Korean J. Pl. Taxon.
44(3): 181-187 (2014)
http://dx.doi.org/10.11110/kjpt.2014.44.3.181

ISSN 1225-8318
Korean Journal of
Plant Taxonomy

Opuntia humifusa (Raf.) Raf. f. *jeollaensis* E. J. Kim & S. S. Whang, a new forma based on three DNA markers

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(Received 31 July 2014; Revised 1 September 2014; Accepted 15 September 2014)

DNA 염기서열에 근거한 선인장과 신품종 왕가시천년초 〔*Opuntia humifusa* (Raf.) Raf. f. *jeollaensis* E. J. Kim & S. S. Whang〕

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ABSTRACT: The taxonomic status of a new forma, *Opuntia humifusa* (Raf.) Raf. f. *jeollaensis* E. J. Kim & S. S. Whang, and the taxonomic relationships of three Korean cladodes were studied based on DNA sequences of three genes. The new forma *O. humifusa* f. *jeollaensis* (Wanggasi-Chunnyuncho) is simlilar to *O. humifusa* (Chunnyuncho), but differ in having both flower with the reddish inner center, and strong and many 3 cm long spines. Molecular studies showed that the new forma grouped into *Macrocentra* series instead of *Humifusa* series which included Korean *O. humifusa*. We designated the new taxa firstly studied here as a new forma, because the taxa have been mainly cultivated in farmlands in Jeolla-do area rather than distributed in nature. The taxonomic relationships of three Korean cladodes are discussed in detail.

Keywords: *Opuntia humifusa* f. *jeollaensis*, a new forma, DNA markers

적 요: 세 개 유전자의 DNA 분자서열을 이용해서 전라도 지역에서 재배되고 있는 신품종 왕가시천년초(*O. humifusa* (Raf.) Raf. f. *jeollaensis* E. J. Kim & S. S. Whang)의 분류학적 지위와 한국산 손바닥 선인장들의 분류학적 유연관계가 조사되었다. 신품종 왕가시천년초는 형태학적으로나 생육 지역이 천년초와 비슷하지만, 노란색 꽃의 안쪽 중심에 붉은 색이 있고, 3 cm 길이의 강모가 많이 발달되어서 차이가 있다. 분자적인 연구결과는 왕가시천년초가 *O. humifusa* complex에서 *Macrocentra* 계열에 포함되어 *Humifusa* 계열에 포함되는 한국산 *O. humifusa*와 다르게 나타났다. 왕가시천년초는 천년초와 마찬가지로 주로 농장에서 재배되고 있어서 품종으로 지위를 주었다. 한국산 손바닥선인장 3종에 대한 분류학적 유연관계가 자세히 논의되었다.

주요어: 왕가시천년초, 신품종, DNA 마커

Cactaceae are a well-supported clade endemic to the New World and comprise ca. 1500~1800 species (Anderson, 2001). Even though some controversy remain in classification of the family into subfamily (Crozier, 2004; Edwards et al., 2005; Bàrcenas et al., 2011), the two major subfamilies, Opuntioideae and Cactoideae are recognized (Nyffeler and Eggli, 2010). Subfamily Opuntioideae encompass five tribes (Wallace and Dickie, 2002), and further the tribe Opuntieae consists of seven genera, *Brasiliopuntia, Consolea, Miqueliopuntia, Opuntia s.s., Salmiopuntia, Tacinga*, and *Tunilla* (Majure et al., 2012a).

Opuntia s.s. consists of around 180~200 species as the largest genus in Opuntioideae (Anderson, 2001; Nyffeler and Eggli,

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http://www.pltaxa.or.kr

2010), and exhibits the widest distribution of any genus in Cactaceae (Benson, 1982; Anderson, 2001). One poorly understood group within *Opuntia s.s.* is the *O. humifusa* complex (Majure et al., 2012a). Species in the *O. humifusa* complex are known to hybridize (Benson, 1982) and contain numerous polyploid entities (Majure et al., 2012b), which has provided for a taxonomically complex history and ambiguous species limits. Though lots of morphological, cytological, and molecular studies have been conducted (Dolyle, 1990; Leuenberger, 2001; Griffith and Porter, 2009; Hernndez-Hernndez et al., 2011; Majure et al., 2012b), the relationship of this complex within *Opuntia* spp. remains to be resolved.

The taxa in *Opuntia* have been both cultivated as long as at least 14,000 year in Mexico (Casas and Barbera, 2002), and also introduced throughout the world for use as foodstuff for humans and animals and as ornamentals (Anderson, 2001; Inglese et al., 2002; Nefzaoui and Salem, 2002). Some *Opuntia* like Chunnyuncho (*O. humifusa*) and Baiknyuncho (*O. ficus-indica*), have also been cultivated in Korea even though it is unknown when they were introduced into Korea (Kim, 2013). Baiknyuncho, only distributed in Jeju Is. where climate is sub-tropical, is weak in cold resistance. On the other hand, Chunnyuncho, cultivated in the inland province where climate is temperate, is found to tolerate severe cold temperature during the winter season. A new taxon of *Opuntia* firstly reported here, similar to *O. humifusa* but differ in having flowers that has yellow petal with red color at the base and the presence of hard spine, is recently selected and widely cultivated in Jeolla-do areas. The origin and taxonomic status of this new taxon, however, is unknown.

Due to the medicinal properties of *Opuntia* species, studies on these taxa have gradually been increasing in oversea (Laurenz et al., 2003; Stintzing and Carle, 2005; Mauseth, 2006; Huang et al., 2008; Ncibi et al., 2008; Majdoub et al., 2010) as well as in Korea (Lee et al., 2004; Choi et al., 2005; Kwon and Song, 2005; Cho et al., 2006; Choi and Shin, 2011; Jung et al., 2012; Jun et al., 2013; Kim, 2013; Yang, 2013; Choi, 2014). It would be problematic if these species would be developed as medical and functional foods without clarifying the taxonomic relationships among three *Opuntia* spp. in Korea. Though there have been several phylogenetic studies of *Opuntia* (Griffith and Porter; 2009; Hernndex et al., 2011; Majure et al., 2012a, b), Korean *Opuntia* spp. have not been included, so the taxonmic relationships of them are unknown so far.

The study aims are to clarify the taxonomic status of a new taxon described above and also to determine the taxonomic relationships of Korean *Opuntia* spp. within the closest *Opuntia* spp. based on several DNA markers.

Material and Methods

We sampled nine plants from three taxa of *Opuntia* in Korea for DNA isolation; three *Opuntia ficus-indica* (L.) Miller collected from Jeju Is., and three *O. humifusa* (L.) and three *O. humifusa* f. *jeollaensis* (a new forma firstly recorded here) respectively collected around Jeolla-do. They were transplanted in the plant growth facility of Chonbuk National University. We mainly used alive plants for studying of morphological and molecular investigations, because *Opuntia* spp. are notoriously difficult to work with herbarium specimens. As methods used to collect specimens are typically inefficient, leading to poor specimen preservation, and the complete loss of most taxonomically useful characters as a result of the succulent nature of the plants and their highly bothersome glochids and spines (Reyes-Aguero et al., 2007).

Total genomic DNA was extracted from fresh cladode (100 mg), ground in liquid nitrogen. Though 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 0.8% agarose gel stained with ethidium bromide. And they were qualified using a spectrophotometer (Nanodrop ND 2000, Nanodrop technologies, USA). The primers used in this study, their sequence information and annealing temperatures are in Table 1. Two plastid genes, *matK* and *trnL-F*, one nuclear gene, nrITS DNA, were amplified. The PCR reaction was carried out in 20 μL reaction containing 25 ng of DNA, 1X PCR reaction buffer, 2.5 mM dNTPs, 20 pmoles of primers, and 1 unit of

Table 1. Genes, primer sequences, annealing temperature for PCR used in this study.

Gene	Sequences	Length amplified	Annealing temperature	Reference
matK	Mat Kx taatttacgatcaattcattc Mat K5 gttctagcaccagaaagtcg	951 bp	48ºC	www.kewgardens.org/barcode/update
trnL-F	trnL ggttcaagtccctctatccc trnlF atttgaactggtgacacgag	466 bp	58ºC	Taberlet et al., 1991
nrITS	ITS4 tcctccgcttattgatatgc ITS5 ggaagtaaaagtcgtaacaagg	685 bp	56ºC	white et al., 1990

tenuto Taq DNA polymerase (Enzynomics, Korea). The PCR reaction was carried out using a GeneAmp PCR system 2700 thermal cycler. The PCR cycling condition was as follows, an initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing for 30 sec, 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 stained with ethidium bromide. A 1-kb DNA ladder was used as size marker. The bands were then eluted from the gel, and cloned into a T-vector (pGEM T 3 easy vector, Promega, USA) and sequenced.

Sequencing was carried out on an ABI prism 3700 sequencer. The sequence chromatogram was edited and assembled using Sequencher (ver. 4.1.1; Genecodes Corporation Inc, USA). In order to find the taxonomic relationships of Korean *Opuntia* spp., we also included the DNA sequences of the closest species in the *Opuntia* s.s. and also used *Tacinga* series as a outgroup followed by Majure et al. (2012a, b). The sequences retrieved from the Genbank database the same as in Table 2. After assembling the sequences of all the regions into one for each species, the sequences along with outgroup were aligned using CLUSTAL X (Thompson et al., 1997) and manually edited using BioEdit (Hall, 1999). A maximum likelihood tree was generated using MEGA 5 (Fig. 3) (Tamura et al., 2011).

Results and Discussion

A new taxon of *Opuntia* firstly studied here, *O. humifusa* f. *jeollaensis* (Wanggasi-Chunnyuncho), is more closer to *O. humifusa* (Chunnyuncho) than *O. ficus-indica* in the sense of both morphological features and distributional range in Korea. This

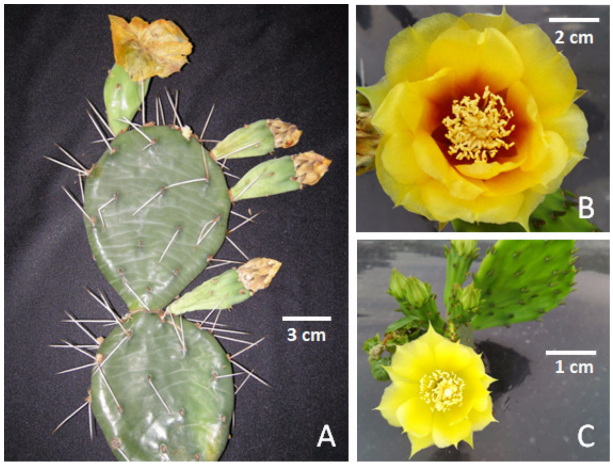

Fig. 1. Photographs of Korean *Opuntia* spp. A-B. *O. humifusa* f. *jeollaensis.* Dried cladodes having flowers and spines with 3 cm long (A). A flower showing yellow petals with raddish center (B). C. *O. humifusa* having young cladodes and a yellow flower.

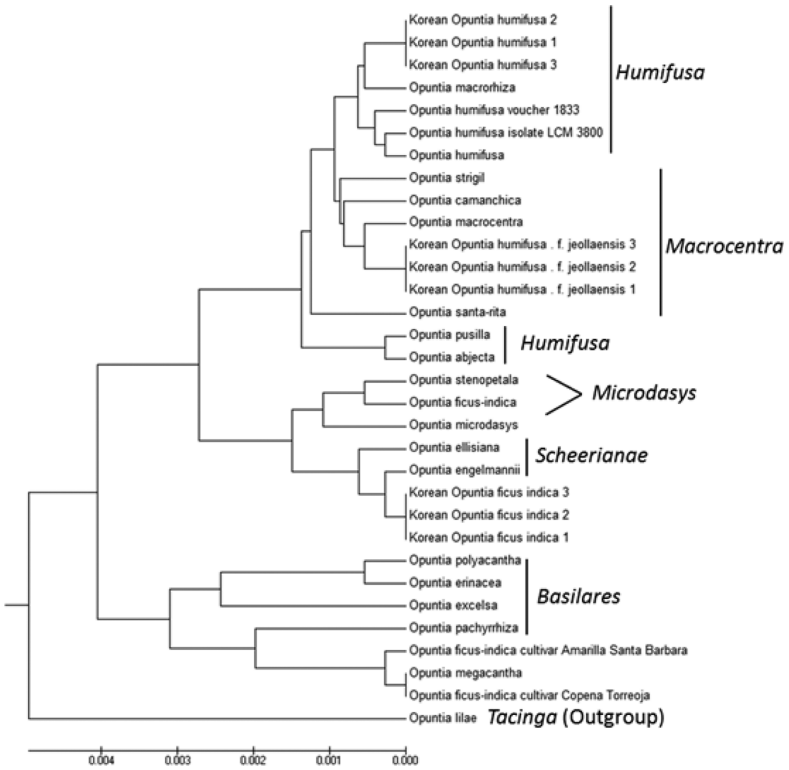

Fig. 2. Dendrogram showing genetic relationships among the *Opuntia humifusa* complex including Korean taxa. The clades are named on the basis of the series recognized by Britton and Rose (1919).

and long spines reaching up to 3 cm and yellow petals with reddish center compared to *O. humifusa,* having small and rare spines reaching less than 2.5 cm and only yellow petals (Fig. 1).

The total length of three genes, *matK*, *trnL-F*, and nrITS, was 2,102 bp. We drew a dendrogram using maximum likelihood method to clarify the taxonomic status of the new forma studied here, and also to determine the taxonomic relationship of Korean *Opuntia* spp. (Fig. 2). The new forma, *O. humifusa* f. *jeollaensis* is closely clustered with the species of the *Macrocentra* series rather than with those of the *Humifusa* series which included Korean *O. humifusa* within the North American Clade by Majure et al. (2012b). As they pointed out, the *Humifusa* complex in North American Clade would be grouped into two large clusters; one comprises the series *Scheerianae*, *Humifusa* and *Macrocentra*, and the other is *Noplaea* and Basilares. We here designated the new taxa as

Table 2. GenBank accession number of the closest *Opuntia* complex followed by Majure et al., (2012a, b) and its Korean OTU studied here.

No	Species	trnL	matK	ITS
1	*Opuntia strigil*	JF712830.1	F786856.1	JF787014.1
2	*Opuntia stenopetala*	JF712825.1	FN997146.1	JF787008.1
3	*Opuntia santa-rita*	JF712818.1	JF786845.1	JF787001.1
4	*Opuntia pusilla*	JF712800.1	JF786828.1	JF786985.1
5	*Opuntia polyacantha*	JF712795.1	FN997449.1	JF786979.1
6	*Opuntia pachyrrhiza*	JF712786.1	JF786813.1	JF786970.1
7	*Opuntia microdasys*	JF712781.1	FN997321.1	JF786966.1
8	*Opuntia megacantha*	JF712778.1	JF786806.1	EU930383.1
9	*Opuntia macrorhiza*	JF712774.1	JF786802.1	JF786960.1
10	*Opuntia macrocentra*	JF712773.1	JF786801.1	JF786959.1
11	*Opuntia lilae*	JF712769.1	F786797.1	JF786955.1
12	*Opuntia humifusa* voucher 1833	JF712712.1	JF786790.1	JF786949.1
13	*Opuntia humifusa* isolate LCM 3800	JF712711.1	EU930379.1	JQ245718.1
14	*Opuntia humifusa*	JF712712.1	JF786739.1	JF786949.1
15	*Opuntia ficus-indica* cultivar Copena Torreoja	JF712711.1	JF786785.1	EU930379.1
16	*Opuntia ficus-indica* cultivar Amarilla Santa Barbara	JF712711.1	JX517861.1	EU930378.1
17	*Opuntia ficus-indica*	JF712757.1	JF786784.1	AB250211.1
18	*Opuntia excelsa*	HM041318.1	HM041737.1	HQ872513.1
19	*Opuntia erinacea*	JF712754.1	JF786782.1	JF786941.1
20	*Opuntia engelmannii*	JF712750.1	FN997517.1	JF786938.1
21	*Opuntia ellisiana*	JF712747.1	JF786775.1	JF786936.1
22	*Opuntia camanchica*	JF712788.1	JF786816.1	JF786973.1
23	*Opuntia abjecta*	JF712838.1	F786865.1	JQ245716.1
24	Korean *Opuntia humifusa* 3	KJ735938	KJ735947	KJ735929
25	Korean *Opuntia humifusa* 2	KJ735939	KJ735948	KJ735930
26	Korean *Opuntia humifusa* 1	KJ735940	KJ735949	KJ735931
27	Korean *Opuntia humifusa* f. *jeollaensis* 3	KJ735941	KJ735950	KJ735932
28	Korean *Opuntia humifusa* f. *jeollaensis* 2	KJ735942	KJ735951	KJ735933
29	Korean *Opuntia humifusa* f. *jeollaensis* 1	KJ735943	KJ735952	KJ735934
30	Korean Opuntia ficus indica 3	KJ735935	KJ735944	KJ735926
31	Korean Opuntia ficus indica 2	KJ735936	KJ735945	KJ735927
32	Korean Opuntia ficus indica 1	KJ735937	KJ735946	KJ735928

a new forma because even though the new taxa clustered to *Macrocentra* more closely than *Humifusa*, they are being mainly cultivated in farmlands of Jeolla-do areas.

Korean *O. ficus-indica* (Baiknyuncho) was closely clustered with *O engelmannii,* but not with the other *O. ficus-indica* sequences downloaded from Genbank database (Table 2). Griffith (2004) had 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-indicia*, but this was disputed by Parfitt and Pinkava (1988). So it is possible that Korean *O. ficus-indica* is conspecific of *O. engelmannii*, but more morphological and chromosome analysis would be necessary before concluding that.

Taxonomic Treatment

***Opuntia humifusa* (Raf.) Raf. f. *jeollaensis* E. J. Kim & S. S. Whang,** for. nov. (Fig. 3)

Korean name: **Wang-gasi-chun-nyun-cho 왕가시천년초**

Shrubs evergreen; Stems jointed orbicular to oblong, 3 to 13 cm long, areoles usually far apart; Leaves subulate, appressed or spreading, early deciduous; Spines often strong, 3 cm long or less, nearly white or sometimes brownish; Glochids numerous, yellow to dark brown; Petals usually yellow with reddish centers, 5 to 8 cm broad; Fruit berries, fleshy; Seeds discoid, circular to broadly oval; Flowering May-July; Fruiting September-November.

Fig. 3. Holotype of *Opuntia humifusa* f. *jeollaensis* E. J. Kim & S. S. Whang.

Holotype: Kumma-myeon, Jeolla-buk Province, Korea. June 17, 2014. *whang30763*. Herbarium of Chonbuk National University (JNU).

Isotype: Kumma-myeon, Jeolla-buk Province, Korea. June 17, 2014. *whang30761, whang30764, whang30765.* Herbarium of Jeonbuk National University (JNU).

Distribution: Jeon-buk Province, Korea

Etymology: The specific epithet is derived from Jeolla-Buk Province where this new taxon is located

Habitats: *Opuntia humifusa* f. *jeollaensis* was cultivated in field and farm at Kumma-myeon, Iksan-si, Jeongeup-si, Jeonju-si in Jeon-buk Province.

Key to *Opuntia* spp. in Korea

1. Cladode not wrinkled during winter season
   ..... *O. ficus-indica* 백년초
1. Cladode wrinkled during winter season
   2. Yellow petals, glochids, small and rare spines
      ..... *O. humifusa* 천년초
   2. Yellow petals with reddish center, many glochids, long and many strong spines
      ..... ***O. humifusa* f. *jeollaensis* 왕가시천년초**

Acknowledgement

We thank to Mr. Sung-Gun Hwang for providing the plant materials. Authors thank the unknown reviewers for their helpful comments.

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