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ORIGINAL ARTICLE

Phylogenetic relationships in *Opuntia* (Cactaceae, Opuntioideae) from southern South America

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Abstract The patterns of relationships between species of *Opuntia* from southern South America are scarcely known in spite of the importance of this region as a diversification center for the Cactaceae. This paper contributes to the better understanding of the genetic and phylogenetic relationships of 15 *Opuntia* species from Argentina, Bolivia, Brazil, Paraguay, and Uruguay by generating new genetic data through Inter-Simple Sequence Repeat (ISSR) genotyping and the sequencing of plastid intergenic spacers *trn*L-*trn*F and *psb*J-*pet*A. The species surveyed are: *O. anacantha, O. arechavaletae, O. aurantiaca, O. bonaerensis, O. colubrina, O. discolor, O. elata, O. megapotamica, O. monacantha, O. penicilligera, O. quimilo, O. salmiana, O. schickendantzii, O. sulphurea,* and *O. ventanensis.* The genetic distance-based analysis of 110 ISSR bands, applying the Neighbor-Joining and NeighborNet algorithms, evidenced considerable intraspecific variation in *O. aurantiaca, O. elata, O. discolor,* and *O. salmiana.* The emergent clustering pattern and the species assignment to taxonomic series show a general agreement for *Armatae* and *Aurantiacae*. The phylogenetic relationships were investigated via haplotype network and maximum likelihood approaches, within a broader sampling that involves most species currently accepted for South America, and samples from throughout the American continent. Hence, 15 haplotypes are recognized for southern South American opuntias whereas eight haplotypes are established for Northern Hemisphere opuntias. Biparentally and maternally inherited genetic data yield partially consistent results, giving genetic support for morphologically defined taxonomic series.

Keywords Cacti · ISSR · Plastid intergenic spacers · Phylogenetic analyses

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Introduction

The cacti of the genus *Opuntia* Mill., commonly known as prickly pears or nopales, are native plants from the American continent, which usually show flattened stem segments or cladodes, and a number of adaptations for drought resistance. Since pre-Hispanic times, *Opuntia* species have had economic relevance throughout its distribution range. Some species are currently commercialized as forage, ornamentals and food source, while others have succeeded as exotic weeds (Anderson 2001).

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The number of species in *Opuntia* sensu stricto has been estimated in 250 (Britton and Rose 1919), 160 (Gibson and Nobel 1986), 181 (Anderson 2001), and lately in 75 (Hunt et al. 2006). The main reasons for such taxonomical conundrum are the paucity of diagnostic morphological characters, the high level of phenotypic plasticity, an alleged recent diversification and the prevalent occurrence of hybridization and introgression between sympatric and allopatric species (Benson and Walkington 1965; Grant and Grant 1971, 1979; Baker and Pinkava 1987, 1999; Griffith 2001a, b, 2004; Wallace and Gibson 2002; Pinkava 2002; Reyes-Agüero et al. 2006; del Castillo and Argueta 2009; Caruso et al. 2010).

Despite the importance of southern South America as a source of cacti biodiversity (Barthlott and Hunt 1993; Anderson 2001; Boyle and Anderson 2002), the *Opuntia* species from this region have received less attention than the opuntias from the Northern Hemisphere. While several molecular phylogenetic studies have focused in the northern species (e.g. Griffith 2004; Griffith and Porter 2009; Bárcenas et al. 2011; Hernández-Hernández et al. 2011; Majure et al. 2012), southern South American (sSA) opuntias have been primarily investigated at the traditional morphological level; also some studies have been achieved on physiological aspects, and on reproductive biology and pollination issues (Britton and Rose 1919; Schlindwein and Wittmann 1997; Leuenberger 2002; Díaz and Cocucci 2003; Hunt et al. 2006; Nattero and Malerba 2011; Lenzi and Orth 2012; F. Font unpubl. data).

In the present study, we aimed at investigating the genetic and phylogenetic relationships of 15 species of *Opuntia* sensu stricto from Argentina, Bolivia, Brazil, Paraguay, and Uruguay (namely *O. anacantha* Speg., *O. arechavaletae* Speg., *O. aurantiaca* Lindl., *O. bonaerensis* Speg., *O. colubrina* A. Cast., *O. discolor* Britton and Rose, *O. elata* Link and Otto ex Salm-Dyck, *O. megapotamica* Arechav., *O. monacantha* Haw., *O. penicilligera* Speg., *O. quimilo* K. Schum., *O. salmiana* J. Parm. ex Pfeiff., *O. schickendantzii* Weber, *O. sulphurea* Gillies ex Salm-Dyck, and *O. ventanensis* Long). For this set of species, the patterns of relationships are scarcely known, and the genetic information available is very limited or inexistent. From a taxonomic perspective, the species surveyed roughly correspond to three taxonomic series (Hunt et al. 2006; F. Font unpubl. data). *Aurantiacae* Britton and Rose, comprises the species: *O. anacantha*, *O. aurantiaca*, *O. colubrina*, *O. discolor*, *O. salmiana,* and *O. schickendantzii*. Also, the recently described *O. ventanensis* has been associated with this series (Long 2012). These species exhibit a prostrate to creeping habit; the cladodes are elongated, lanceolate, flattened to cylindroid or terete, highly branched, and easily removable. Another taxonomic series is *Armatae* Schum. (= *Elatae* Britton and Rose), revised by Leuenberger (2002), Hunt et al. (2006) and F. Font unpubl. data. This series includes the species *O. arechavaletae*, *O. bonaerensis*, *O. elata*, *O. megapotamica*, *O. monacantha,* and *O. penicilligera*. These cacti are tall bushes with large, orbicular to elliptic cladodes, except for *O. penicilligera* which shows a prostrate habit. The series *Sulphureae* Britton and Rose solely includes *O. sulphurea,* which has a prostrate habit with thick, oval and tuberculate cladodes, and morphological variable populations that develop from southern Bolivia and western Paraguay to northern Argentinean Patagonia. The elusive species *O. quimilo* is now considered as an isolate entity, though it was previously placed in *Streptacanthae* Britton and Rose, and more recently within an *Armatae* clade (Majure et al. 2012). *Opuntia quimilo* is a perennial shrub with a wide geographical distribution in Argentina, Paraguay, and Bolivia, and a particular reproductive biology for a cactus: gynodioecy (Díaz and Cocucci 2003; Nattero and Malerba 2011).

To explore the interspecific genetic relationships among these *Opuntia* species, we carried out a genotyping with Inter-Simple Sequence Repeat markers (ISSR; Zietkiewicz et al. 1994), followed by genetic distance-based analyses. Besides the use of ISSR markers in microevolutionary studies, this technique has also been used in the estimation of genetic diversity and differentiation among individuals and closely related plant species (Ruas et al. 2003; Mansyah et al. 2010; Gaiero et al. 2011; Wang 2011; Rana et al. 2012). However, few studies have applied ISSR fingerprinting in *Opuntia.* For instance, Luna-Páez et al. (2007) employed this technique for variety distinction, whereas Souto Alves et al. (2009) used it to develop cultivar-specific markers. This PCR-based technique generates multilocus highly polymorphic dominant genomic markers without the need of prior DNA sequence knowledge (Zietkiewicz et al. 1994; Mishra et al. 2003).

To accomplish a probabilistic phylogenetic analysis within a broader sampling framework, we generated chloroplastic nucleotide sequences. In phylogenetic studies concerning taxa prone to hybridization processes, such as cacti, the chloroplast DNA with its uniparental inheritance, haploidy and lack of recombination, becomes the preferred source of nucleotide data (Porter et al. 2000; Nyffeler 2002; Arias et al. 2005; Butterworth and Wallace 2004; Ritz et al. 2007; Bonatelli et al. 2013). Herein, we have surveyed most of the species currently accepted for South America (Hunt et al. 2006; F. Font unpubl. data) excepting 5–7 entities for which living plant materials are inaccessible.

Table 1 Southern South American species of *Opuntia* from which fresh cladodes were used for DNA extraction and subsequent processing as explained in "Materials and methods" section

Species	Voucher number	Collection number (FF)[a]	Collection site	Genbank accession number	
				trnL-trnF	*psbJ-petA*
O. anacantha Speg.	Font 525	242	Vera, Prov. Santa Fe, Argentina		
	Font 476	839	Ibarreta, Patiño, Prov. Formosa, Argentina	KJ914581	KJ914602
O. arechavaletae Speg.	Font 441	553	Luján, Prov. Buenos Aires, Argentina	KJ914582	KJ914603
	Font 544	799	Minas do Camaquá, Rio Grande do Sul, Brazil	KJ914583	
O. aurantiaca Lindl.	Font 202	231	San Andrés de Giles, Prov. Buenos Aires, Argentina		
	Font 224	564	Colón, Prov. Entre Ríos, Argentina		KJ914604
	Font 537	795	Valle Edén, Tacuarembó, Uruguay	KJ914584	
O. bonaerensis Speg.	Font 442	230	San Andrés de Giles, Prov. Buenos Aires, Argentina	KJ914585	KJ914605
	Logarzo 551	871	Conhello, Prov. La Pampa, Argentina		
O. colubrina A. Cast.	Font 454	750	Los Pioneros, Presidente Hayes, Paraguay	KJ914586	KJ914606
	Font 461	757	Boquerón, Paraguay		
O. discolor Britton and Rose	Font 440	122	Chacabuco, Prov. Chaco, Argentina		
	Font 508	782	La Paz, Prov. Catamarca, Argentina		
	Font 502	777	La Paz, Prov. Catamarca, Argentina	KJ914587	
	Font 305	718	Villamontes-Taringuiti, Tarija, Bolivia	KJ914588	KJ914607
O. aff. *discolor*	Font 492	768	Ancasti, Prov. Catamarca, Argentina		
O. elata Link and Otto	Font 254	692	Los Pioneros, Presidente Hayes, Paraguay	KJ914591	
ex Salm-Dyck	Font 291	711	Villamontes-Taringuiti, Tarija, Bolivia	KJ914594	KJ914610
	Font 607	822	Near Alegrete, Rio Grande do Sul, Brazil	KJ914593	KJ914609
	Font 536	793	Ruta 26, km 108, Paysandú, Uruguay	KJ914592	
	Font 606	680	Mercedes, Prov. Corrientes, Argentina	KJ914590	
	Font 212	27	Moreno, Prov. Buenos Aires, Argentina	KJ914589	KJ914608
O. megapotamica Arechav.	Font 621	823	Punilla, Prov. Córdoba, Argentina	KJ914595	KJ914611
	Font 433	655	Diamante, Prov. Entre Ríos, Argentina		
O. monacantha Haw.	Font 214	70	Castelar, Prov. Buenos Aires, Argentina	KJ914596	KJ914612
O. penicilligera Speg.	Font 531	740	Olavarría, Prov. Buenos Aires, Argentina	KJ914597	KJ914613
O. quimilo K. Schum.	Font 499	774	La Paz, Prov. Catamarca, Argentina	KJ914598	KJ914614
O. salmiana J. Parm. ex Pfeiff.	Font 581	652	Diamante, Prov. Entre Ríos, Argentina	KJ914599	
	Font 304	717	Villamontes-Taringuiti, Tarija, Bolivia		
	Font 494	770	Ancasti, Prov. Catamarca, Argentina		
O. schickendantzii Weber	Font 660	838	Cuesta El Lajar Prov. Salta, Argentina	KJ914600	KJ914615
O. sulphurea Gillies ex Salm-Dyck	–	682	Barranca Yaco, Prov. Córdoba, Argentina		KJ914616
O. ventanensis Long	Font 196	150	San Alberto, Prov. Córdoba, Argentina	KJ914601	

The voucher and collection numbers, geographical origin of the specimens and Genbank sequence accession numbers are indicated

Prov. Province

[a] Cultivated plants from F. Font's collection

Materials and methods

Fresh plant materials

The list of the fresh plant materials used, together with the accession number of each specimen, collection sites, and GenBank accession numbers of the nucleotide sequences generated here, are presented in Table 1. These materials are from the personal collection of F. Font (cited as FF in Table 1). Species identification, based on morphological grounds, follows the taxonomic treatment of Leuenberger (2002), Hunt et al. (2006) and F. Font (unpubl. data).

Voucher specimens are deposited at the BAF Herbarium (Herbario del Museo de Farmacobotánica Juan A. Domínguez, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Argentina). Figure 1 indicates the collection sites of the specimens listed in Table 1.

DNA extraction

Total genomic DNA was extracted from photosynthetic tissue of specimens listed in Table 1, using Wizard Genomic DNA Purification kit (Promega Corp.) following manufacturer's protocol.

ISSR genotyping

Among eight ISSR primers tested, the primers $(AG)_8T$ and $(AG)_8YT$ were selected; the other six primers rendered bands of poor quality or did not produce any amplification products. PCR runs were performed in 25 µl reactions, containing 2.5 µl buffer 10X (Invitrogen Life Technologies), 1.5 mM $MgCl_2$, 200 µmol/L dNTPs, 100 ng of primer, and 0.5 units of Taq DNA polymerase (Invitrogen Life Technologies) and 1 µl of genomic DNA (1:100 dilutions). Amplification conditions were as follows: 94 °C for 1 min, followed by 30 cycles at 94 °C for 45 s, 44 °C for 45 s, and 72 °C for 45 s, and a final extension of 5 min at 72 °C. PCR products were checked on 1.5 % (w/v) agarose gel electrophoresis. Aliquots of each positive sample were mixed with an equal volume of dye reagent (98 % [v/v] formamide, 10 mM EDTA, 0.025 % [w/v] bromophenol blue, and 0.025 % [w/v] xylene cyanol), heat denatured and the amplicons separated through 6 % (v/v) high resolution, denaturing (8 M urea) polyacrylamide gel electrophoreses (PAGE) with 1X TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8). Electrophoreses were carried out at constant power (55 W) in a Model S2 sequencing gel electrophoresis apparatus (Life Technologies). Visualization of the bands was achieved by staining the gels with the Silver Sequence DNA Sequencing System kit (Promega Corp.) following manufacturer's protocol. Randomly selected specimens were PCR amplified in duplicates or triplicates, and used for screening the stability of the banding patterns. A molecular weight marker (100 bp DNA Ladder, Solis Biodyne) was incorporated as

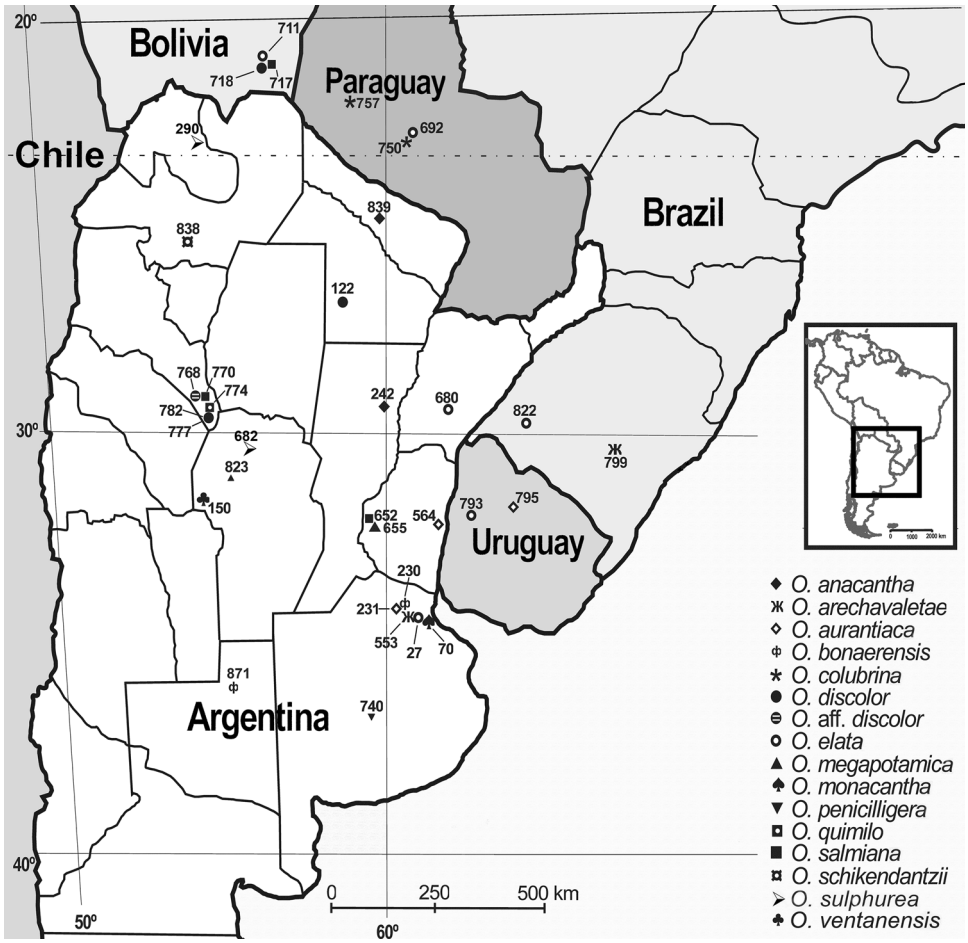

Fig. 1 Geographic location of southern South American *Opuntia* specimens sampled and listed in Table 1

size reference, in each PAGE run. Gels were air dried and digitalized using a conventional scanner.

Nucleotide sequencing

We attempted the PCR amplification of the nuclear internal transcribed spacer (ITS) region of the ribosomal DNA using universal primers ITS4 and ITS5 (White et al. 1990). For the amplification of the plastidic *trn*L-*trn*F intergenic spacer, we used primers *trn*E and *trn*F (Taberlet et al. 1991) and for the *psb*J-*pet*A intergenic spacer we used the primers described in Majure et al. (2012). Both intergenic regions are located in the Large Single Copy (LSC) region of the chloroplast. PCR amplifications of nuclear and plastidic regions were done using 1 µl aliquots of the total genomic DNA (1:100 dilutions) in a final volume of 25 µl, as described above. PCR were carried out at 94 °C for 4 min, followed by 30 cycles of 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 45 s, and a final elongation step at 72 °C for 5 min. PCR products were electrophoresed on 1 % (w/v) agarose gels; bands were excised and purified using the QIAquick Gel Extraction kit (QIAgen Inc.). Purified *trn*L-*trn*F PCR products were directly sequenced, whereas *psb*J-*pet*A products were cloned using the pGEM-T Easy Vector Cloning Kit (Promega Corp.) following manufacturer's instructions. Purification of the plasmidic vector plus the insert was achieved using the QIAprep Spin Miniprep kit (QIAgen Inc.). Nucleotide sequences were obtained at the "Servicio Interno de Genotipificación y Secuenciación de ADN-INTA" (Castelar, Buenos Aires, Argentina) using the corresponding amplification primers, and the universal primers T7 and SP6 for the cloned fragments. Resulting sequences were edited with BioEdit program (Hall 1999); sequence identity was confirmed by comparison with sequences deposited in public databases, using the BlastN search algorithm (http://www.ncbi.nlm.nih.gov/BlastN). All sequences generated were deposited at the GenBank (Table 1).

Data analysis

For ISSR genotyping, only stable unambiguous bands between 200 and 800 bp in size were used to generate a binary matrix based on the presence (1) or absence (0) of each band. The binary matrix was imported into the SplitsTrees4 program (Huson and Bryant 2006) to create a distance-based unrooted tree diagram by means of the Neighbor-Joining algorithm (Saitou and Nei 1987). The distance matrix was obtained with the Hamming distance index (Hamming 1950) and the option: ignore ambiguous states. In addition, the ISSR distance matrix was analyzed using a network approach on the basis that cacti tend to hybridize and introgress and that genomic DNA undergoes recombination, thus being likely that the assumption of evolution in a strictly bifurcated fashion is violated. Therefore, alternative interspecific relationships were visualized with the NeighborNet algorithm (Bryant and Moulton 2004) implemented in SplitsTrees4 with the following settings: edge fitting as ordinary least squares, equal angle as the chosen splits transformation, least squares to modify weights, and four maximum dimensions as the filtering option. The split graph generated yields a visual representation of conflicting signals in the data by presenting them as a series of parallel edges. For both the Neighbor-Joining and the NeighborNet, the SplitsTrees4 program computes the least squares fit (LSfit) between the pair-wise distances from the graph and the distances from the matrix. Internal node supports were estimated by 1,000 bootstrap pseudoreplicates (Felsenstein 1985) with the same program.

PCR amplification of nuclear ITSs yielded, in most cases, a major band with 2–3 secondary bands. In other cases, up to three bands of equal intensities were detected in agarose gels (data not shown). PCR conditions assayed were sufficiently stringent (annealing at 55 °C), and thus we consider the multiple ITS banding patterns as data in support of polyploidy and/or of a lack of concerted evolution. To avoid the concomitant difficulty in detecting the orthologous copies required for any phylogenetic analysis, we desisted from using this nuclear region.

Plastid sequence data were analyzed with two approaches to investigate haplotype variation and to the inference of the phylogenetic relationships. These analyses were carried out incorporating sequence data from 30 species of *Opuntia* from North and South America, which were generated by Hernández-Hernández et al. (2011) and Majure et al. (2012) (Online Resource 1). Firstly, to diminish either taxa or sequence redundancy, all the sequences that belonged to the same nominal species were aligned and compared pairwise by calculating the percentage of similarity (uncorrected p-distance) in MEGA5.05 (Tamura et al. 2007). Then, identical sequences were condensed into the corresponding single operational terminal. Being the chloroplast a single hereditary unit, the two non-coding plastid regions were concatenated (i.e. *trn*L-*trn*F followed by *psb*J-*pet*A). The final alignment was obtained using the MUSCLE algorithm with default parameters, as implemented in MEGA5.05 (Online Resource 3). Haplotype detection and analysis were performed in NETWORK 4.6.1.1 program (fluxus-engineering.com) using only parsimony informative sites and applying the Median-Joining algorithm (Bandelt et al. 1999) with default parameters. Since there is no recombination within the LSC region of the chloroplast genome, each detected variant is considered as a unique chloroplastidic haplotype. For the probabilistic phylogenetic inference, the best fitting evolutionary model,

namely Tamura 3 parameters (Tamura 1992) plus Gamma distribution (5 categories, gamma value = 0.5437), were selected according to the information criteria implemented in MEGA5.05. This model was incorporated into the Maximum Likelihood analysis, which was accomplished using all sites, with the same program. The sequences of two Opuntioideae were included in this analysis for rooting purposes (*Consolea* spp. JF712688, JF787472 and *Maihueniopsis* spp. JF787479). We generated *trn*L-*trn*F sequence data for *Maihueniopsis* spp. (GenBank accession KJ914617) as described above, to complement the data available. Support for internal nodes was estimated by 1,000 bootstrap pseudoreplicates in MEGA5.05.

Results

The ISSR genomic fingerprints for 24 specimens (13 species) were recorded, yielding 110 variable bands showing a frequency range of 0.042–0.875 (Online Resource 2). Most bands (45.5 %) show a frequency ≤0.25, whereas ca. 27 % of the bands show a frequency range of 0.3–0.42 and other ca. 27 % a frequency ≥0.46. Two constant bands were registered in the size range considered. On average, 36 variable bands were recorded per specimen (range 8–63). The mean genetic distance (Hamming 1950) is 0.124; being *O. elata* 680- *O. aurantiaca* 564 the most distant pair (0.498), whereas the latter specimen and *O. aurantiaca* 231 have null distance. The unrooted Neighbor-Joining diagram (LSfit = 94.202) shows that most specimens from identical nominal species fail to cluster together (Fig. 2). For instance, the specimens *O. discolor* 122 and 782, which show a bootstrap support value (BSV) of 67 %, appear more closely related (with BSV = 72 %) to two *O. aurantiaca* specimens (231 and 564; BSV = 100 %), than to the other *O. discolor* specimens. Likewise, the specimens of *O. salmiana, O. aurantiaca,* and *O. elata* appear dispersed over the phylogram. The NeighborNet split graph (Fig. 3) shows a better adjustment of the data (LSfit = 98.434) than the strictly bifurcating Neighbor-Joining, though both diagrams show groupings and partitions that are concordant.

Twenty-one *trn*L-*trn*F and 16 *psb*J-*pet*A nucleotide sequences were obtained (average length: 427 and 1,112 bp, respectively). Sequences generated from specimens *O. arechavaletae* 553 and 799 are identical, as are the sequences obtained from *O. discolor* 718 and 777, and those of *O. elata* 680, 692, 793, and 822. Thus, in subsequent analyses these nominal species were represented by corresponding consensus sequence. Likewise, when the similarity of the sequences retrieved from GenBank (Online Resource 1) was checked, all identical sequences were condensed. As a result, in the final dataset *O. echios, O. excelsa, O. macbridei, O. megasperma* and *O. microdasys* are represented by corresponding consensus sequences. However, we kept two *O. salmiana* terminals to account for the variation present in *psb*J-*pet*A, although *trn*L-*trn*F sequences of *O. salmiana* from GenBank and from our specimen 652 are identical. Similarly, *O. arechavaletae, O. sulphurea, O. quimilo,* and *O. monacantha,* are represented by two terminals each because sequence identity between our data and that downloaded from GenBank is <95 %. The *psb*J-*pet*A sequence retrieved for *O. schickendantzii* (JF787582) was excluded from the analysis due to its incompleteness (208 bp in total). Therefore, the final dataset consists of 44 ingroup opuntias and 2 outgroup terminals; the alignment spanned 1,625 positions, with 150 variable sites and 108 singletons.

The haplotype network was constructed using 34 parsimony informative sites. The analysis recognizes 26 haplotypes and postulates five putative ancestral or missing haplotypes and two loops (Fig. 4). Sixteen characters appear as homologous changes, whereas 18 characters have a homoplasious distribution, changing their states 2–6 times. The smaller loop is caused by a single homoplasious position, while the other is caused by six homoplastic sites. The central haplotype (ht 1) involves 12 species, of which seven are originated in the Northern Hemisphere (*O. chaffeyi, O. depressa, O. erinacea, O. excelsa, O. ficus-indica, O. megacantha,* and *O. robusta*) and the rest from northern and southern South America (*O. arechavaletae, O. aurantiaca, O. bella, O. macbridei,* and *O. schumannii*). Remaining sSA specimens radiate in four haplogroups (Hg 1–4) showing 14 homologous changes. *O. arechavaletae* 553, 799 and both *O. monacantha* form the haplogroup 1 (Hg 1); whereas *O. bonaerensis* 230, 871, *O. elata* 27, *O. elata* 680, 692, 793, 822, *O. megapotamica* 823, 655 and both sequences of *O. quimilo,* form haplogroup 2 (Hg 2). The haplotype 2 (ht 2) comprises *O. anacantha* 839, *O. colubrina* 750, 757, *O. discolor* 777, 718, *O. elata* 711 and *O. sulphurea* 682. All these sequences, together with *O. sulphurea* from the GenBank, represent haplogroup 3 (Hg 3). Then, the haplogroup 4 (Hg 4) is formed by the two *O. salmiana* and *O. schickendantzii* 838. Haplogroups 5 and 8 (Hg 5, 8) mainly involve specimens from North America with the exception of *O. penicilligera*; whereas haplogroups 6 and 7 (Hg 6–7) engage taxa from northern South America, the Galapagos Islands and Colombia, respectively.

The highest log likelihood phylogram (Fig. 5) shows that sSA *O. anacantha* 839, *O. colubrina* 750, 757, *O. discolor* 777, 718, *O. elata* 711, *O. sulphurea* 682, and *O. sulphurea* from the GenBank, form a clade with the highest BSV (98 %), in agreement with Hg 3. Both *O. arechavaletae* and *O. monacantha* appear intermingled with *O. macbridei* and *O. strigil*—the most divergent terminal

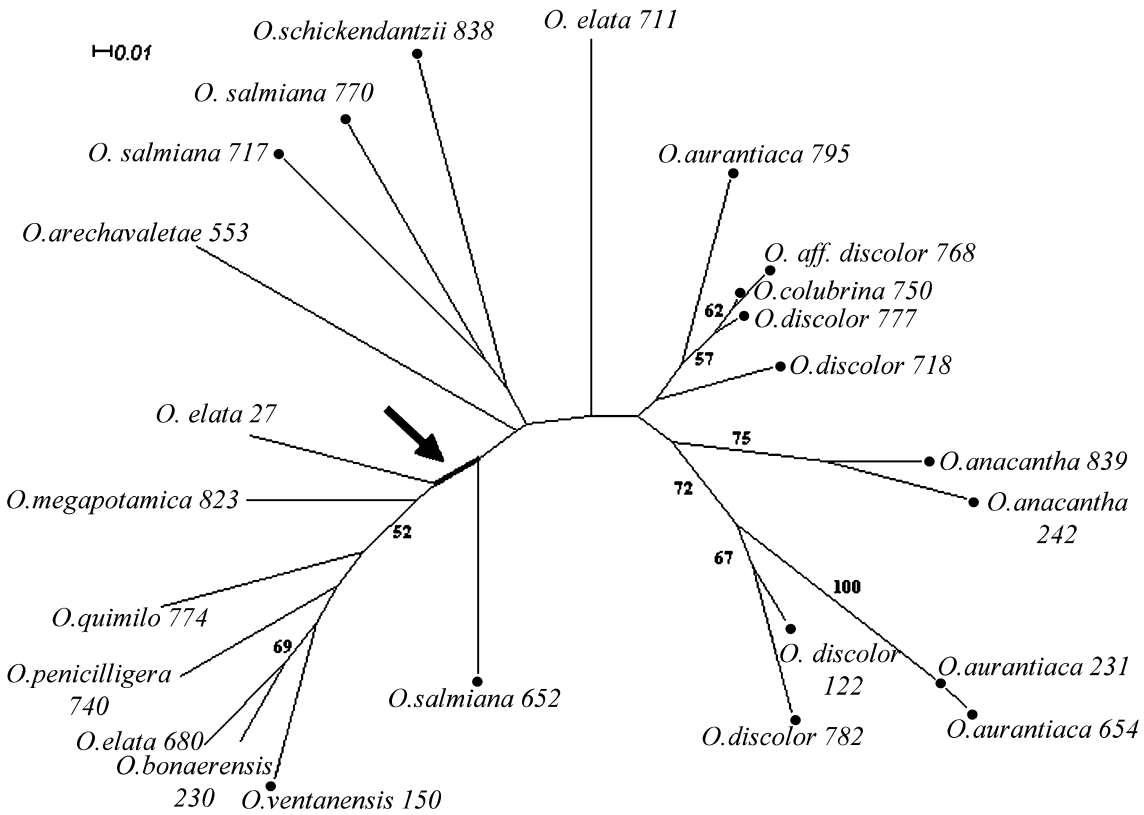

Fig. 2 Neighbor-joining unrooted tree of southern South American *Opuntia* species based on ISSR markers and Hamming distance (1950). Bootstrap values ≥50 % are indicated in *bold type*. Species assigned to the series *Aurantiacae* are labeled with a *black circle* at the branch tips, whereas *Armatae* species and *O. quimilo* appear unlabeled. The *scale bar* is in genetic distance units. *Arrow*, see "Discussion"

based on accumulated changes on the *psb*J-*pet*A spacer—though with low support (BSV <50 %). *O. engelmanii* and *O. phaecantha* (BSV = 64 %), plus *O. penicilligera, O. camanchica,* and *O. macrocentra* appear related as in Hg 8, though with BSV <50 %. In correspondence with the haplogroups defined above, on one hand *O. salmiana* and *O. schickendantzii* (BSV = 59 %), and *O. echios, O. galapageia,* and *O. megasperma* (BSV <50 %) on the other, form two separate clades. As in Hg 2, *O. bonaerensis, O. elata, O. megapotamica,* and *O. quimilo* appear closely related (BSV = 64 %).

Discussion

The genetic and phylogenetic relationships of *Opuntia* s.s. were inferred using distance and character-based methodological approaches applied onto molecular data with biparental and maternal inheritance. A special focus was placed on *Opuntia* species from southern South America. New sequence data is contributed herein for *O. anacantha, O. aurantiaca, O. bonaerensis, O. colubrina, O. discolor, O. megapotamica, O. penicilligera* and *O. ventanensis.* The information produced for *O. arechavaletae, O. elata, O. schickendantzii*, and *O. sulphurea,* is a contribution of more complete sequences and of new intraspecific nucleotidic variants. Instead, the data generated for *O. monacantha, O. salmiana* and *O. quimilo,* are concurrent with data derived from other studies. It is worth mentioning that in the present survey we have dealt with 15 southern South American opuntias, whereas previous studies have considered only seven taxa for the region.

A tendency of association between groupings and species assignment to a taxonomic series may be derived from the analyses of the genomic ISSR fingerprints, if a root is placed in the Neighbor-Joining (arrow in Fig. 2). In that case, we could distinguish two clusters (though with BSV <50 %). One cluster is formed by 15 specimens from the series *Aurantiacae* plus two specimens from the series *Armatae* (*O. elata* 711 and *O. arechavaletae* 553). The other cluster is formed by six specimens from *Armatae* plus *O. quimilo* and *O. ventanensis*. Similarly, these two clusters appear when considering the highlighted split in the NeighborNet (Fig. 3). This suggests that morphological

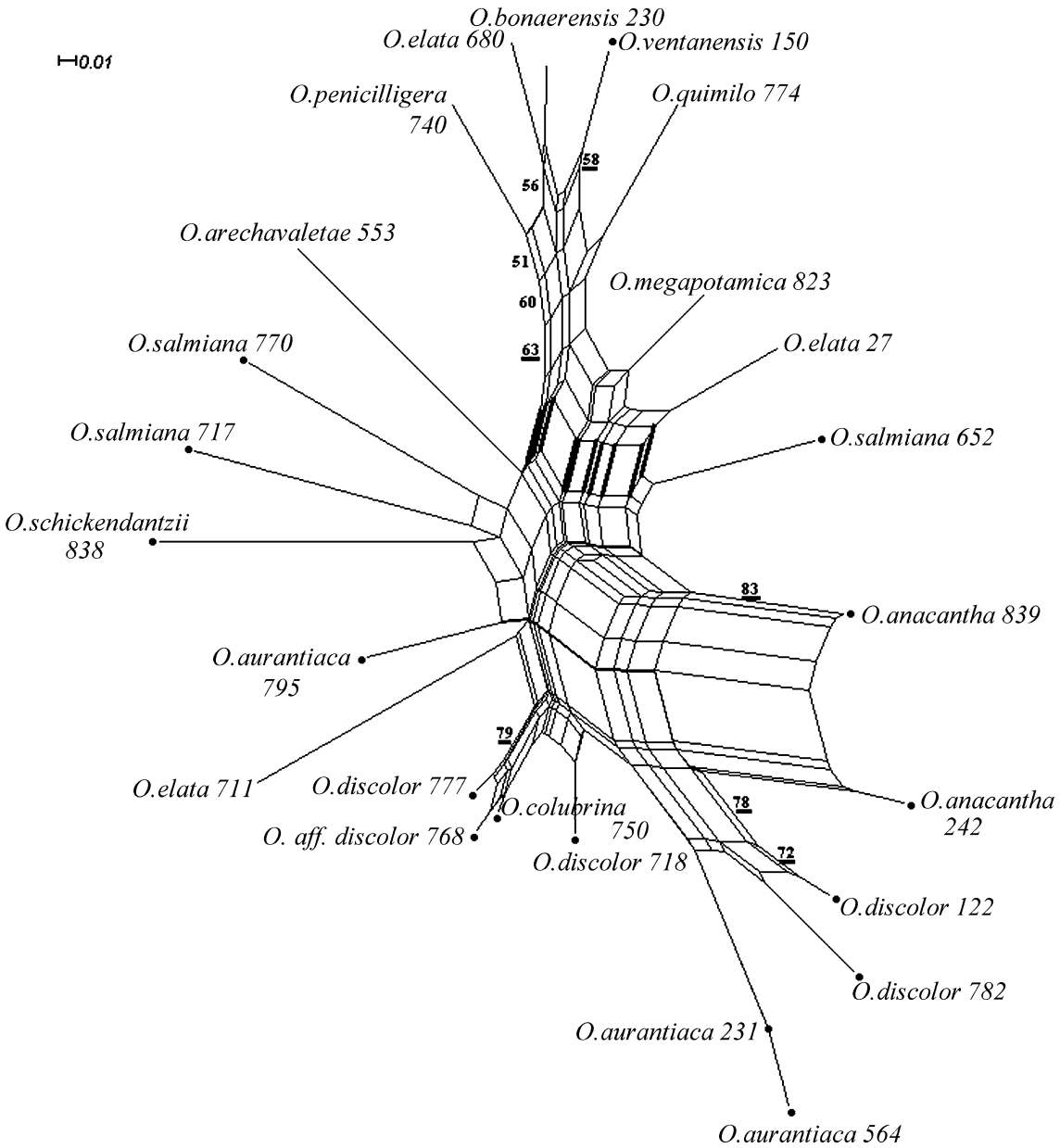

Fig. 3 Neighbor-net graph of southern South American *Opuntia* species based on ISSR markers and Hamming distance (1950). Bootstrap values ≥50 % are in *bold type*; *underlined values* support the same partitions as in Fig. 2. Species assigned to the series *Aurantiacae* are labeled with a *black circle* at the branch tips, whereas *Armatae* species and *O. quimilo* appear unlabeled. The *scale bar* is in genetic distance units; the length of each split is proportional to its weight. *Highlighted split*, see "Discussion"

features considered to distinguish the two series may have a genetic basis that deserves further scrutiny, and should be verified on additional samples. Even though we are aware that the number of ISSR primers employed is low, the number of variable bands recorded (55 bands per primer) is higher than the values reported in other fingerprinting studies of *Opuntia* species. For instance, Luna-Páez et al. (2007) and Souto Alves et al. (2009) found 14 bands per ISSR primer, and Griffith (2004) obtained 3.53 bands per RAPD primer. The use of high resolution sequencing-size PAGE coupled with silver nitrate staining, allowed us to reveal such a high number of bands throughout a wide size range. At the time the ISSR fingerprints were recorded, the ploidy level of the species involved was mostly unknown. The characterization of sSA *Opuntia* species by means of cytogenetic techniques, indicates that five species are diploids ($2n = 2x = 22$) and nine polyploids (from triploids with $2n = 33$, to a pentaploid with $2n = 55$) (M. F. Realini et al. unpubl.). The reconsideration of our raw data in the light of this knowledge, suggests that the relationship

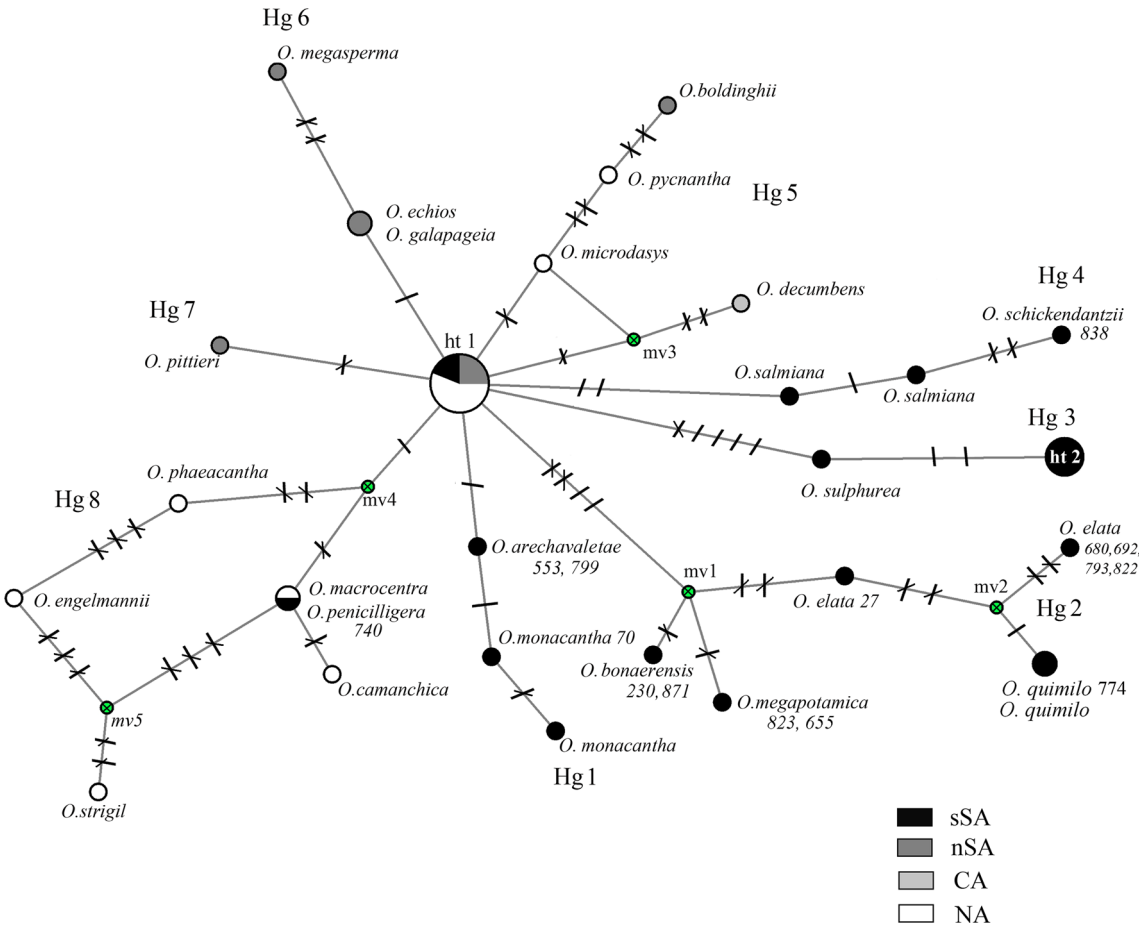

Fig. 4 Haplotype network of *Opuntia* maternal lineages. Each haplotype is represented by a *circle*; its size is proportional to its frequency. *Colors* indicate individual's origin: *black* southern South America; *dark grey* northern South America; *light grey* Central America; *white* North America. *Dashes* indicate nucleotide changes between two haplotypes linked by an edge; *simple dashes* denote homologous changes; *crossed dashes* denote homoplasious changes. Inferred median vectors are indicated by *crossed circles* and initials "mv". For species composition of major haplotypes (ht), see text. *Hg* haplogroup

between the ploidy level and the number of bands effectively registered per specimen is not straightforward, at least in the present case.

Partially consistent results were found between the analyses of biparentally inherited molecular markers (ISSR) and those maternally inherited (plastidic sequences). Both datasets underscore the close relationship of *O. salmiana* and *O. schickendantzii,* on the one hand, and of *O. anacantha*, *O. aurantiaca*, *O. colubrina,* and *O. discolor,* on the other. Also *O. bonaerensis, O. elata, O. megapotamica,* and *O. quimilo,* appear related in those analyses. The fact that *O. quimilo* emerges more related to *Armatae* species than to *Aurantiacae* agrees with Leuenberger (2002). As to the specimen *O. elata* 711, it appears nested within *Aurantiacae* with both data sets; a re-evaluation of this particular specimen would aid at detecting a misidentification or mislabeling. The placement of *O. penicilligera,* by means of its maternal lineage, in alliance to North American species is an unexpected outcome that contrasts with ISSR results, where it neither shows an isolated location nor particularly long branches (i.e. with large genetic distances) that would set it apart from other South American species. At the morphological level, there are several features that connect *O. penicilligera* with *O. megapotamica* more than with other species of *Opuntia*, for instance, sub-orbicular green-bluish articles, well-developed ferruginous glochids, purple inner pericarpelar tissue and green stigmas (F. Font personal observation). The formal description of *O. ventanensis* states its phenotypic resemblance to *O. salmiana* and *O. aurantiaca* (Long 2012). Our results are not conclusive for *O. ventanensis* given that the genomic data suggest a connection with *Armatae* species, whereas the maternal *trn*L-*trn*F lineage relates it to *Aurantiacae* (data not shown). The lack of complete agreement between ISSR and chloroplastidic DNA results, evidenced in *O. penicilligera* and *O. ventanensis*, could be based on past or recent occurrence of reticulation and cross hybridization, introgression and/or

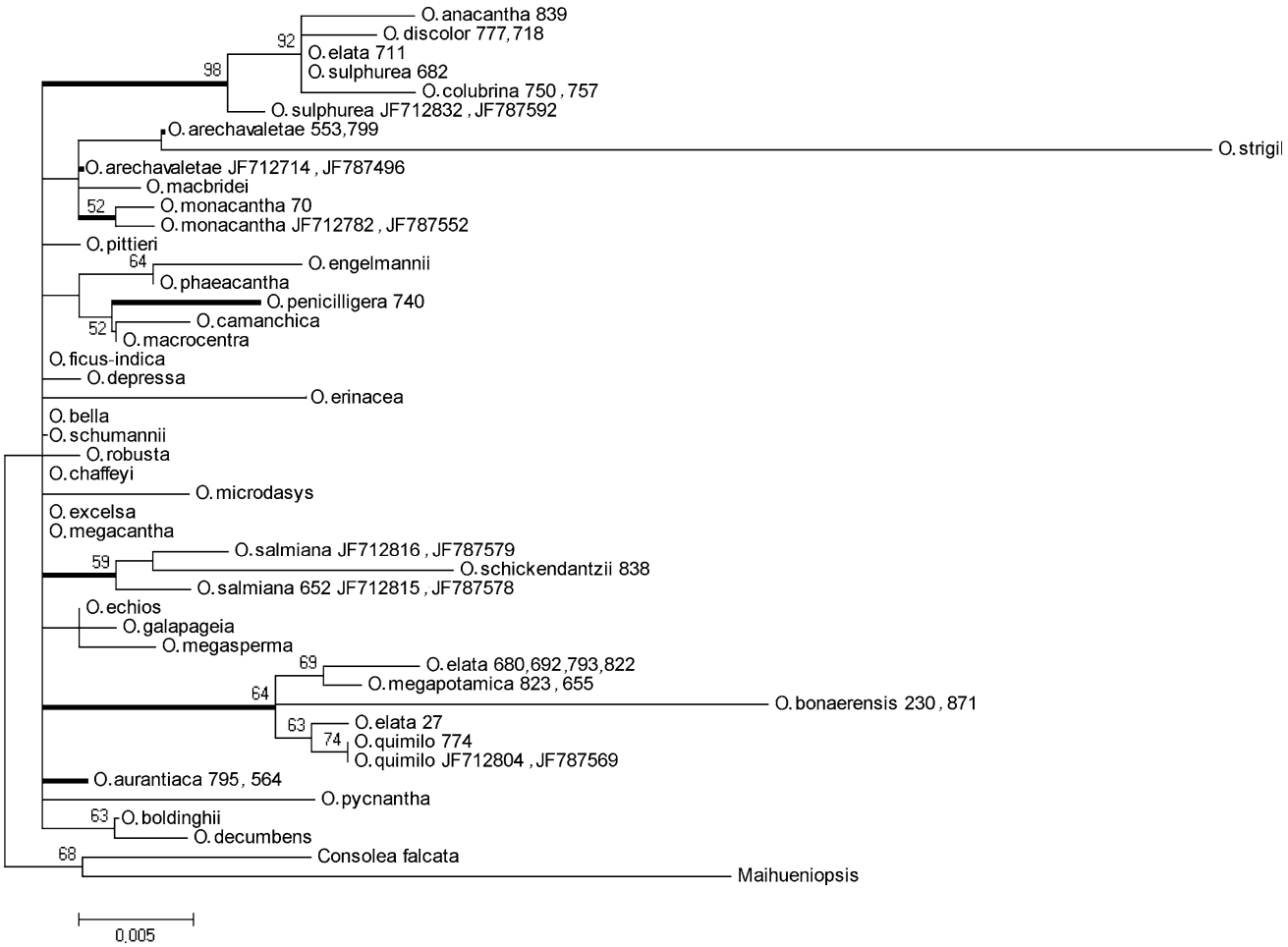

Fig. 5 Maximum likelihood phylogram derived from *trn*L-*trn*F plus *psb*J-*pet*A concatenated sequences, using the Tamura (1992) model of molecular substitution (Ln = −3,402.43). The *numbers above branches* are bootstrap support values ≥50 %. The *scale bar* is in number of substitutions per site. *Thick branches* indicate clades of southern South American taxa

on the occurrence of chloroplast capture (Stegemann et al. 2012). However, additional samples should be examined before attempting any robust conclusions on the ongoing evolutionary processes.

Concerning previously analyzed sSA species, our results agree with Griffith and Porter (2009) in that the terete-stemmed *O. salmiana* is allied with flat-stemmed opuntioids, but differ in its relative phylogenetic placement. Herein, *O. salmiana* appears in an intermediate position in the Neighbor-Joining and NeighborNet analyses and in the chloroplastidic haplotype network (forming part of Hg 4); still, its placement relative to remaining sSA opuntias is uncertain, as shown by the maximum likelihood analysis. In the study of Griffith and Porter (2009), based on ITS and *trn*L-*trn*F sequence data, *O. salmiana* forms part of a basal polytomy (a trichotomy). Later, Guiggi (2010) erected the genus *Salmiopuntia* Frič ex Guiggi for *O. salmiana* without indicating the basis of this splitting. Caution should be taken not to confuse the unresolved position shown by *O. salmiana,* in the tree depicted by Griffith and Porter (2009), as an indication in support of its splitting since that polytomy has three potential resolutions. Hence, until new evidence may be gathered we continue considering *O. salmiana* as another *Opuntia* s.s. Consequently, our results disagree with those of Majure et al. (2012) since they expressly employed *O. salmiana* (under *Salmiopuntia*) to root all their phylogenies. As mentioned above, with ISSR and plastid data sets we detected a relationship between *O. salmiana* and *O. schickendantzii*, whereas Majure et al. (2012) recovered a *Brasiliopuntia brasiliensis* + *O. schickendantzii* clade, even though no evident morphological characters support this relationship. On the other hand, the intraclade phylogeny of Majure et al. (2012) (Fig. 3, page 855, op. cit.), should be interpreted cautiously as it is the majority rule consensus tree derived from a rapid bootstrap analysis. As such, it neither represents the phylogram with the maximum likelihood, nor a phylogenetic hypothesis. Resampling methods give an idea of how much evidence is in favor or against particular groups (Goloboff 1998), and cannot replace the phylogenetic inferences.

The maximum likelihood topology presented here resembles those of Griffith and Porter (2009) and of Bárcenas et al. (2011) in lack of back-bone resolution, but a close examination of the latter shows disagreement in the placement of *O. quimilo*. In the *trn*K-*mat*K phylogenetic analysis of Bárcenas et al. (2011), *O. quimilo* appears as sister group to all (*Opuntia* + *Nopalea*) plus *Tunilla,* and thus it was considered outside of a core *Opuntia* clade. In contraposition, all of our results support *O. quimilo* as related to *Armatae* [= *Elatae*] species. This is in agreement with Majure et al. (2012), even though they consider *O. elata* as a diploid species, and our cytogenetic survey indicates polyploidy (M. F. Realini et al. unpubl.). As to *O. sulphurea*, our results indicate that it is allied to *Aurantiacae* species, contrasting with Griffith and Porter (2009) who recovered it within a Galapagos Islands clade, and with Majure et al. (2012) who recovered it within an Elatae clade. Concerning the remaining species included in our analysis, we found concordance with Griffith and Porter (2009) and Majure et al. (2012), in that the cacti from the Galapagos (*O. echios, O. megasperma* and *O. galapageia*) form a clade. Particularly with Griffith and Porter (2009), we agreed in the recovery of *O. phaecantha, O. engelmanii, O. camanchica,* and *O. macrocentra,* as a group, but not with Majure et al. (2012) where *O. engelmanii* forms part of another clade.

The unresolved placement of the species shown in the plastidic genealogy may be solved using several highly variable regions; however, any taxonomic proposal should also be based on substantial nuclear information. Arakaki et al. (2011) postulated a recent origin for *Opuntia* species (7.5 ± 2.3 to 5.6 ± 1.9 million years old), and suggested that the cactus floras of the three main centers of biodiversity are extremely young, and more or less contemporary. This may explain the poor resolution shown by the gene trees derived from the present and previous studies (e.g., Griffith and Porter 2009; Bárcenas et al. 2011; Majure et al. 2012). In concurrence, the star-like shape of the haplotype network, with a central, most probable, ancestral haplotype (Ht 1) not geographically localized (that is, shared between species without overlapping distribution areas) could be explained by a recent dispersal and/or diversification of few maternal lineages towards North and South America, and the persistence of ancient polymorphism, as was seen in *Hordeum* (Jakob and Blattner 2006). It is worth noting that even though a similar number of North and South American taxa have been analyzed here, 1.8 times more haplotypes were detected for sSA taxa (i.e., 15 haplotypes in sSA plastid lineages compared to eight haplotypes in northern hemisphere lineages), evidencing that southern opuntias harbor significant genetic variation, not previously considered.

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