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Genetic diversity and phylogenetic relationship among Tunisian cactus species (*Opuntia*) as revealed by random amplified microsatellite polymorphism markers

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ABSTRACT. *Opuntia ficus indica* is one of the most economically important species in the Cactaceae family. Increased interest in this crop stems from its potential contribution to agricultural diversification, application in the exploitation of marginal lands, and utility as additional income sources for farmers. In Tunisia, *O. ficus indica* has been affected by drastic genetic erosion resulting from biotic and abiotic stresses. Thus, it is imperative to identify and preserve this germplasm. In this study, we focused on the use of random amplified microsatellite polymorphisms to assess genetic diversity among 25 representatives of Tunisian *Opuntia* species maintained in the collection of the National Institute of Agronomic Research of Tunisia. Seventy-two DNA markers were screened to discriminate accessions using 16 successful primer

combinations. The high percentage of polymorphic band (100%), the resolving power value (5.68), the polymorphic information content (0.94), and the marker index (7.2) demonstrated the efficiency of the primers tested. Therefore, appropriate cluster analysis used in this study illustrated a divergence among the cultivars studied and exhibited continuous variation that occurred independently of geographic origin. *O. ficus indica* accessions did not cluster separately from the other cactus pear species, indicating that their current taxonomical classifications are not well aligned with their genetic variability or locality of origin.

Key words: Cluster analysis; Molecular markers; Polymorphism; *Opuntia* germplasm; Random amplified polymorphic DNA; Tunisian collection

INTRODUCTION

The *Opuntia* genus (Cactaceae family) is native to Mexico and includes approximately 200 species that are widely dispersed throughout arid and semi-arid areas of the world. *O. ficus indica* represent the most common culinary species of this genus. The largest cactus collection is known to be located in Mexico, where the greatest diversity is observed for the native cactus pear and is represented by wild populations. In Tunisia, approximately 500.000 ha are now planted with cactus (Nefzaoui and Ben Salem, 2001). However, a major limitation in the development of cactus pear fruit and fodder varieties is the lack of characterization and evaluation of the available germplasm. Taxonomic evaluation of *Opuntia* is complicated by the relationship between phenotypic variation and ecological conditions, polyploidy, vegetative or sexual reproduction, and hybridization between species (Scheinvar, 1995). In addition, phenotypic variability is the most frequently observed in fruit size and color, cladode size, morphology, and phenology (fruit ripening time) (Pimienta-Barrios and Muñoz-Urias, 1995). Although morphological traits are easily monitored, they are inadequate for characterizing the germplasm, as they can be influenced by the environmental conditions. Germplasm characterization using molecular fingerprinting has become increasingly used for crop improvement. The application of genetic markers has also been successfully used for resolving taxonomic and evolutionary problems of several crop plants, assess the structure of genetic variability, establish genetic relationships among accessions (Andersen and Lübberstedt, 2003), and identify genes that express potentially useful traits for agricultural production or crop improvement (Wang et al., 1998). Recently, several molecular markers have been shown to be useful for classifying *Opuntia* species and cultivars. Random amplified polymorphic DNA (RAPD) was successfully applied for the molecular characterization of Mexican accessions (Mondragón-Jacobo, 2003), to identify cultivars and recognize duplicate accessions in collections (Wang et al., 1998), and to assess the genetic diversity within Tunisian Barbary figs *O.ficus indica* L. Mill. (Zoghlami et al., 2007). Amplified fragment length polymorphism have been applied to verify the identity of *O. ficus indica* and *O. megacantha* (Labra et al., 2003), as well as to characterize the 3 Tunisian collections of cactus (Snoussi Trifa et al., 2009). The objective of this study was to use the random amplified microsatellite polymorphism (RAMPO) technique to generate useful molecular markers, investigate polymorphisms, and understand the genetic relationships among accessions of Tunisian cactus. We examined the

level of differentiation among cactus pear genotypes, including the most widespread cultivars and their relationship with wild accessions and related species. Our study also resolved some of the discrepancies that exist when *Opuntia* germplasm was classified based only on morphological features. Characterization and evaluation of the available cactus pear gene pool are essential for future breeding programs. We focused on the advancements made in the application of molecular markers for germplasm characterization. We also examine the potential for applying functional marker-based molecular tools to evaluate agronomically important traits in the germplasm.

MATERIAL AND METHODS

Plant materials

A set of 25 cactus cultivars, belonging to 7 *Opuntia* species (*O. ficus indica*, *O. engelmannii*, *O. tomentosa*, *O. undulata*, *O. ellisiana*, *O. streptacantha*, and *O. robusta*), were sampled from a collection established at the National Institute of Agronomic Research of Tunisia. The main characteristics (code, ecotype, and species) for the considered cultivars are summarized in Table 1. For each cultivar, young cladodes collected from adult trees were stored at -20°C until DNA extraction.

Table 1. *Opuntia* species used for random amplification microsatellite polymorphism analysis and their country of origin.

Code	Species	Ecotype	Origin
Oac	*Opuntia ficus indica*	Caref 58	Algeria
Oms	*Opuntia ficus indica*	Sefrou	Morocco
Omc	*Opuntia ficus indica*	Carroii	Morocco
Osa	*Opuntia ficus indica*	Chico	South Africa
Ome	*Opuntia ficus indica*	El Bouroug	Morocco
Ots	*Opuntia ficus indica*	Sbeitla	Tunisia
Ott	*Opuntia ficus indica*	Thala	Tunisia
Otmr	*Opuntia ficus indica*	Mornag	Tunisia
Ost	*Opuntia ficus indica*	Tronzara	Sicily
Onm	*Opuntia ficus indica*	Leavis	New mexico
Oab	*Opuntia ficus indica*	Burbank Azrou	Algeria
Oan	*Opuntia ficus indica*	Nopalitas	Argentine
Oet	*Opuntia ficus indica*	Ethiopia	Ethiopie
Otd	*Opuntia ficus indica*	Djebel Bargou	Tunisia
Otmo	*Opuntia ficus indica*	Montarnaud	Tunisia
Oto	*Opuntia ficus indica*	Oueslatia	Tunisia
Omm	*Opuntia ficus indica*	Morocco	Morocco
Omb	*Opuntia ficus indica*	Bab Toza	Morocco
Osb	*Opuntia ficus indica*	Bianca	Sicily
Op.ro	*Opuntia robusta*	Camuesa	Mexico
Op.st	*Opuntia streptacantha*	Mexico	Mexico
Op.to	*Opuntia tomentosa*	Carthage	Algeria
Op.un	*Opuntia undulata*	France	France
Op.el	*Opuntia ellisiana*	P. Felk	Texas
Op.eg	*Opuntia engelmannii*	Caref 1	Algeria

DNA extraction

Genomic DNA was extracted from the frozen cladodes following the procedures described by Dellaporta et al. (1984), with some modifications because of problems arising from the interference of mucilage with DNA. DNA quality was estimated on a 0.8% agarose gel and

DNA quantity was determined spectrophotometrically by measuring absorbance at 260 nm.

Primers and polymerase chain reaction (PCR) amplification

RAMPO is a PCR-based technique that combines the advantages of inter-simple sequence repeat (ISSR) and RAPD analysis as described by Chatti et al. (2007) and Rhouma et al. (2008). The nucleotide sequences of ISSR and RAPD primers used in the present study are listed in Table 2. RAPD-PCRs were performed in a 25-µL volume reaction containing 20 ng DNA template (1.5 µL), 50 pM primer (1 µL), 2.5 µL Taq DNA polymerase buffer, 1.5 U *Taq* DNA polymerase (QBIOgène, Illkirch, France), and 200 mM of each dNTP (DNA polymerization mix; Pharmacia). PCRs were conducted in a DNA thermocycler (Biometra, Göttingen, Germany) and performed for 5 min at 94°C for initial denaturation, followed by 35 cycles for 30 s at 94°C, 1 min at 35°C, and 1 min at 72°C, with a final extension for 5 min at 72°C. ISSR-PCR amplifications were performed in a total volume of 25 µL containing 2 µL RAPD-PCR product, 120 pg ISSR primers (2 µL), 200 µM of each dNTP, 2.5 µL 10 Taq DNA polymerase buffer, and 1.5 U *Taq* DNA polymerase. PCRs were monitored under the same conditions as RAPD, with appropriate hybridization temperatures for each ISSR primer. Sixteen primer combinations were used in the present study. For each combination, 2 independent RAMPO reactions were performed for each DNA sample to ensure the reproducibility of the generated banding patterns. Reaction products were separated by 1.5% agarose gel electrophoresis containing ethidium bromide. The sizes of the amplified fragments were estimated by comparison with a 1-kb ladder loaded simultaneously with the amplified products (Sambrook et al., 1989).

Table 2. Characteristics of random amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR) primers used in this study.

Primer	Label	Sequence (5'-3')	Tm (°C)
RAPD	OPA-03	AGTCAGCCAC	35
	OPA-06	GGTCCCTGAC	35
	OPM-20	AGGTCTTGGG	35
	OPN-11	TCGCCGCAAA	35
ISSR	ISSR1	$(AG)_{10}G$	60
	ISSR2	$(AG)_{10}T$	57
	ISSR3	$(CT)_{10}A$	57
	ISSR4	$(CT)_{10}G$	60

Statistical analysis

Reproducible and clear bands were scored as either present (1) or absent (0) to create a binary matrix. For each primer, the total number of bands and the percentage of polymorphic bands (PPB) were calculated. The ability of the most informative primers to differentiate between accessions was assessed by estimating their resolving power (R*p*) (Prevost and Wilkinson, 1999). R*p* = ΣIb, where Ib = 1 - (2 x |0.5 - p|), where *p* is the proportion of accessions containing the I band. Furthermore, the discriminating power of the derived markers was calculated by estimating the polymorphic information content (PIC), using the following formula: PIC = 1 - $\sum fi$, where *f*i is the frequency of the ith allele (Lynch and Walsh, 1998). In addition, the marker index (MI), which is used to provide a suitable estimate of marker utility

(Powell et al., 1996), was calculated using the following formula: MI = PIC x n x β, where β is the fraction of polymorphic markers and is estimated after considering the polymorphic loci (n_p) and non-polymorphic loci (n_{np}) as $\beta = n_p / (n_p + n_{np})$. The multiplex ratio (n) is the average number of DNA fragments amplified/detected per genotype using a marker system.

Cluster analysis

Nei and Li's (1979) genetic distances between pairs of accessions were calculated and used to construct an unweighted pair group method with arithmetic mean (UPGMA) dendrogram. The reliability of the nodes of the tree was tested by bootstrap analysis with 1000 replicates. All analyses were carried out using the Free-Tree software (Pavlicek et al., 1999). The Tree View program was used to draw a phylogenetic dendrogram from the obtained tree file. Variation among cultivars was also estimated by principal component analysis using the XLSTAT program (AddinSoft, Paris, France).

RESULTS

Efficiency of the primer combinations

For all cactus cultivars, 16 primer combinations (RAPD primers x ISSR primers) were tested. Except for the OPA-06 x ISSR3 primer combination, 15 primer combinations generated clear and reproducible RAMPO profiles (Table 3). A total of 115 reproducible RAMPO fragments were resolved and 72 bands were polymorphic. The number of bands varied from 3 (OPN-1 x ISSR3) to 8 (OPM-20 x ISSR1), with a mean of 5.14 bands per primer combination. The PPB for all accessions varied from 42.85% (OPA-03 x ISSR1) to 100% (OPA-06 x ISSR4), with an average of 67.52% (Table 3).

Table 3. List of random amplified microsatellite polymorphism (RAMPO) primer combinations used for detecting the genetic diversity of *Opuntia* accessions.

Primer combination	Bands number		PPB	Rp	PIC	MI
	Total	Polymorphic				
OPA-06 x ISSR1	8	7	87.50	2.96	0.93	6.23
OPA-06 x ISSR2	7	5	71.42	3.68	0.81	5.14
OPA-06 x ISSR4	4	4	100.0	5.68	0.94	7.20
OPA-03 x ISSR1	7	3	42.85	1.12	0.91	2.98
OPA-03 x ISSR2	8	6	75.00	3.60	0.83	4.76
OPA-03 x ISSR3	6	4	66.66	1.44	0.78	4.70
OPA-03 x ISSR4	9	6	66.66	2.32	0.92	4.65
OPM-20 x ISSR1	11	8	72.72	1.12	0.79	5.12
OPM-20 x ISSR2	9	6	66.66	4.24	0.92	4.65
OPM-20 x ISSR3	10	7	70.00	0.88	0.98	4.93
OPM-20 x ISSR4	9	4	44.44	2.80	0.89	2.99
OPN-01 x ISSR1	7	5	71.42	4.8	0.68	4.97
OPN-01 x ISSR2	8	4	50.00	2.8	0.64	3.52
OPN-01 x ISSR3	5	3	60.00	1.44	0.21	3.15
OPN-01 x ISSR4	7	0	-	-	-	-
OPA-06 x ISSR3	Smear	-	-	-	-	-
Total	115	72	-	-	-	-
Average	7.66	5.14	67.52	2.77	0.80	4.64

Percentage of polymorphic bands (PPB), resolving power (R*p*), polymorphism information content (PIC), and marker index (MI) of the primers tested.

Based on these results, the tested primers were sufficiently powerful to detect DNA polymorphisms in *Opuntia* crops. This is strongly supported by the high values for R*p*, which varied from 1.12 (OPA-03 x ISSR1, OPM-20 x ISSR1) to 5.68 (OPA-06 x ISSR4), with a mean of 2.77 (Table 3). Moreover, as shown in Figure 1, PIC values varied from 0.21-0.94, with a mean of 0.81. In contrast, 63 of the 72 RAMPOs exhibited high PIC values (from 0.8-0.9). The MI for individual primer combinations were recorded, the overall MI values ranged from 2.98 (OPA-03 x ISSR1) to 7.2 (OPA-06 x ISSR4), with an average of 4.7 per primer combination (Table 3). These results showed that the (OPM-06 x ISSR4) primer combination was the most appropriate for examining genetic polymorphisms in *Opuntia* crops as it showed the highest values for PPB (100%), R*p* (5.68), and MI (7.2). Thus, a large amount of genetic diversity at the DNA level characterizes the Tunisian *Opuntia* germplasm. Our data suggested that RAMPO is an efficient and informative procedure for determining the genetic diversity of *Opuntia* species as well as discriminating between *Opuntia* genotypes.

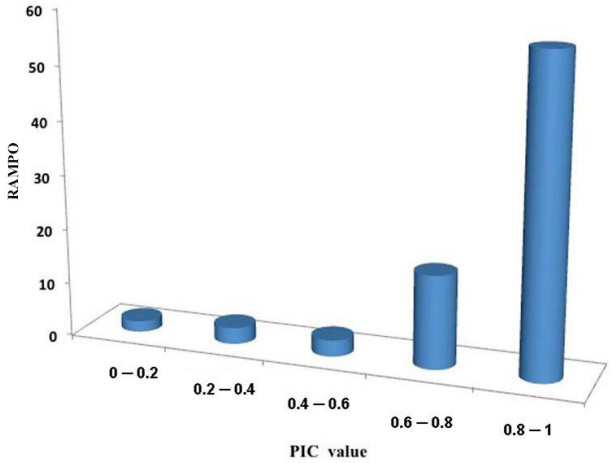

Figure 1. Distribution of the polymorphic information content (PIC) data obtained using random amplified microsatellite polymorphisms (RAMPO) markers.

Genetic diversity and phylogenic relationships

Based on the Nei and Li's formula (1979), the resulting genetic matrix exhibited values ranging from 0.04-0.83, with a mean of 0.43, and showed a relatively high degree of genetic diversity in the collection studied (Table 3). The lowest distance (0.04) was observed between *O. ficus indica* Bianca-Sicily and *O. robusta*, suggesting that they were very similar at the DNA level. The highest distance (0.83) was estimated between the *O. ficus indica* Thala-Tunisia and *O. ellisiana* accessions. This result suggested the presence of a high level of genetic divergence between the species examined. All remaining genotypes displayed intermediate levels of similarity. The UPGMA dendrogram, based on the pairwise genetic distance values, showed 2 groups of accessions (Figure 2). The first group (A) could be subdivided into 2 sub-clusters; the first (A1) included 7 *O. ficus indica* cultivars such as *O. ficus indica* Thala-Tunisia, *O. ficus indica* El Bouroug-Morocco, *O. ficus indica* Ethiopia-Ethiopie, and only 1 different species represented by *O. engelmannii*, while the second subcluster (A2) regrouped

the remaining cultivars such as *O. robusta*, *O. undulata*, *O. ellisiana*, *O. streptacantha*, and *O. ficus indica* Bab Toza-Morocco. The second group (B) was represented by *O. ficus indica* Leavis-New Mexico. As illustrated by the UPGMA dendrogram, clusters were independent of the geographical origin of the studied *Opuntia* species, suggesting that a common genetic basis characterizes these genotypes despite their phenotypic divergence. Thus, no genotype groups were assigned to any particular region. To evaluate genetic differentiation among accessions, RAMPO data were computed to perform a principal component analysis (Figure 3). The three principal component analysis axes accounted for simultaneously 66.01, 20.48, and 13.51% of the observed variation. The most important variables integrated positively by the first axis were bands generated using the primer combinations OPM-20 x ISSR1 and OPN-01 x ISSR1 and negatively using markers generated by the OPN-01 x ISSR2 combination. The second axis was defined positively by bands amplified by OPA-03 x ISSR1 and OPA-03 x ISSR4 and negatively by the OPA-06 x ISSR4 combinations.

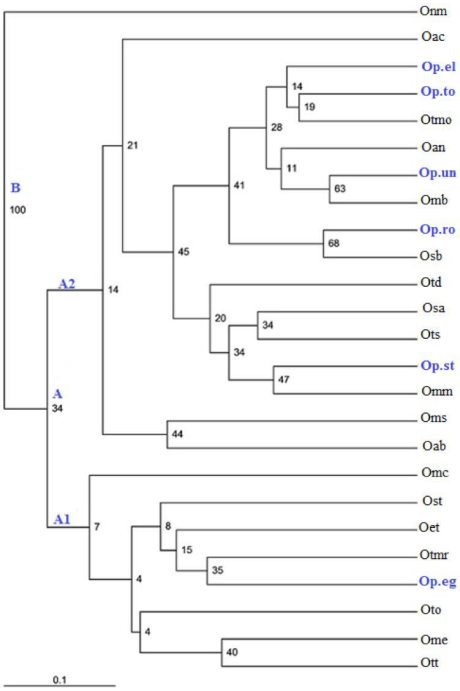

Figure 2. Cluster analysis of the 25 *Opuntia* accessions constructed by UPGMA dendrogram using Nei and Li's genetic distances and based on 115 RAMPOs. Variation among the *Opuntia* accessions was assessed after 1000 permutation bootstrap analysis (Table 1 for ecotype codes).

The plot obtained according to axes 1 and 2 revealed 3 clusters of accessions (Figure 3). The first cluster was represented by *O. streptacantha*, *O. robusta* (Mexico), and *O. ficus indica* from Sicily and Morocco, while the second cluster was represented by *O. undulata*, *O. ellisiana*, *O. tomentosa*, and heterogene group of *O. ficus indica* cultivars from Tunisia and Morocco. The third was well represented by *O. ficus indica* cultivars from South Africa, Sicily, Algeria, Morocco, Tunisia, Ethiopia, and New Mexico with *O. engelmannii* from Algeria.

These results reveal the dispersion of the cultivars and suggest that substantial genetic differentiation exists among them. The distribution occurred independently of their geographic origin, confirming the results of cluster analysis. This was also confirmed by the high bootstrap values calculated.

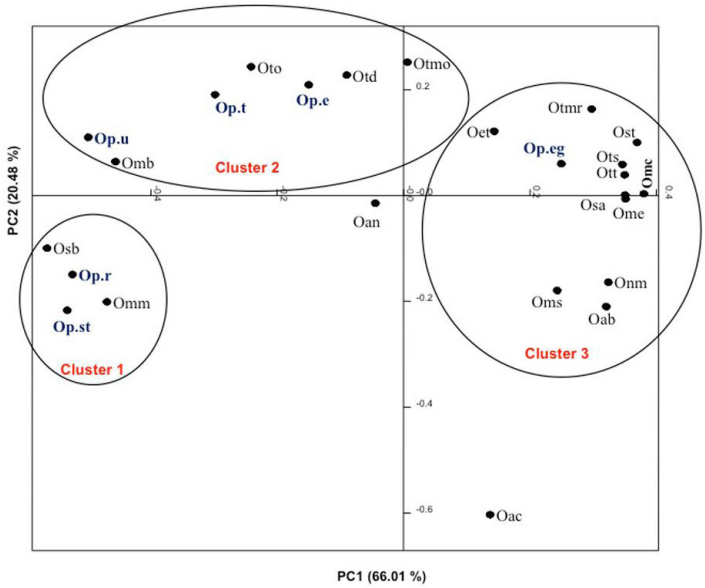

Figure 3. Principal component (PC) analysis of traits based on 3 clusters of 25 accessions of *Opuntia* spp. The distribution of accessions on the first 2 component scores was in agreement with cluster analysis and based on 115 RAMPOs (Table 1 for ecotype codes).

DISCUSSION

In this study, we used the RAMPO procedure to generate new molecular markers that were suitable for assessing the genetic diversity and structure of *Opuntia* species. The primers tested in this study were used to amplify a total of 72 polymorphic bands over 115 generated bands. These primers are characterized by high PIC and R*p* rates as well as by PPB, but with lower values than those described by Chatti et al. (2007) and Rhouma et al. (2008) for figs and date palm cultivars, respectively. For figs, resources the 16 used primer combinations generated 63 RAMPO markers with a PPB of 45.65% and a collective rate of 26.96 for R*p* parameters as described by Chatti et al. (2007). Rhouma et al. (2008) reported the ability of 18 primer combinations to produce 186 reproducible bands scored as RAMPO markers with 88.57% of polymorphic bands and an R*p* rate of 4.06 for the date palm crop. Therefore, compared with data previously reported for *Opuntia* species (Labra et al., 2003; Griffith., 2004; Zoghlami et al., 2007; Helsen et al., 2009; Snoussi Trifa et al., 2009; Caruso et al., 2010), the designed procedure was used to detect the highest level of polymorphism in these species. Nagaty and Rifaat (2012) suggested that the RAPD markers correlated with biochemical and morphological traits to characterize 2 red and yellow prickly pear cultivars.

Here, cluster and multivariate analyses illustrated a common genetic basis for characterizing *Opuntia* genotypes despite their phenotypic divergence. The level of DNA variation among the 7 species from different countries revealed that even at this early stage of domestication, there might already have been a considerable genetic bottleneck in the gene pool of fruit cacti. Ours results suggest the polyphyly of *O. ficus indica*. In fact, *O. ficus indica* accessions did not cluster separately from the other cactus pear species, indicating that their current taxonomical classifications do not fit with their genetic variability. The species concept of this tree may consist of multiple unique clones derived from various parental stocks as suggested by Griffith (2004). The taxonomic statute of *O. ficus indica* may define a group of convergent cultivars derived from different parental species (Griffith, 2004) caused by hybridization, which is well documented for the opuntioid cacti. Hence, application of this designed method would be useful for characterizing the local *Opuntia* germplasm and refining classification obtained using internal-transcribed sequences (Griffith, 2004; de Lyra et al., 2013). Substantial genetic divergence and differentiation among accessions were observed. The genetic similarity between *O. engelmannii* and *O. ficus indica* accessions from Tunisia suggests that *O. engelmannii* cannot be considered a different species but supports that it represents the domesticated spined form of *O.ficus indica* as suggested by Snoussi Trifa et al. (2009). A similar conclusion was reached by Labra et al. (2003), who found that *O. ficus indica* could be considered to be a domesticated form of *O. megacantha*. This agrees with the results of Griffith (2004), who suggested that Barbary fig (*O. ficus indica*) is one of several long-domesticated cactus species (Casas and Barbera, 2002) in central Mexico and diffused throughout several warm regions of the world by European travelers beginning in the late 15th century. As reported by Britton and Rose (1919), *Opuntia* species can be grouped in a total of 29 series defined by their morphological structure, i.e., stems, joints, plant branching, epidermis, areoles, spines, flowers, and fruits. The present study revealed high similarity levels between *O. ficus indica* and *O. undulata*, and the classification and groupings for these 2 species were confirmed in the same series of *O. ficus indica* based on their common morphological traits (Labra et al., 2003). As suggested by Labra et al. (2003), high genetic similarity was detected between *O. ficus indica* and *O. undulata* based on amplified fragment length polymorphism analysis. The high genetic similarity between *O. ficus indica* Bianca-Sicily and *O. robusta* (Mexico) and the low level of divergence between these cultivars and other closely related species such as *O. streptacantha*, *O. undulata*, *O. tomentosa*, and *O. ellisiana* were in accordance with the results of Labra et al. (2003), by using the chloroplast simple sequence repeat technique to study the genetic diversity between *O. ficus indica*, *O. robusta*, and other unclassified genotypes and reported high similarity between these accessions. In addition, our data demonstrated that, typically, continuous genetic diversity characterizes the *Opuntia* accessions and the topology of the derived UPGMA dendrogram strongly supported this assumption. In fact, genotypes were clustered independently either from their geographical origin, suggesting a narrow genetic basis among the ecotypes studied despite their phenotypic distinctiveness. The large diffusion of *Opuntia* outside their native area has allowed the conservation of their original large genetic variability and the development of new variability, resulting from adaptations to new environments. Similarly, Wang et al. (1998) used RAPD markers, morphological traits, and physiological parameters and found the same results, and did not differentiate cactus accessions with reference to their geographic origin. Thus, the application of the RAMPO method is of great interest for local *Opuntia* germplasm characterization.

CONCLUSIONS

In this study, we report the analysis of genetic diversity within a set of 30 *Opuntia* species using RAMPO markers. Our goal was to develop reliable molecular markers for exploring genetic diversity and establishing phylogenetic relationships in a set of Tunisian cactus collection germplasm. We combined the RAPD and ISSR procedures to develop RAMPO markers; studies based on RAMPO technique and the sustainability of this method for surveying genetic diversity in figs (Chatti et al., 2007) and the date palm (Rhouma et al., 2008; Rhouma-Chatti et al., 2011) have been reported previously. Importantly, this method has been used in other plant species and has been used to examine the DNA in various cultivated crops (Richardson et al., 1995; Ramser et al., 1997; Udupa et al., 1998).

This is the first study to apply RAMPO markers in the assessment of genetic diversity of *Opuntia* species. Our results indicate that the level of polymorphism among cactus species is appreciably high and that the RAMPO procedure constitutes a useful approach for characterizing germplasm molecular polymorphisms and may be useful for accelerating the transfer of economically important traits from wild germplasms to cultivated *Opuntia* species through marker-assisted selection. This method can also be used to verify the hybrid statute among cacti species. The experiment presented herein demonstrates the potential usefulness of RAMPO for classifying cactus accessions and for determining relationships among species. Further studies will be carried out to better define the genetic relationships among and within *Opuntia* species and cultivars. The use of different molecular tools can be used to analyze cactus pear genetic diversity for different purposes, such as variety selection and genotype identification and certification. Moreover, the establishment of coordinated conservation actions of cactus pear genetic resources can reduce the risk of genetic erosion in natural and cultivated populations through an understanding of available genetic variability. A larger number of primer combinations and/or the ecotypes should be used to gain deeper insight into the genetic diversity of this crop. Thus, the genetic diversity of cactus pear should be evaluated to provide information that can be used for crop improvement strategies and to determine whether to increase the Tunisian cactus gene pool. Studies are currently underway for the molecular characterization and rational conservation of Tunisian landraces.

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