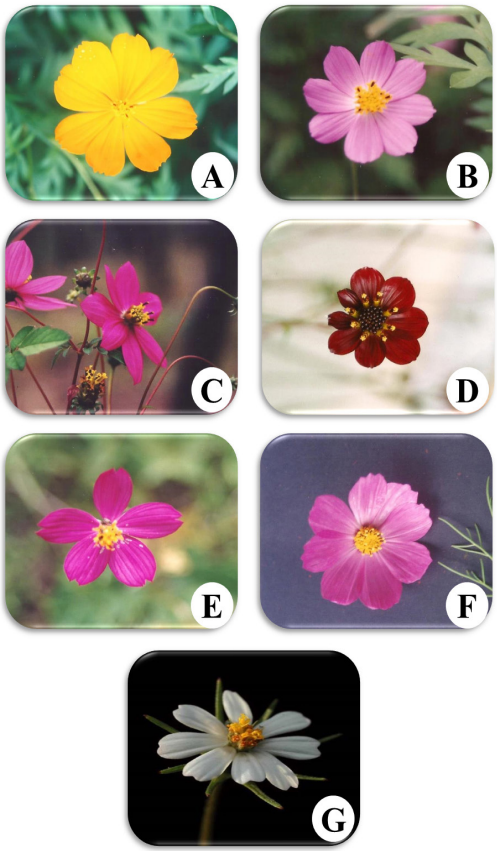

Figure 1. *Cosmos* species used in the genetic diversity study. **A.** *Cosmos sulphureus*; **B.** *Cosmos pacificus*; **C.** *Cosmos diversifolius*; **D.** *Cosmos purpureus*; **E.** *Cosmos crithmifolius*; **F.** *Cosmos bipinnatus*; **G.** *Cosmos parviflorus* (Tenorio, 2001).

Table 1. List of *Cosmos* species used and details of collection sites. All species were collected at Estado de México, México.

No.	Scientific name	Municipality	Altitude	Latitude and longitude
1	*C. sulphureus*	Zumpahuacán	1644	18°49'N 99°36'E
2	*C. pacificus*	Ixtapan del Oro	1760	19°11'N 100°15'E
3	*C. diversifolius*	San Simón de Guerrero	1889	18°56'N 100°06'E
4	*C. purpureus*	Ocuilan	2086	18°58'N 99°27'E
5	*C. crithmifolius*	Ocuilan	2086	18°58'N 99°27'E
6	*C. bipinnatus*	Toluca	2640	19°23'N 99°42'E
7	*C. parviflorus*	Jocotitlán	2835	19°43'N 97°47'E

DNA isolation and PCR amplification

The genomic DNA was extracted from approximately 100 mg leaf tissue from each *Cosmos* species. The extraction procedure was the cetyltrimethylammonium bromide method that was reported by Arzate-Fernández et al. (2005). The DNA samples were stored at -20°C prior to analysis. The PCR was made in a final volume of 10 µL with 1 µL 10 ng DNA, 1 µL 10X 15 mM PCR buffer with ammonium, 0.5 µL 15 mM $MgCl_2$, 1 µL 10 mM dNTPs, 1 µL 20 mM primer, and 0.1 U enzyme Taq DNA polymerase.

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 and Mattiesch, 1998) were used (Table 2). The amplification conditions for 10-base RAPD primers were reported by Yamagishi (1995). The program for 15-base primers followed that of Yamagishi et al. (2002) with the following minor modifications: for the primers P619 and P635, the program consisted of 30 cycles of 94°C for 1 min, 53°C for 3 min, and 72°C for 2 min; whereas the program was the same for the primers P625, P628, and P647 except with an annealing temperature of 56°C. Finally, the PCR cycle conditions for 20-base primers were those according to Debener and Mattiesch (1998).

ISSR markers

For ISSR markers, 5 primers of the anchored microsatellites type were used (3'-ASSR) (Yamagishi et al., 2002). In each primer, the anchor consisted of a triplicate of a distinct sequence (Table 2). The amplification conditions were those that were used by Yamagishi et al. (2002) with minor modifications (45 cycles of 94°C for 1 min, 48°C for 1 minute, and 72°C for 1 min).

The amplification of DNA fragments for RAPD and ISSR markers was performed in a thermocycler (Mastercycler Gradient, Eppendorf, Germany). The separation of the fragments was by horizontal electrophoresis. A molecular marker of 100 to 3000 bp 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.

Statistical analysis

For RAPD and ISSR markers, each band that was generated by each primer was

considered to be an independent locus and was calculated manually; that is, the value of "1" was assigned for the presence of a band and "0" for its absence. All of the calculations were performed using the tools for population genetic analysis (POPGENE ver 1.32).

We obtained the total amplified fragments (AF), polymorphic fragments (PF), and percentage of polymorphism (%P) using the 95% criterion. Genetic diversity among populations was analyzed by Nei's measures of total genetic diversity (H_T) and genetic distance (G_D).

In both markers, to determine the genetic relationships among all populations and how these were clustered, a dendrogram was constructed with the data using the unweighted pair group method of averages (UPGMA) based on Nei's G_D using POPGENE.

To determine if the genetic variation among the evaluated species was related to an altitudinal gradient, the values of the genetic distances that were derived from the RAPD and ISSR markers were correlated with those of the geographic altitude that corresponded to each of the cosmos populations. For this, a Mantel test was performed using the XLSTAT program version 2011.3.02.

Table 2. GC content (%GC), total amplified fragments (AF), polymorphic fragments (PF) and percentage of polymorphism (%P) for each RAPD and ASSR primer used.

Primer	%GC	AF	PF	%P
Y24*	70	9	9	100
Y29*	70	5	5	100
Y37*	70	10	10	100
Y38*	70	8	2	25
Y41*	80	4	3	75
Mean	72	7.2	5.8	80
P619**	66	10	8	80
P625**	66	14	13	92.8
P628**	60	12	12	100
P635**	73	8	8	100
P647**	60	11	10	90.9
Mean	65	11	10.2	92.7
P495***	60	10	10	100
P496***	60	13	13	100
P497***	60	14	13	92.8
P498***	60	10	10	100
P500***	60	8	8	100
Mean	60	11	10.8	98.5
3'-ASSR02		5	2	40
3'-ASSR15		6	5	83.3
3'-ASSR20		8	5	62.5
3'-ASSR29		5	5	100
3'-ASSR35		8	4	50
Mean		6.4	4.2	67.1

*10 base (Yamagishi, 1995). **15 base (Yamagishi et al., 2002). ***20 base (Debener and Mattiesch, 1998).

RESULTS AND DISCUSSION

In this study, RAPD and ISSR markers were successfully applied to assess the genetic diversity of 7 *Cosmos* species, constituting the first report to describe the genetic diversity of cosmos in México.

RAPD markers

PCR amplification with 10-, 15-, and 20-base RAPD primers led to reproducible

fragment patterns for all of the evaluated *Cosmos* species. The majority of those RAPD fragments ranged from 350 to 3000 bp (Figure 2A, B and C). For the 10-base primers, the mean of total, and the polymorphic fragments that were generated per primer were 7.2 and 5.8, respectively, whereas those values were 11 and 10.2, respectively, for 15-base primers and 11 and 10.8, respectively, for 20-base primers (Table 2).

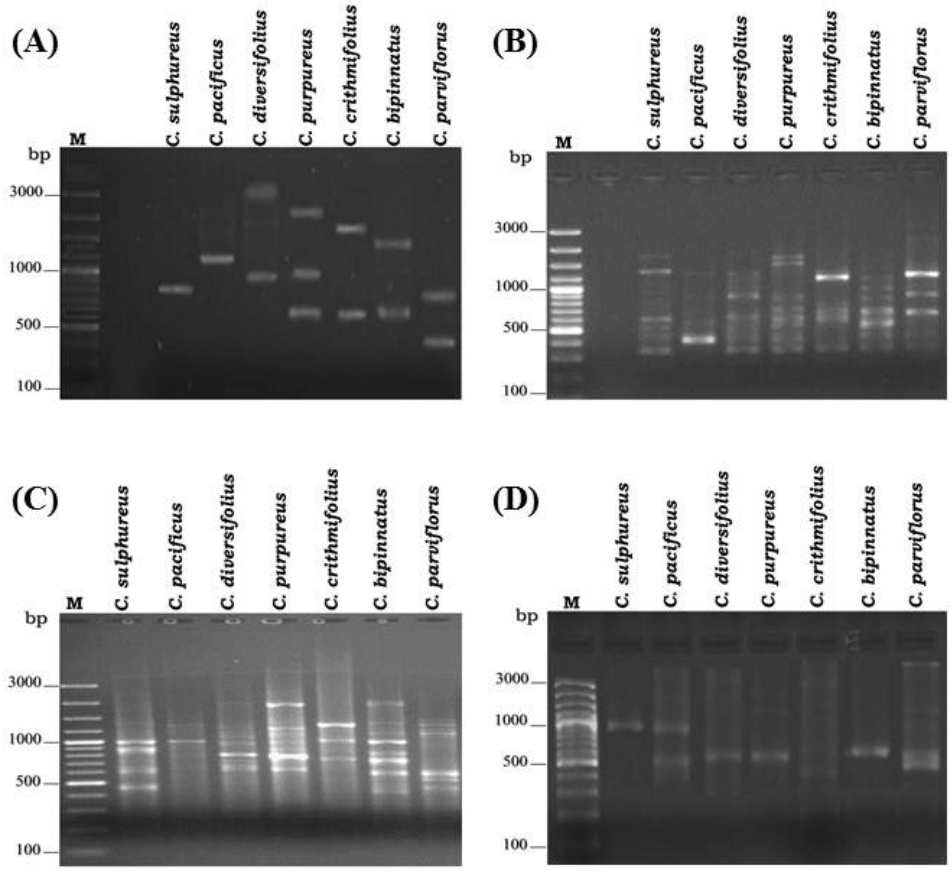

Figure 2. RAPD and ISSR profiles of seven species of *Cosmos* generated by the 10 base primer Y37 (**A**), 15 base primer P625 (**B**), 20 base primer P497, (**C**) and 3'ASSR-29 (**D**).

The primer efficiency is measured according to the polymorphic bands that were amplified by the same primer. In this study, our results showed that the efficiency of primers to generate polymorphic fragments increased with primer length. Thus, 15- or 20-base primers generated 55 DNA fragments each and included a greater number of polymorphic fragments, 51 with the 15-base primer and 54 by the 20-base primer, than the total and polymorphic bands generated by the 10-base primers (36 and 29, respectively) (Table 2). There is not a clear reason why the long primers produced more polymorphic bands; however, it has been reported that the GC content may be a factor that determines the efficiency of a primer (Solouki et al., 2007) because GC content is associated with annealing temperature and is related to the generation of

more DNA fragments. Thus, it has been observed that the efficiency for amplifying polymorphic bands is higher with long primers with a lower GC content than with short primers with a higher GC content (Solouki et al., 2007). According to this, it is possible that the GC content favored the major efficiency of the 15- (65% GC) and 20-base primers (60% GC) that were used in this study because more DNA fragments were amplified and also a greater percentage of polymorphism was detected (92.7 and 98.5, respectively) compared with those that were amplified with 10-base primers, which had 72% GC content and 80% polymorphism (Table 2). Our results are similar to those that were reported by Solouki et al. (2008) and Piña-Escutia et al. (2010b), where more DNA fragments and 100% polymorphism were obtained using a long primer with low GC content.

ISSR markers

In this study, reproducible polymorphic banding patterns were obtained with ISSR markers in the 7 *Cosmos* species (Figure 2d). The mean of total and the polymorphic fragments that were generated per primer were 6.4 and 4.2, respectively. ISSR markers have been successfully used to estimate the extent of genetic diversity in a wide range of crop and wild species, including lily (Arzate-Fernández et al., 2005), jacaranda (Escandón et al., 2005), citronella (Bhattacharya et al., 2010), and ginseng (Li et al., 2011). The ISSR marker efficiency has been attributed to motif sequences, as well as the sequence of its anchor. The CT motif sequences produce higher polymorphism than the AT replicates (Hu et al., 2003). Despite being the most abundant sequences in plant genomes, these sequences have the disadvantage that the amplification of the DNA fragments is low; this may be because of the semi-complementarity of the primer in the annealing stage of the PCR. In this study, with the primer 3'-ASSR29, whose sequence is GTA, it was possible to obtain 100% polymorphism (Table 2). This suggests that the ISSR markers can be a highly informative, fast, and reliable system for the genetic diversity studies as reported by Liu et al. (2011). Our results also suggest that the CT sequences may be very abundant in the genomes of wild ornamental species because the primers that were used here have been also useful for determining the genetic diversity of other ornamental wild species such as *Lilium maculatum* (Arzate-Fernández et al., 2005), *Tigridia pavonia* (Piña-Escutia et al., 2010a), and *Sprekelia formosissima* (Bautista-Puga et al., 2011).

Genetic diversity and clustering analysis

When dominant markers are used, the estimates of genetic diversity are usually also quite similar and closely correlated both for within population diversity and for population differentiation, suggesting that enough primers and primer combinations are employed to ensure sufficient numbers of polymorphic bands and good genome coverage (Nybom, 2004). In this study, similar results were found because the polymorphism that was obtained was 91.7% and the H_T was 0.33 for pooled RAPD markers (10, 15, and 20 bases), whereas these values were 65.6%, and 0.22 for ISSR markers, respectively, indicating the presence of high genetic diversity among the *Cosmos* species that were analyzed. It is known that the mating system and mode of reproduction affect significantly the extent and distribution of the genetic diversity. In self-compatible species, increased homozygosity results in low levels of genetic variation, whereas species with predominantly outcrossing mating systems exhibit higher levels of genetic variation. *Cosmos* species have an outcrossing mating system and can be reproduced by

seeds. Therefore, the mating system and mode of reproduction could explain the high genetic diversity values that were found in this study. Our results are similar to those that were reported in previous studies of wild species, such as *Liparis* (Chung et al., 2007) and *Tadehagi* (Liu et al., 2011), wherein a very high level of genetic diversity has also been reported.

The UPGMA dendrograms that were obtained with the pooled RAPD and ISRR markers (Figure 3) showed that there is a clear genetic differentiation among the *Cosmos* species that were evaluated. In both dendrograms, the species grouping was very similar and revealed 2 clusters. The first cluster comprised the species *C. sulphureus*, *C. pacificus*, and *C. diversifolius*, while the second cluster included the species *C. purpureus*, *C. crithmifolius*, *C. bipinnatus*, and *C. parviflorus*. Overall, for RAPD markers, the G_D range among the species ranged from 0.27 to 0.63 with an average of 0.45. For ISSR markers, the G_D ranged from 0.09 to 0.57 with an average of 0.33. It is known that low genetic distances among the populations indicate a close genetic relationship, whereas the genetic relationship is more distant in populations with higher genetic distances. One factor that enhances the gene exchange between individuals of geographically separated populations is the wide spreading of seeds and pollen (Byrne et al., 2008). According to this, it is possible that the low average G_D values that were found in our study can be result of the gene exchange among the *Cosmos* species because they employ pollen dispersal by insects and have widely spreading seeds by wind.

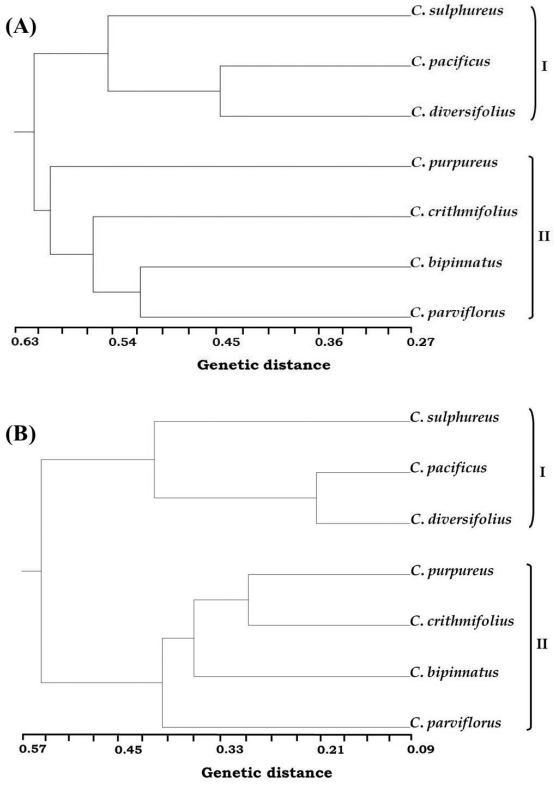

Figure 3. Dendrograms generated using UPGMA analysis, showing relationships among seven *Cosmos* species, based on RAPD pooled (10, 15, and 20b) (**A**), and ISSR data (**B**).

Hamrick and Godt (1989) reported a strong correlation between geographical range and genetic diversity. This indicates that populations may differ with respect to all aspects of diversity and show variation in the number of alleles, the identity of those alleles, and the effect they have on the characteristics in the population. In fact, different geographic locations nearly always differ with respect to some potentially significant ecological characteristic (such as latitude, altitude, temperature, and moisture availability) (Rao and Hodgkin, 2002). In this study, similar results were found: the clustering 7 *Cosmos* species appeared to be influenced by the geographic altitude of each species (Table 1; Figure 3), which was confirmed by the positive correlations between genetic distances and geographic altitude that were revealed for RAPD pooled ($r = 0.60$, $P \leq 0.001$) and ISRR ($r = 0.76$, $P \leq 0.001$) markers.

The species of cluster I (*C. sulphureus*, *C. pacificus*, and *C. diversifolius*) were collected in lower altitudes (Table 1) than the other species that were assessed. Apart from its flower color distinctiveness (Figure 1), these species have an annual growth habit, trilobed leaves with acuminate apex, and abundant flower number, which makes this cluster morphologically distinct from the other *Cosmos* species. Rao and Hodgkin (2002) mentioned that under natural conditions, there is a close relationship between the morphological and the physiological traits of plants and of habitats in which the traits have evolved and are expressed. In accordance with these findings, the genetic differentiation of the first cluster of *Cosmos* species could be determined by environmental homogeneity in their collection sites, where topography, temperature, and climate were very similar. These results are similar to those that were reported by Piña-Escutia et al. (2010a) and Liu et al. (2011), who also found that the genetic variation was influenced by the environmental homogeneity.

In cluster II, 2 subgroups can be observed. *C. purpureus* and *C. crithmifolius* were clustered in the first subgroup, whereas *C. bipinnatus* and *C. parviflorus* were clustered in the second subgroup. It has been reported that long-lived and outcrossing species tend to be more genetically diverse (Hamrick and Godt, 1996). In addition, the breeding system and altitude of origin of the species are very important in determining the differences between populations from different geographic locations (Jordano and Godoy, 2000; Rao and Hodgkin, 2002). Among the *Cosmos* species that were assessed in this study, only *C. purpureus* and *C. crithmifolius* have a perennial growth habit; also, they have solitary inflorescence and a low flower number. In addition, both species were collected in the same site (Ocuilan) at 2086 m, where the climate is cooler than other sites. Considering the above information, we assumed that the breeding system, growth habit, and altitude of collection site enhance the gene exchange between these species (both shared 13 morphological characters, data not shown) and determined that the genetic differentiation was the same.

Wen and Hsiao (2001) mentioned that populations at high altitudes experience greater annual and diurnal climatic variation. In this study, *C. bipinnatus* and *C. parviflorus* were collected in the high altitudes (2640 and 2835 m, respectively). In their collection sites, the daytime temperature in summer can be greater than 30°C, whereas the nighttime temperature in winter is usually below 0°C and frost often occurs. According to this, it is possible that these factors can enhance that the plants are less affected by any disturbance; therefore, the gene flow can be higher among them. This could explain the genetic differentiation of *C. bipinnatus* and *C. parviflorus*. In fact, both species share a large number of morphological characteristics (14, data not shown) in comparison to other *Cosmos* species. Our results also agree with those that were reported by Jordano and Godoy (2000), who mentioned that the difference in the altitude of origin of the populations was related to a marked difference in the phenology of each species.

CONCLUSIONS

Effective conservation of plant genetic resources requires a sound scientific and technical basis (Rao and Hodgkin, 2002). In Mexico, cosmos have been unexploited because of the lack of information about the resource abundance and distribution. This is the first report on the genetic characterization of 7 *Cosmos* species. RAPD and ISSR markers revealed the genetic diversity of the assessed species. The genetic relation among them was visualized in the dendrograms that were generated by both types of marker. Furthermore, the *Cosmos* species clustered according their collection sites, and we found a positive correlation between the genetic distance and geographic altitude of each *Cosmos* species. From an ecological perspective, the genetic differentiation of *Cosmos* species indicates that it is necessary to preserve them in their natural habitat; besides this, it is possible to preserve them through the germoplasm collection for *ex situ* conservation approaches.

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