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Phylogeny of Korean *Opuntia* spp. based on multiple DNA regions

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Abstract: Although *Opuntia* species are of high agronomic value in Korea, the taxonomic position of Korean *Opuntia* species has never been investigated. The taxonomic position of Korean *Opuntia* spp. within the tribe *Opuntieae* was examined based on DNA sequence analysis of *matK*, *trnL-F*, *atpB-rbcl*, and ITS regions. The total amplified sequence length was 2977 bp; only 18 parsimonious informative sites were present, even though they belonged to different species. A phylogenetic tree using both the maximum likelihood method with 2000 bootstrap replications and Bayesian posterior probabilities was constructed. The new forma, *Opuntia humifusa* f. *jeollaensis*, used in this study was placed within the Macrocentra clade rather than the Humifusa clade. The genetic distance between *O. humifusa* f. *jeollaenis* and *O. camanchica* was the lowest among all *Opuntia* spp. analyzed in this study. Korean *O. ficus-indica* was genetically closer to *O. engelmannii* than to *O. ficus-indica* previously reported. *Opuntia engelmannii* and *O. ficus-indica* have been considered conspecific previously, and so it is likely that the Korean *O. ficus-indica* used in this study may be a relative of *O. engelmannii* or may have arisen from a lineage different to the *O. ficus-indica* used in the analysis.

Key words: Korean *Opuntia*, phylogeny, *matK*, *trnL-F*, *atpB-rbcL*, ITS region

1. Introduction

Cactaceae comprises between 1438 (Hunt, 2006) and 1850 (Nyeffler and Eggli, 2010) species. Opuntioideae is a subfamily of Cactaceae; *Opuntia s.s* is one of the largest genera, with around 180 to 200 species (Anderson, 2001; Nyffeler and Eggli, 2010). There are roughly 26 series of subgenus conforming to *Opuntia s.s.* (Majure et al., 2012b). They are widely distributed and occur in subtropical dry forests, moderate deserts, and temperate forests (Benson, 1982).

Some *Opuntia* species are widely cultivated (Inglese et al., 2002) for consumption. *Opuntia s.s.* were domesticated around 8000 years ago in Mexico (Ostolaza, 1994). They have several medicinal properties such as heptaprotective (Ncibi et al., 2008), hypoglycemic (Trejo-Gonzalez et al., 1996; Laurenz et al., 2003), antimicrobial (Lee et al., 2004), antioxidative (Stintzing et al., 2005), neuroprotective (Go et al., 2003), and wound healing (Park et al., 2001), and they protect the brain from glucose and oxygen deprivation (Huang et al., 2008). They are also used in traditional oriental folk medicines to treat diabetes, indigestion, edema, burns, wounds, etc. (Ahn, 1988; Go et al., 2003).

In Korea, *O. ficus-indica* (Baiknyuncho) and *O. humifusa* (Chunnyuncho) are cultivated in large quantities. They are members of the subfamily *Opuntioideae*. *O. ficus-indica* and *O. humifusa* are native to South and Central America; when they were introduced into Korea is still unknown (Kim and Park, 2009). *O. ficus-indica* is grown only on Jeju Island, where the climate is subtropical, while *O. humifusa* is grown on the Korean mainland and is found to withstand severe cold temperatures. A new forma of *Opuntia* was identified by us; it was named *O. humifusa* f. *jeollaensis*. It has phenotypic similarity to *O. humifusa*, but differs by having red centered yellow flowers and the presence of hard spines in the cladodes (Kim et al., 2014). The origin of this forma and its taxonomic position are not known, but it is widely cultivated in the Jeollabuk-do Province of Korea.

Although there have been several studies on the phylogeny of *Opuntia* (Griffith and Porter., 2009; Hernandez et al., 2011; Majure et al., 2012a, 2012b), Korean *Opuntia* spp. have not been included in these studies, and so their taxonomic position is not known. Due to their medicinal properties and horticultural importance, *Opuntia* spp. are increasingly becoming important in Korea (Go et al., 2003; Lee et al., 2004; Stintzing et al., 2005), and so it is necessary to clarify their taxonomic relationship. The phenotypic plasticity among *Opuntia* spp. can confound taxonomic circumscription (Barthlott and Hunt, 1993; Stuppy, 2001; Wallace and Gibson, 2002), and so the use of molecular

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data for phylogenetic study can give a clear understanding of their relationship.

The present study was carried out with the premise that the forma studied here could be a new taxon introduced into Korea, or a mutant of *O. humifusa*, or a hybrid of *O. ficus-indica* and *O. humifusa*. Hybridizations are very common in *Opuntia*, and these hybridizations have resulted in many new species (Pinkava, 2003). *Opuntia ficus-indica* itself was shown to have originated from the hybridization of species belonging to the *Nopalea* series and *Basilares* series (Majure et al., 2012b).

The aim of this study was to find out the origin of the new forma used in this study and to determine the taxonomic position of Korean *Opuntia* taxa within the closely related *Opuntia* spp. occurring worldwide, based on several DNA regions.

2. Materials and methods

2.1. Species sampling

The collection data of plant samples used in this study are given in Table 1. Three individuals from each species were used in the analysis. The plants were grown at the growth facility of Chonbuk National University, Republic of Korea. Fresh samples were used for DNA isolation. GenBank accession numbers of sequences amplified in this study and GenBank accession numbers of previously published sequences retrieved from the NCBI database and used in this study are given in Table 2.

2.2. Isolation of DNA

Total genomic DNA was extracted from fresh cladodes (100 mg). Although they are highly mucilaginous, their DNA was successfully isolated using a modified CTAB method (Doyle and Doyle, 1990). The quality of the isolated DNA was checked on a 0.8% agarose gel stained with ethidium bromide, and they were quantified using a spectrophotometer (Nanodrop ND 2000, Nanodrop technologies, USA).

2.3. PCR amplification and sequencing

The primers used in this study, their sequence information, and annealing temperatures are given in Table 3. Three plastids and one nuclear region were amplified. The PCR reactions were carried out as 20 μL reactions containing 25 ng of DNA, 1X PCR reaction buffer, 2.5 mM dNTPs, 20 pmoles of primers, and 1 unit of tenuto Taq DNA polymerase (Enzynomics, Korea). The PCR reactions were carried out using a GeneAmp PCR system 2700 thermal cycler. The PCR cycling conditions were as follows: initial denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing (Table 2) for 30 s, and extension at 72 °C for 2 min, and a final extension step at 72 °C for 10 min. The PCR products were resolved on an agarose gel and stained with ethidium bromide. A 1-kb DNA ladder was used as size marker. The bands were then eluted from the gel, cloned into a T-vector (pGEM T easy vector, Promega, USA), and sequenced.

2.4. Sequencing, alignment, and data analysis

Sequencing was carried out on an ABI prism 3700 sequencer. The sequence chromatograms were edited and assembled using the program Sequencher (ver. 4.1.1; Genecodes Corporation Inc., USA). The sequences amplified in this study were then compared with the nucleotide database in NCBI using BLAST, and the sequences of *Opuntia* species available for all the regions amplified in this study were retrieved and used for further analysis. After assembling the sequences of all the regions into one for each species, the sequences were aligned using CLUSTAL X (Thompson et al., 1997) and manually edited using BioEdit (Hall, 1999). Parameters like conserved sites, variable sites, parsimony informative sites, GC content, and genetic distance were estimated using MEGA5 software (Tamura et al., 2011). The combined data analysis was justified based on a congruence test using partitioned Bremer support (De Salle and Brower, 1997) on separate plastid and nuclear data using the program TreeRot ver. 3 (Sorenson and Franzosa, 2007). No significant incongruence was detected. Visual analysis of trees generated from separate nuclear and plastid regions was also done to check for strong incongruence. jModeltest v1.1 (Posada, 2008) was used to find the model of molecular evolution that best fits our sequence data under the Akaike information criterion (AIC). The GTR+G (base frequencies: A = 0.318, T = 0.357, C = 0.168, G = 0.157; gamma distribution = 0.36 for plastid, and base frequencies: A = 0.209, T = 0.151, C = 0.332, G = 0.309; gamma distribution = 0.27, for ITS) was found to be the best fit model for both plastid and ITS datasets. A maximum likelihood tree with 2000 bootstrap

Table 1. Some morphological characteristics of Korean *Opuntia* spp. used in this study.

Taxa	Flower color	Glochid color	Location	Cladode during winter
O. ficus indica	Yellow	White	Jeju Island	Not wrinkled
O. humifusa	Yellow	Stramineous	Jeolla-buk-do	Wrinkled
O. humifusa f. *jeollaensis*	Red centered Yellow	Red	Jeolla-buk-do	Wrinkled

Table 2. GenBank accession number of DNA sequences used here.

Species name	*trnL- trnF*	*atpB - rbcL*	*matK*	*ITS*
Tacinga palmadora	JF712845.1	JF787307.1	JF786872.1	JF787028.1
T. inamoena	JF712843.1	JF787305.1	JF786870.1	JF787027.1
T. lilae	JF712769.1	JF787233.1	JF786797.1	JF786955.1
Salmiopuntia salmiana	JF712815.1	JF787279.1	JF786843.1	JF786999.1
Opuntia. humifusa f. *jeollaensis* 3*	KJ735941	KJ735959	KJ735950	KJ735932
O. humifusa f. *jeollaensis* 2*	KJ735942	KJ735960	KJ735951	KJ735933
O. humifusa f. *jeollaensis* 1*	KJ735943	KJ735961	KJ735952	KJ735934
O. tomentosa	JF712834.1	JF787298.1	JF786861.1	JF787067.1
O. strigil	JF712830.1	JF787291.1	JF786856.1	JF787014.1
O. stenopetala	JF712825.1	JF787287.1	FN997146.1	JF787008.1
O. schumannii	JF712821.1	JF787283.1	JF786849.1	JF787004.1
O. santa-rita	JF712818.1	JF787280.1	JF786845.1	JF787001.1
O. rufida	JF712813.1	JF787277.1	FN997506.1	JF786997.1
O. retrorsa	JF712814.1	JF787274.1	JF786839.1	JF786995.1
O. quimilo	JF712804.1	JF787267.1	AY015279.1	JF786988.1
O. pusilla	JF712800.1	JF787264.1	JF786828.1	JF786985.1
O. polyacantha	JF712795.1	JF787259.1	FN997449.1	JF786979.1
O. pachyrrhiza	JF712786.1	JF787250.1	JF786813.1	JF786970.1
O. microdasys	JF712781.1	JF787246.1	FN997321.1	JF786966.1
O. megasperma	HM041324.1	JF787245.1	HM041743.1	JF786965.1
O. megacantha	JF712778.1	JF787243.1	JF786806.1	EU930383.1
O. macrorhiza	JF712774.1	JF787240.1	JF786802.1	JF786960.1
O. macrocentra	JF712773.1	JF787238.1	JF786801.1	JF786959.1
O. macbridei	HM041323.1	JF787236.1	HM041742.1	JF786958.1
O. humifusa	JF712712.1	JF787178.1	JF786739.1	JF786949.1
O. ficus-indica	JF712757.1	FJ026615.1	JF786784.1	AB250211.1
O. excelsa	HM041318.1	JF787220.1	HM041737.1	HQ872513.1
O. erinacea	JF712754.1	JF787219.1	JF786782.1	JF786941.1
O. engelmannii	JF712750.1	JF787217.1	FN997517.1	JF786938.1
O. ellisiana	JF712747.1	JF787213.1	JF786775.1	JF786936.1
O. echios	HM041317.1	JF787209.1	HM041736.1	JF786932.1
O. camanchica	JF712788.1	JF787195.1	JF786816.1	JF786973.1
O. basilaris	JF712722.1	JF787189.1	JF786750.1	JF786913.1
O. aureispina	JF712718.1	JF787185.1	JF786746.1	JF786910.1
O. abjecta	JF712838.1	JF787300.1	JF786865.1	JQ245716.1
O. humifusa 3*	KJ735938	KJ735956	KJ735947	KJ735929
O. humifusa 2*	KJ735939	KJ735957	KJ735948	KJ735930
O. humifusa 1*	KJ735940	KJ735958	KJ735949	KJ735931
O. ficus-indica 3*	KJ735935	KJ735953	KJ735944	KJ735926
O. ficus-indica 2*	KJ735936	KJ735954	KJ735945	KJ735927
O. ficus-indica 1*	KJ735937	KJ735955	KJ735946	KJ735928
Nopalea karwinskiana	JF712707.1	JF787174.1	HM041732.1	JF786899.1
N. hondurensis	JF712704.1	JF787172.1	JF786732.1	JF786896.1
N. gaumeri	HM041311.1	JF787170.1	HM041731.1	JF786894.1
N. dejecta	HM041310.1	JF787168.1	HM041730.1	JF786893.1
N. cochenillifera	JF712700.1	JF787166.1	HM041729.1	EU559672.1

The asterisk indicates sequences amplified in this study.

Table 3. DNA regions and associated primers used in this study.

Region	Sequences	Length amplified	Annealing temperature	Reference
atpB- rbcL	atbp R GTAGTAGGATTGGTTCTCAT rbcl F TAGTCTCTGTTTGTGGTGACAT	875bp	54 °C	Janzen DH et al., 2005
matK	MatKx TAATTTACGATCAATTCATTC Mat K5 GTTCTAGCACCAGAAAGTCG	951 bp	48 °C	www.kewgardens.org/barcode/update
trnL-F	trnL GGTTCAAGTCCCTCTATCCC trnlF ATTTGAACTGGTGACACGAG	466 bp	58 °C	Taberlet et al., 1991
nrITS	ITS4 TCCTCCGCTTATTGATATGC ITS5 GGAAGTAAAAGTCGTAACAAGG	685 bp	56 °C	White et al., 1990

replications was generated using MEGA5 (Tamura et al., 2011). Nodes were considered highly supported when bootstrap values were more than 70% (Hillis and Bull, 1993). A Bayesian tree was also constructed, using the Markov chain Monte Carlo (MCMC) analysis in MrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003), using the same evolutionary model that was used for the ML analysis. Three replicate analyses were run for 5 million generations each to ensure that the runs were converging on the appropriate posterior probability distribution. Nodes were considered highly supported when pp values were higher than 0.95 (Felsenstein, 1985). The tree was later visualized in FigTree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/). A phylogenetic tree based on maximum likelihood analysis with 2000 bootstrap replications based on the Tamura 3-parameter (T92) model (Tamura, 1992) involving only the Korean *Opuntia* spp. was also generated.

Genetic divergence was calculated using maximum likelihood analysis. For the analysis, data with less than 95% site coverage were eliminated, i.e. fewer than 5% missing data, alignment gaps, and ambiguous bases were allowed at any position. Several sequences of closely related Korean *Opuntia* spp. were downloaded from the GenBank database for the analysis; their accession numbers are given in Table 2. Taxa belonging to the *Tacinga* series were used as out-groups. The out-groups were not involved in the genetic divergence estimations; they were only used in the phylogenetic analysis.

3. Results

The new forma of *Opuntia* used in this study, *O. humifusa* f. *jeollaensis*, was phenotypically similar to *O. humifusa* (Chunnyuncho), although with some minor differences (Table 1) in the color of the flowers and glochids. During the winter months the cladodes of *O. humifusa* and *O. humifusa f. jeollaensis* wrinkle, whereas the cladode of *O. ficus-indica* does not wrinkle.

The total length of the sequence amplified by PCR was 2977 bp, with 875 bp of *atpB-rbcL* region, 951 bp of *matK* region, 466 bp of *trnL-trnF* region, and 685 bp of nr ITS region from the three species. All the sequences above were combined for analysis after no incongruence was found in the major nodes of the plastid and nuclear trees. Their combined GC content was 39.3%. ITS sequences had the highest GC content. Eighteen variable and parsimony informative sites were revealed by the analysis (Table 4). A maximum likelihood tree involving only the Korean taxa showed that *O. humifusa f. jeollaensis* was genetically closest to *O. humifusa*. The tree also showed that the forma was not a hybrid between *O. ficus-indica* and *O. humifusa*. Moreover, between *O. humifusa* and the new forma, there were only 3 parsimonious informative sites. The mean genetic distance among the three species was only 0.003.

In order to find the taxonomic position of Korean *Opuntia* spp. within the tribe *Opuntieae*, the DNA sequences of *Opuntia* species occurring worldwide were downloaded from the GenBank database and were compared with the Korean *Opuntia* spp. studied here. The mean genetic distance was 0.056. *O. basilaris* and *O. quimilo* were found to be genetically the least similar.

Phylogenetic analysis using the maximum likelihood method with 2000 bootstrap replications and Bayesian posterior probability analysis (Figure) resulted in fourteen major clades, out of which only nine were recovered with high support by both BI and ML analysis. The genus *Tacinga* was used as the out-group. The new forma, *O. humifusa* f. *jeollaensis*, was placed in the *Macrocentra* group but with low support, a posterior probability value of 0.66. The Korean *O. humifusa* was placed in the same clade as that of a previously reported *O. humifusa* with a bootstrap value of 90. The new forma, *O. humifusa* f. *jeollaensis*, was found to be close to *O. camanchica*; only 3 variable sites were found on sequence analysis between the new forma and *O. camanchica*. The Korean *O. ficus-indica* used in this

Table 4. Statistics of regions used in this study.

Data used	Total sequence length	No. of variable sites	No. of parsimony information sites	No. of conserved sites	GC content (%)	Best fit model
Only Korean *Opuntia* spp. sequenced in this study	2977 bp	18	18	2959	39.3	T92
Korean *Opuntia* and all *Opuntia* spp. sequences downloaded from GenBank	2681 bp	375	61	2274	39.5	GTR+G (Based on AIC)

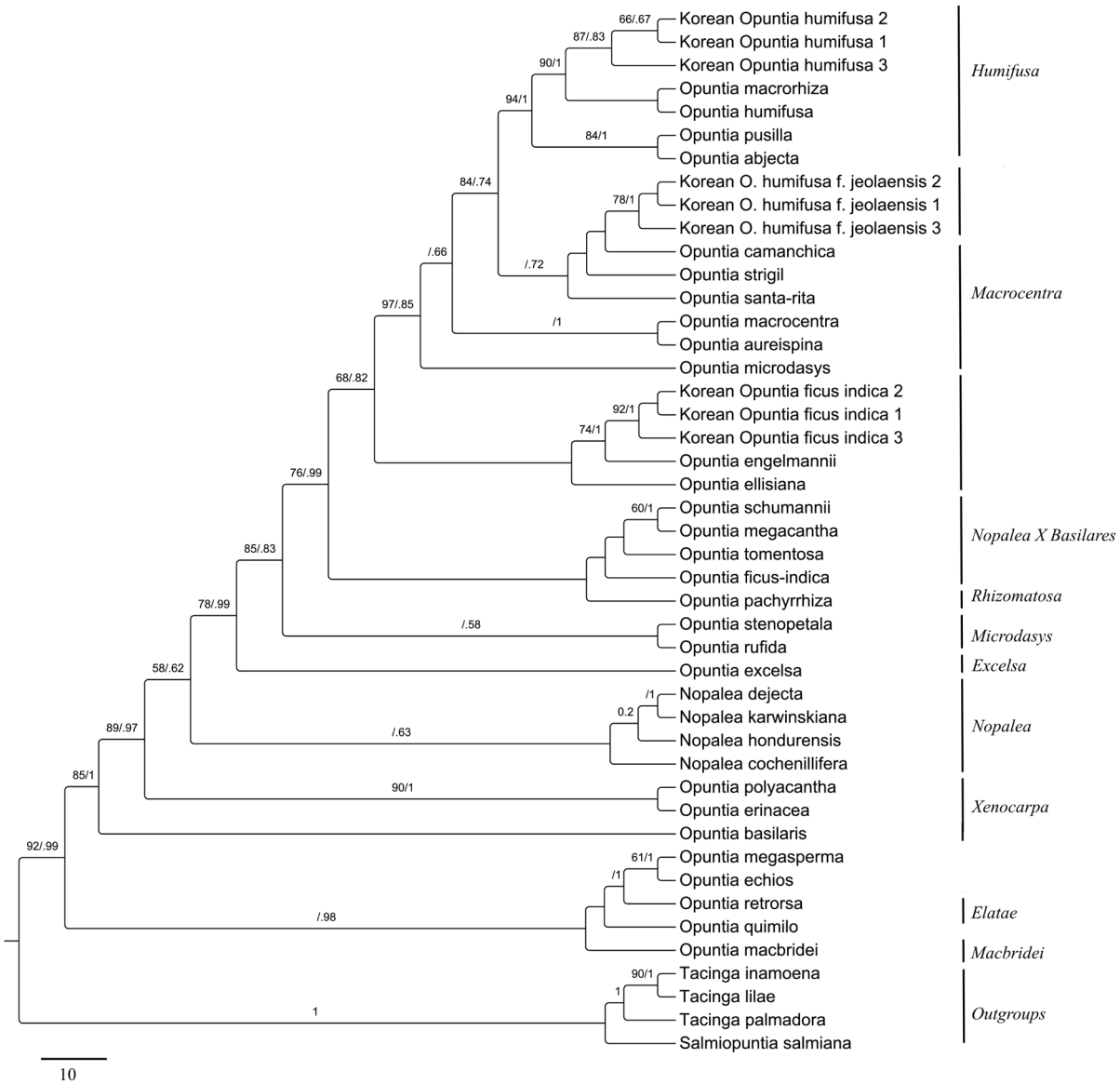

Figure. Phylogeny of *Opuntia* s.s. The maximum likelihood tree (–ln likelihood = –3411.1858) for the concatenated data (*atpB-rbcL*, *matK*, and *trnL-F*) set under the GTR+G model of sequence evolution (Nei and Kumar, 2000). The value above branches denotes the bootstrap values (left) and the Bayesian posterior probability values (right). Clades are named after the series recognized by Britton and Rose (1920) and Engelmann (1856), and the hybrid (Nopale × Basilares) identified by Majure et al. (2012b). Bootstrap values <50 and posterior probabilities <0.5 are not given.

study was found to be close to *O. engelmannii*, with strong clade support shown by ML and BI analysis. The previously reported *O. ficus-indica* and other allopolyploids like *O. megacantha* and *O. schumannii* were placed in a separate clade. The clades formed were largely specific to the series recognized by Britton and Rose (1920), Englemann (1856), and Majure et al. (2012b).

4. Discussion

The total length of the DNA sequences of *matK*, *trnL-F*, *atpB-rbcL*, and ITS regions amplified was 2977 bp. The GC content of the combined sequences was 39.3%. ITS sequences had the highest GC content; this is consistent with other plant taxa (White et al., 1990; Baldwin, 1992). Low sequence divergence (Table 4) was observed among Korean *Opuntia* spp. Majure et al. (2012b) also observed low sequence divergence in *Opuntia* s.s.

The phylogenetic tree based on maximum likelihood analysis and Bayesian analysis (Figure) placed Korean *O. humifusa* within the Humifusa clade. The tree (Figure) showed that the forma was close to *O. camanchica* belonging to the *Macrocentra* series. There was a clear delineation of the Macrocentra clade from the Humifusa clade with strong bootstrap and posterior probability support. The sequence chromatogram and the phylogeny clearly showed that the forma is not a hybrid or mutant of Korean *O. humifusa* spp. as was initially hypothesized. Further, morphologically, the flowers of *O. camanchica* are also similar, with a red base on a yellow petal (Griffith, 2003; Pinkava, 2003); their glochids are also red, the same as the forma, but the spine color is different. *O. camanchica* spines are dark brown and they occur only on the upper half of the cladode (Griffith, 2003), but the spines of the forma are white and occur throughout the cladode. The new forma was grouped within the Macrocentra clade.

Korean *O. ficus-indica* was genetically close to *O. engelmannii* and not to the *O. ficus-indica* sequence downloaded from the GenBank database. *Opuntia ficus-indica* is a domesticated cactus. Griffith (2004) and De Lyra et al. (2013) reported *O. ficus-indica* as polyphyletic as they included individual clones from multiple lineages. Furthermore, Benson and Walkington (1965) had placed *O. engelmannii* as a synonym under *O. ficus-indica*, but this was refuted by Parfitt and Pinkava (1988). Therefore, it is possible that the *O. ficus-indica* used in this study is conspecific to *O. engelmanii*, and might have been derived from a different lineage from the earlier reported *O. ficus-indica*, but further morphological and karyotype analysis will be necessary before concluding so.

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