# Genetic diversity in *Opuntia spp*. cultivated for forage production

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## ABSTRACT

*Opuntia* genus belongs to the *Cactaceae* and includes a range of species from 75 to 250 originated from the Americas. Several *Opuntia* species represent useful sources of forage in many arid areas, and their cultivation is drastically increasing. However the taxonomic classification of these species is complicated by the lack of reliable morphological descriptors, the relevant phenotypic plasticity, and the frequency of natural hybridization and of polyploidy. In this study 31 genotypes selected for forage production including *Opuntia ficus-indica* and some related species were analyzed using SSR markers to establish their genetic variability and to elucidate phylogenetic relationships among cultivated genotypes and related species. The analysis of six loci allowed to univocally identify most of the accessions and confirmed the fact that their taxonomical classification is not congruent with the observed patterns of genetic diversity. Most of the accessions selected for forage production were grouped exhibiting a narrow genetic variation level and clustered separately from two reference *O. ficus-indica* genotypes, used for fruit production in Mediterranean area. NeighbourNet analysis displayed a low level of diversification among the forage Opuntia from Brazil suggesting they probably have common ancestors. This information will be useful to plan future breeding strategies aimed at the selection of improved genotypes to be cultivated in different areas.

***Keywords*:** cactus pear, breeding, cladodes, phylogenetic relationships, SSR markers.

## INTRODUCTION

*Opuntia* genus belongs to the *Cactaceae* and includes a wide number of species ranging from 75 (Hunt *et al*. 2006) to 250 (Britton and Rose, 1963) according to different classification criteria. These species originated from the Americas. The taxonomic classification of the genus is drastically complicated by the reduced number of reliable morphological descriptors, the relevant phenotypic plasticity, the high frequency of polyploidy and the intra and inter-generic

hybridization (Scheinvar, 1995; Wallace and Gibson, 2002; Caruso *et al*. 2010; Majure *et al*. 2012).

Several *Opuntias* have been cultivated since centuries and they have gained popularity for fruit and forage production but also for ornamental purposes and derivate products for cosmetic and medical uses (Mondragón-Jacobo, 2001). Most of the varieties used for fruit production, in native lands as well as in other productive areas, belong to the species *Opuntia ficus-indica* (L. Mill.) and are known with different common names (Caruso *et al*. 2010).

Forage derived from *Opuntia spp* is an important source of food to feed cattle, sheep, goat, particularly in dry seasons (Mulas and Mulas, 2004; Nefzaoui, 2010; Beccaro *et al*. 2015) and in semi-arid environments. The cladodes can be used either as fresh, or stored as silage before their usage (Castra *et al*. 1977). The cultivation for forage production is increasing, reaching 2.6 million hectares cultivated in the world. Brazil and Tunisia (with 600,000 ha, respectively), followed by South Africa (525,000 ha), Ethiopia (355,000 ha), Mexico (230,000 ha) and Algeria (150,000 ha) are the greatest users of cactus for forage or fodder (Grünwaldt *et al*. 2015 and articles cited therein) and an increasing interest is reported for many other countries (FAO, 2013).

*Opuntia* genotypes used for forage production are referred to a number of different species. In particular, Felker *et al*. (2006) refer to about 200 spiny and spineless accessions belonging to 17 species. Among those, the most important forage clones identified by USDA belong to *O. ficus-indica*, *O. robusta*, *O. cochinillifera*, *O. ellisiana* and *O. engelmannii* species cultivated in Brazil, South Africa and USA. De Lyra *et al*. (2015) analyzed 28 accessions from a collection of the germplasm bank of the Agronomic Institute of Pernambuco by ribomosal RNA ITS markers, and separated the accessions according to their classified botanically as *Nopalea cochenillifera*, *O. robusta* and *O. ficus-indica*.

The absence of clear morphological descriptors for each species, and high frequency of natural hybridization have led for *Opuntias* to a controversial taxonomic identification that is still debated. During the last years, a number of studies have been performed using various random and specific molecular markers including RAPDs, nrITS, AFLP, ISSR, and SSR, for genetic diversity studies, cultivar identification, phylogenesis analysis, etc. (Wang *et al*. 1998; Labra *et al*. 2003; Mondragón-Jacobo, 2003; Griffith, 2004; Zoghlami *et al*. 2007; Luna-Paez *et al*. 2007; García-Zambrano *et al*. 2009; Souto Alves *et al*. 2009; Bendhifi-Zarroug *et al*. 2013 and 2015; Ganopoulos *et al*. 2015; Realini *et al*. 2015; Valadez-Moctezuma *et al*. 2015; Samah *et al*. 2016).

In the present study, forage prickly pears belonging to the Brazilian germplasm and to the germplasm collection of Catania University (Department of Agriculture, Food and Environment), Italy, were analyzed using SSR markers to establish the genetic variability among selected genotypes used for forage production and to elucidate phylogenetic relationships among cultivated genotypes and related species.

## MATERIALS AND METHODS

### *Plant material, morphological analysis and DNA extraction*

Twenty-three cactus pear genotypes were obtained from the Instituto Agronômico de Pernambuco (IPA), in Brazil. Eight genotypes belonging to seven *Opuntia* genotypes belonging to six related species were used as reference and collected from the experimental farm of Catania University (Table 1). In this work we analyzed cultivars and selections of *O. ficus-indica* and other related species mainly used for forage production. Morphological traits were visually estimated by recording cladodes shape and spinescence. DNA was isolated from young cladodes following the protocol described in Caruso *et al*. (2010).

### *Microsatellite analysis*

In this study, eight *Opuntia*-specific microsatellite primers, described in Caruso *et al*. (2010) were used. Polymerase chain reaction and capillary electrophoresis analysis were performed according to the protocols described in Las Casas *et al*. (2014).

### *Genetic distance and clustering*

The different alleles were considered as dominant markers, and a 0/1 matrix was then created. An unweighted Neighbor-joining (NJ) tree (Saitou and Nei, 1987) was constructed as described in Caruso *et al*. (2010), using the Dice coefficient and the Dissimilarity Analysis and Representation for Windows (DAR-win5) software version 5.0; 1,000 bootstraps analysis was performed for branches robustness test. In addition SplitsTree version 4.11.3 (Huson and Bryant, 2006) was used to perform a NeighbourNet (NN) analysis and for generating a network (Bryant and Moulton, 2004).

## RESULTS

Twenty-three cactus-pear genotypes from IPA collection field and seven genotypes belonging to six related species were analyzed using eight SSRs. According to preliminary morphological characterization, 19 out of the 23 Brazilian genotypes were with cladodes of big size (data not shown), of variable shape and spineless (Table 1), confirming their aptitude for forage production. Capillary electrophoresis analysis produced clear genotyping profiles for six SSRs (Table 2) while two primer pairs (Opuntia12 and Opuntia9) were discarded from further analyses due to their low amplification rate. In total, we detected 82 alleles and 19 unique alleles in the investigated population.

The number of alleles and unique alleles for genotype ranged, respectively, from 1 to 8 and from 0 to 3 (Table 1); an average value of 13.7 alleles for each marker was observed (Table 2). In such a condition it was hard to define the status of each SSR (if single or multi-locus). However we found as the different ploidy level of the species influenced the number of peaks for each SSR as already reported by Caruso *et al*. (2010). Nine genotypes revealed the presence of unique alleles. The identified genotypes ranged from 8 (Opuntia3 and Opuntia5)

**Table 1.** Genotypes of *Opuntias* and related genera used for the SSR-based analysis.

| | Taxonomic classification* | Spines | Cladode shape | No. of alleles | Average no. of alleles | No. of unique alleles |
|---|---|---|---|---|---|---|
| Orelha de elefante Mexicana | *O. stricta* - (*O. ficus-indica*) | Many | Ovate | 1 – 3 | 2.2 | 2 |
| Orelha de elefante Africana | *O. undulata* | Absent | Ovate | 2 – 8 | 4.3 | 0 |
| IPA-Sertânia | *N. cochenillifera* | Absent | Ovate | 1 – 3 | 1.8 | 0 |
| Orelha de onça | *O. ficus-indica* (*N. cochenillifera*) | Absent | Ovate | 1 – 3 | 1.8 | 0 |
| Gigante | *O. ficus-indica* (*O. robusta*) | Absent | Ovate | 2 – 6 | 3.8 | 0 |
| Clone IPA-20 | *O. ficus-indica* | Intermediate | Ovate | 2 – 7 | 4.0 | 0 |
| Palma F8 | *O. atropes*- (*O. robusta*) | Many | Elliptic | 2 – 5 | 3.5 | 0 |
| Palma Redonda | *O. ficus-indica* | Many | Round | 2 – 7 | 4.0 | 0 |
| Copena F1 | *O .ficus-indica* (*O. robusta*) | Absent | Elliptic | 2 – 8 | 4.3 | 1 |
| Copena V1 | *O. ficus-indica - O. robusta* | Few | Ovate | 2 – 4 | 3.2 | 3 |
| IPA-90-18 | | Few | Ovate | 1 – 8 | 4.0 | 1 |
| IPA-90-92 | (*N. cochenillifera*) | Few | Ovate | 2 – 7 | 4.2 | 0 |
| México vegetable 1294 | | Absent | Elliptic | 2 – 8 | 4.2 | 0 |
| Additional cv. 1258 | | Few | Elliptic | 1 – 3 | 1.4 | 2 |
| México Fodder/1278 | *N. cochenillifera* | Absent | Elliptic | 2 – 5 | 3.4 | 0 |
| IPA 90-156 | | Absent | Ovate | 2 – 8 | 4.7 | 0 |
| Marmillon Fodder-1327 | *O. ficus-indica* (*N. cochenillifera*) | Absent | Ovate | 2 – 8 | 4.5 | 0 |
| IPA 90-115 | | Few | Ovate | 2 – 8 | 4.7 | 0 |
| IPA 90-73 | | Few | Ovate | 2 – 8 | 4.5 | 0 |
| IPA 90-111 | | Absent | Ovate | 2 – 8 | 4.6 | 0 |
| V-19 | | Absent | Round | 1 – 4 | 2.2 | 3 |
| Jalpa | *O. ficus-indica* | Absent | Ovate | 3 – 8 | 6.0 | 2 |
| Mão de Moça | *N. cochenillifera*** | Absent | Ovate | 1 – 4 | 2.2 | 0 |
| *O. ficus-indica 1* | *O. ficus-indica* | Absent | Ovate | 1 – 7 | 3.2 | 0 |
| *O. ficus-indica* 2 | *O. ficus-indica* | Absent | Ovate | 1 – 6 | 3.0 | 0 |
| *O. oligacantha* | *O. oligacantha* | Many | Ovate | 1 – 5 | 2.5 | 0 |
| *O. streptacantha* | *O. streptacantha* | Few | Ovate | 2 – 8 | 4.4 | 0 |
| *O. elizondoana 29* | *O. elizondoana* | Many | Ovate | 1 – 7 | 3.8 | 2 |
| *O. spinulifera* | *O. spinulifera* | Many | Ovate | 1 – 5 | 3.2 | 3 |
| *O. vulgaris* | *O. vulgaris* | Absent | Ovate | 1 – 5 | 3.0 | 0 |
| *O. joconostle* | *O. joconostle* | Intermediate | Ovate | 1 – 5 | 2.8 | 0 |

* The classification in brackets is the one recently proposed by de Lyra *et al*. (2015), diverging from all other hypotheses.
** (originated from IPA-Sertania).

to 19 (Opuntia9). The mean of PIC values of the six SSRs was 0.82, ranging from 0.74 to 0.91 (Table 2).

**Table 2**. SSR primers used for the molecular analysis of the *Opuntia* genotypes.

| Primer* | Core | No. of alleles | PIC value | No. of unique alleles** | No. of identified genotypes |
|---|---|---|---|---|---|
| Opuntia3[a] | $(AG)_{19}$ | 6 | 0.78 | 0 | 8 |
| Opuntia5[a] | $(TA)_5$ | 6 | 0.74 | 1 | 8 |
| Opuntia9[a] | $(AG)_{15}$ | 19 | 0.91 | 4 | 19 |
| Opuntia11[a] | $(CT)_{13}TT(CT)_2$ | 10 | 0.81 | 4 | 15 |
| Opuntia13[a] | $(AG)_{12}$ | 25 | 0.81 | 7 | 16 |
| Ops.24[b] | $(CT)_{24}$ | 16 | 0.90 | 3 | 14 |
| Average | | 13.7 | 0.82 | 3.17 | |

*M13F sequence was added at the 5' end of each forward primer.
**Private alleles obtained from each primer.
[a] Helsen *et al*. (2007).
[b] Caruso *et al*. (2010).

Among the analyzed SSRs, Opuntia9 and Opuntia13 were the most polymorphic, revealing 19 and 25 alleles and four and seven unique alleles, respectively, allowing the identification of 19 and 16 genotypes; their PIC values were of 0.91 and 0.81, respectively.

The least polymorphic markers were Opuntia3 and Opuntia5, with 6 alleles, and 0 and 1 unique alleles respectively, and with PIC values of 0.78 and 0.74. The most and the least polymorphic analyzed genotypes were respectively 'Jalpa' with 6 alleles and 2 unique alleles, and 'Addicional cv. 1258' with 1.4 alleles and 2 unique alleles on average. The 6 SSRs exhibited a high level of poliymorphism and allowed to discriminate the analyzed genotypes with a few exceptions.

The genetic distance among the analyzed *Opuntias* was calculated using the Dice coefficient. In the dendrogram based on Neighbor-joining (Figure 1), two main clusters are present (A and B), plus a genotype ('Jalpa') which was not included in any cluster. Cluster A included three genotypes belonging to *Nopalea cochenillifera* ('Orelha de Onça' and 'IPA-Sertania', undistinguished, and 'Mão de Moça') and the genotypes 'Additional cv. 1258' and 'V19', both of unknown origin and classification. Cluster A also included 11 accessions belonging to the seven different species we used as reference (*O. ficus-indica*, *O. oligacantha*, *O. streptacantha*, *O. elizondoana*, *O. spinulifera*, *O. vulgaris* and *O. joconostle*). Also the genotypes 'Orelha de Elephante Mexicana', ascribed to the species *O. stricta*, 'Palma F8' ascribed to the species *O. atropes,* and 'Copena V1' were clustered in this group.

Cluster B is composed of 14 genotypes including most of the IPA selections. Few genotypes belonging to this cluster were not discriminated. In particular, the following accessions showed the same allelic profiles: 'Palma redonda' and 'Gigante'; 'Marmillon fodder 1327' and 'IPA 90-73'; 'IPA 90115 and 'IPA 90-156'. The recorded bootstrap values showed the reliability of the obtained clusters.

A NeighborNet (NN, Figure 2) network analysis was also performed in order to ascertain variability patterns and reticulate evolution occurring in *Opuntia*. Most of the clusters found in NJ dendrogram were also evidenced in NN analysis where many accessions selected in Brazil clustered together, whereas the related species included as references segregated and displayed a wider range of variability.

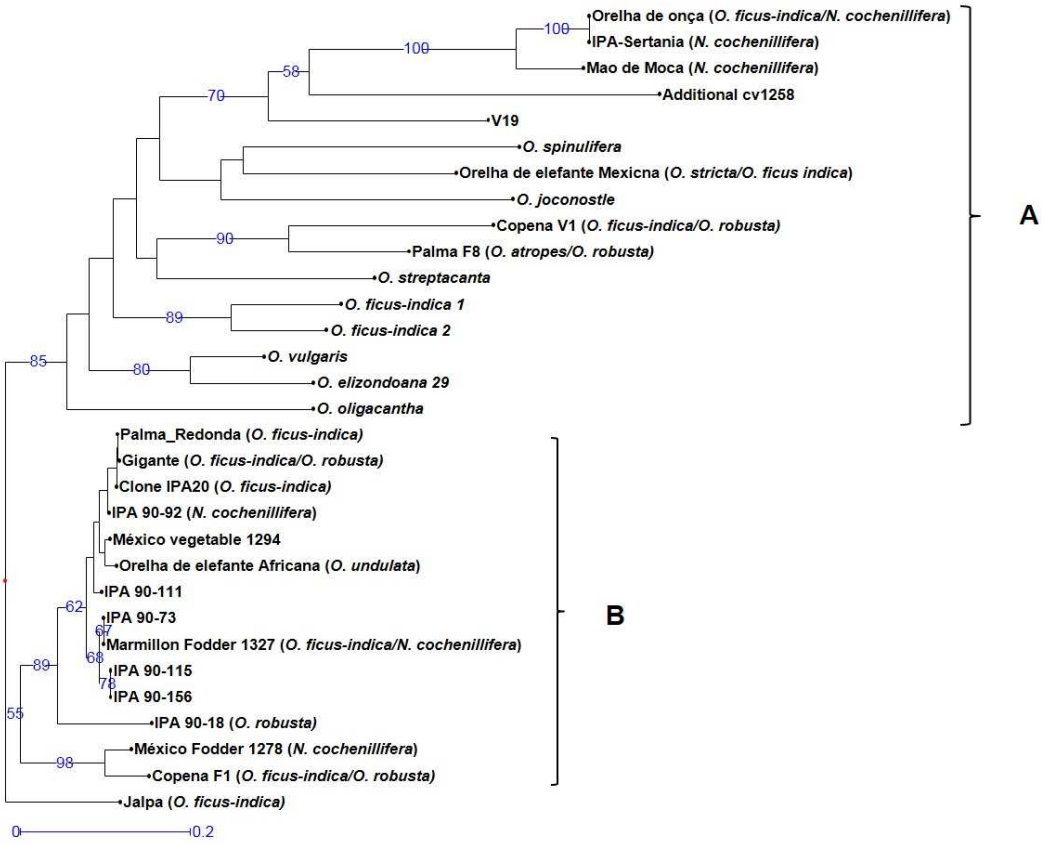


**Figure 1.** Unweighted Neighbor-Joining tree based on the Dice distance. The numbers at the branch points indicate bootstrap support values >50% (1,000 replicates).

## DISCUSSION

Several *Opuntia* species represent important sources of forage and fodder for livestock in many dry areas of the world, and their cultivation is drastically increasing especially in Brazil and Mexico, but also in western Asia and northern and southern Africa (Samah *et al*. 2016). Therefore, it is necessary to understand the pattern of genetic diversity among the best performing cultivated accessions, and characterize them at molecular level before their large scale propagation. Morphological analysis allows to identify species and varieties according to the main important agronomic traits (cladode size and spines absence). Spine absence/presence has been considered a relevant trait for phenotypic characterization and for the agronomic evaluation of varieties for forage production. Lack of thorns is a valuable trait

both for varieties used for forage and fruit production, but it shows a great variability between mother plants and their progenies (Nieddu *et al*. 2006) probably due to genetic, epigenetic and environmental factors (Nieddu and Chessa, 1997; Labra *et al*. 2003). In the case of *Opuntias*, only few reliable morphological descriptors are available making it difficult the characterization and selection of different accessions to be used for different purposes. The availability of new DNA-based analyses has drastically changed the methods for characterization being the new molecular methods independent from environmental conditions and making it possible the attaining of univocal description of the plant material.

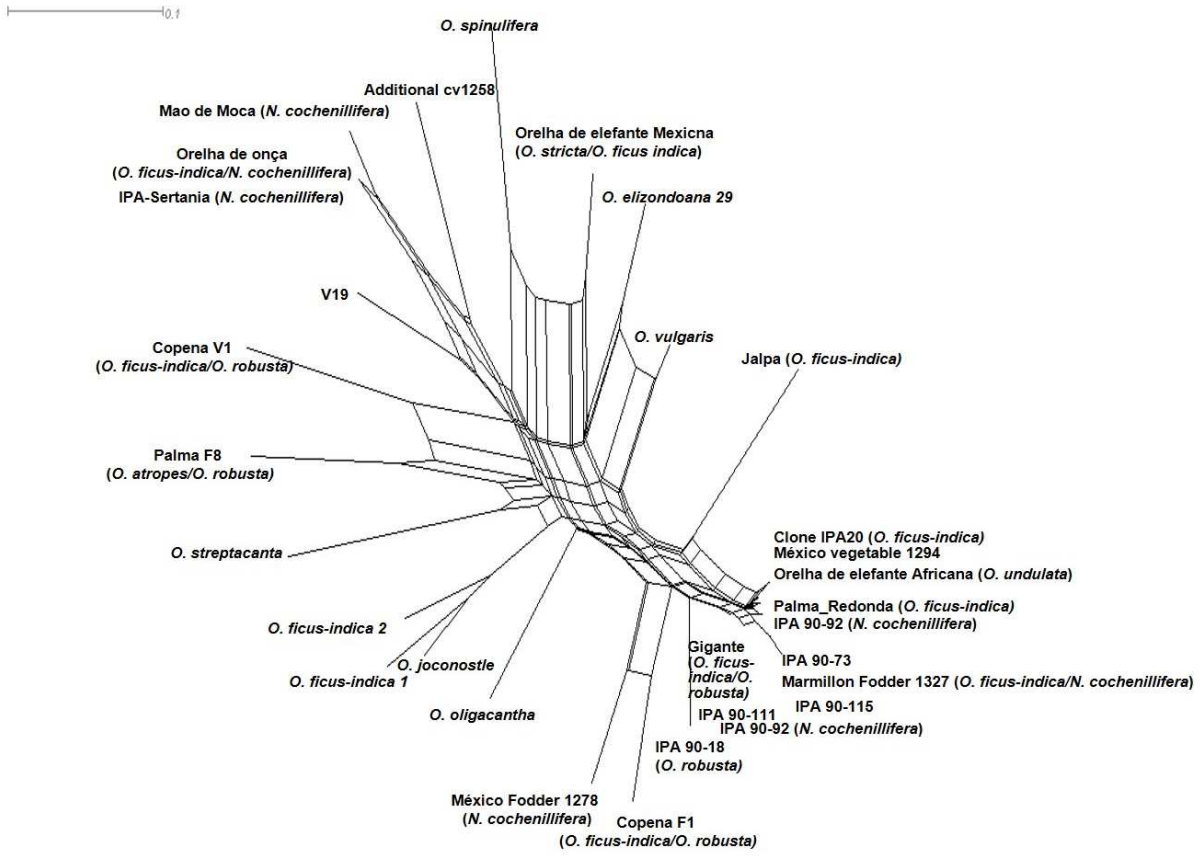


**Figure 2.** NeighborNet network of the 31 *Opuntia* genotypes based on Dice dissimilarity index.

In this work, we used microsatellite markers to estimate genetic differentiation within a group of genotypes selected for forage production in comparison with related species. On the whole, the analysis covered genotypes belonging to at least 10 different species. Most of the accessions studied in this work were not previously investigated at molecular level. The species included as references grouped together (cluster A). Regarding the genotypes classified as *Nopalea cochellinifera,* we found three of them ('IPA-Sertania', 'Orelha de Onça' and 'Mao de Moca') belonging to a specific subgroup of cluster A, while others ('IPA 9092', 'Mexico Fodder 1278' and 'Marmillon Fodder 1327') described as *N. cochellinifera* (de Lyra *et al*. 2015)

clustered with other *Opuntias*. This result confirms, as current taxonomical classification of these species is not congruent with the observed patterns of genetic diversity.

Most of the accessions selected in Brazil for forage production were grouped in cluster B. This cluster exhibited a narrow genetic variation level and comprises 3 pairs of undistinguished genotypes; namely 'Palma Redonda' was undistinguished from 'Gigante' whereas the latter was distinguished from the derived genotype 'Clone IPA20'; actually 'Palma Redonda' and 'Gigante' are morphologically distinct, at least for some cladodes characters. The other two undistinguished pairs ('IPA 9073' and 'Marmillon Fodder 1327'; 'IPA 90115' and 'IPA 90156') did not displayed clear morphological differences. As for uncharacterized genotypes, a higher number of markers would likely make their genetic discrimination possible.

Overall, the groups of NJ dendrogram are coherent with NN analysis. In NN many parallel edges are evident, especially for the *Opuntia* species used as reference; this result confirms those of Caruso *et al*. (2010) about the existence of incompatible splits among these *Opuntia* species. NeighbourNet analysis showed a low level of variation among the Brazilian selected genotypes, indicating that they probably have common ancestors. On the other hand, most of the forage *Opuntias* from Brazil clustered separately from two reference *O. ficus indica* genotypes (*O. ficus indica* 1 and *O. ficus indica* 2), used for fruit production in Mediterranean area, but at the same time having large and spineless cladodes.

## CONCLUSION

The present work contributed for the first time to shed light on the phylogenetic relationships of a wide group of accessions selected in Brazil for forage production. On the whole, these results can be useful for the setting up of conservation protocols of *Opuntia* and related genera genetic resources, based on their molecular characterization. The univocal identification of most of the analyzed accessions will be useful for planning future breeding strategies aimed at the selection of improved genotypes to be cultivated in different areas and for a reliable management of such an important source of variability within the *Opuntia* genus.

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