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Matias Köhler, Luis J. Oakley, Fabián Font, M. Laura Las Peñas & Lucas C. Majure

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**Research Article**


# On the continuum of evolution: a putative new hybrid speciation event in *Opuntia* (Cactaceae) between a native and an introduced species in southern South America

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MATIAS KÖHLER<sup>1,2</sup> , LUIS J. OAKLEY<sup>3,4</sup> , FABIÁN FONT<sup>5</sup> , M. LAURA LAS PEÑAS<sup>6</sup>  & LUCAS C. MAJURE<sup>2</sup> 

<sup>1</sup>Programa de Pós-Graduação em Botânica, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

<sup>2</sup>University of Florida Herbarium (FLAS), Florida Museum of Natural History, Gainesville, FL, USA

<sup>3</sup>Cátedra de Botánica, Facultad de Ciencias Agrarias, Universidad Nacional de Rosario, Santa Fe, Argentina

<sup>4</sup>Red List Authority Coordinator for the Temperate South American Plant Specialist Groups – International Union for Conservation of Nature (IUCN), Gland, Switzerland

<sup>5</sup>Herbario Museo de Farmacobotánica “Juan A. Domínguez” (BAF), Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires, Argentina

<sup>6</sup>Instituto Multidisciplinario de Biología Vegetal (IMBIV), Facultas de Ciencias Exactas, Físicas y Naturales, Universidad Nacional de Córdoba – CONICET, Córdoba, Argentina

Hybridization plays a fundamental role in plant evolution and diversification, promoting gene flow, morphological novelties, and plant speciation. Here, we integrated fieldwork, collections-based research, morphological observations, molecular systematics, and cytogenetic data to investigate the identity of a previously unidentified taxon of *Opuntia* (the prickly pear cacti) observed in the north-east region of Argentina, southern South America. Our analyses revealed a cytonuclear phylogenetic discordance among nuclear and plastid genomes, as well as a polyploid nature of the studied taxon. Combining our molecular phylogenetic and cytogenetic analyses with morphological observations, we suggest that hybridization events between the native *Opuntia rioplatensis* and the North American introduced species, *O. ficus-indica*, likely produced the taxon here described as *O. × cristalensis*. *Opuntia* is the most widespread genus of Cactaceae, and many species have been introduced worldwide for an array of different purposes. Our report proposes the putative first hybridization event in *Opuntia* between a North American species with a southern South American lineage, which may shed light on more complex evolutionary scenarios, speciation within the group, and the impacts of species introduction.

**Key words:** *Opuntia*, Cactaceae, polyploidy, cytogenetics

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## Introduction

Hybridization is one of the key topics in plant studies. This phenomenon – typically considered to represent crossing between species, but also extended to include crossing between genetically differentiated races or populations within a species – plays a crucial role in plant evolution and diversification, as well as in agriculture (Goulet et al., 2017; Rieseberg & Carney, 1998; Zirkle, 1935). Through hybridization, a significant portion of speciation events occurs in plants, most commonly via allopolyploidy (by the fusion of unreduced gametes, genome doubling following hybridization, or via a triploid bridge) or much more rarely at the same ploidy

level (homoploid hybrid speciation) (Ramsey & Schemske, 1998; Soltis & Soltis, 2009).

*Opuntia* (L.) Mill. (prickly pear cacti, *tunas*, or *nopales*) is the most widespread genus of Cactaceae, with ~150 species naturally occurring from southern South America to northern North America (Anderson, 2001; Britton & Rose, 1919; Majure et al., 2012a). The group represents a recent evolutionary radiation which resulted in expansive morphological diversity with marked species formation through reticulate evolution and polyploidy (Rebman & Pinkava, 2001; Pinkava, 2002; Griffith, 2003; Majure et al., 2012a; Majure & Puente, 2014; Köhler et al., in prep.).

The genus consists of eight major clades, with two essentially encompassing South American species whereas the other six contain mainly Central and North

Correspondence to: E-mail: [matias.k@ufrgs.br](mailto:matias.k@ufrgs.br)

American lineages (Majure *et al.*, 2012a; Majure & Puente, 2014; Köhler *et al.*, in prep.). The southern South American lineages are embedded within the Elatae clade (*sensu* Majure *et al.*, 2012a), representing ~30 native species occurring in the Chaquenan biogeographic domain throughout different biogeographic provinces, such as the Chacoan, Espinal, Monte, and Pampean (*sensu* Cabrera & Willink, 1980; Leuenberger, 2002; Font, 2014; Las Peñas *et al.*, 2017; Köhler *et al.*, 2020a; Köhler *et al.*, 2020a; Köhler *et al.*, in prep.). In the Pampean and Espinal provinces, *Opuntia rioplatensis*<sup>1</sup> Font is one of the most conspicuous prickly pears of those vegetation types (Font, 2014; Köhler *et al.*, 2018; Las Peñas *et al.*, 2017).

The prickly pear cacti are not only culturally important – notably in Mexico (Anderson, 2001; Chávez-Moreno *et al.*, 2009) – but are also ecologically and economically prominent (Inglese *et al.*, 2017; Nobel, 2002). Members of the group are cultivated worldwide for a multitude of purposes, such as for food, ornamentals, biofencing, fodder, as well as for production of natural dye from cochineal insects (Nobel, 2002; Ranjan *et al.*, 2016). This has been accompanied by the selection and development of several cultivars and/or varieties with specific traits, with *Opuntia ficus-indica* (L.) Mill., from Mexico, the most well known (Cervantes-Herrera *et al.*, 2006; Griffith, 2004; Kiesling, 1998; Kiesling & Metzger, 2017; Reyes-Agüero *et al.*, 2005).

During a broad floristic, taxonomic, and systematic study of the southern South American species of *Opuntia*, a distinct morphotype was observed in the Chaquenan region of the Santa Fe province, Argentina (Fig. 1). Our preliminary observations revealed it as an unidentified taxon of what morphologically appeared to be the introduced *O. ficus-indica* (provisionally *O. aff. ficus-indica*), however, which differed from *O. ficus-indica* in its stem, pericarpel, flower, and fruit features (see Fig. 4, and discussion). Here, we present additional studies combining our fieldwork, herbarium research, and morphological observations with molecular phylogenetics and cytogenetic analyses to better assess the identity of that taxon.

## Materials and methods

### Studied area, plant materials, and taxonomic work

Comprehensive fieldwork was carried out in southern South America spanning the major biogeographic regions

to obtain data about the natural populations of several species of *Opuntia*. The region is represented by different vegetation types in several biogeographic units within the Chaquenan domain – i.e. ‘Pampeana’, ‘Espinal’, ‘Chaquena’ and ‘Monte’ (*sensu* Cabrera & Willink, 1980; Pennington *et al.*, 2000; Prado, 2000; Oyarzabal *et al.*, 2018). We studied materials housed in the major herbaria of the region to check specimen identification and records of *Opuntia* species, i.e. BA, BAF, BCWL, CGMS, COR, CORD, CTES, HAS, ICN, LIL, LP, MBM, MCN, MVFA, MVJB, MVM, SI, UNR (all herbarium acronyms follow *Index Herbariorum* (Thiers, 2021+), except BCWL, non-indexed herbarium, of the Biological Control of Weeds Laboratory (FuEDEI), Hurlingham, Buenos Aires, Argentina), as well as digital materials available at GBIF.org (2021) and SpeciesLink platform (2021). We surveyed the major bibliographic literature that comprises classical treatments, which include the diversity of *Opuntia* species (Anderson, 2001; Backeberg, 1958, 1966; Bravo-Hollis, 1937; Britton & Rose, 1919; Hunt *et al.*, 2006; Leuenberger, 2002; Ritter, 1979, 1980; Schumann, 1899) to assess morphological and diagnostic features of the hitherto known species, mostly delimited using the morphological species concept (Stace, 1989). From that literature, a second revision was taken accessing the magna opera that contains further original descriptions through the use of electronic libraries and virtual databases (i.e. Tropicos.org, Botanicus.org, International Plant Names Index, JSTOR Global Plants, Biodiversity Heritage Library, and other specific journals). By checking the affinities of the studied morphotype (*Opuntia aff. ficus-indica*) to *O. ficus-indica* and *O. rioplatensis* (see details in the Results section), we further conducted additional observations on representative specimens of those species, as well as specialized literature (Adli *et al.*, 2017; Cervantes-Herrera *et al.*, 2006; Font, 2014; Kiesling & Metzger, 2017; Las Peñas *et al.*, 2017; Reyes-Agüero *et al.*, 2005a,b; Wiersma, 2008) to assess morphologically distinctive characters (habit, cladodes, spine production, pericarpel, flower and fruit features) checking descriptions and illustrations associated with the species and specimens.

### DNA sampling, sequence alignment, and phylogenetic analyses

We first gathered molecular sequences available on GenBank. The previous sequenced materials from Köhler *et al.* (2020b) were downloaded using functions of R package ape (Paradis *et al.*, 2004, R Core Team, 2018), and imported into Geneious 11.1.5 (Biomatters, Auckland, New Zealand). These sequences correspond to the chloroplast genes markers *ccsA*, *rpl16*, *trnK* including *matK* (*trnK/matK*), and the intergenic spacer

<sup>1</sup>*Opuntia rioplatense* in original spelling (Font, 2014). However, *Opuntia* is a feminine genus, thus, the epithet must combine with this gender with the *ensis* ending: *Opuntia rioplatensis*.

*trnL-trnF*, covering a selected taxon sampling of the southern South American Elatae clade of *Opuntia*, the North American Humifusa clade, and the outgroup *Brasiliopuntia brasiliensis* (Willd.) A. Berger (sensu Majure et al., 2012a). We newly sequenced five other taxa to better cover the previously mentioned clades, two accessions of *Opuntia* aff. *ficus-indica*, and one accession of *Opuntia ficus-indica* (information for all sampled materials and sequences analysed are available in Supplemental Table S1). All new sequences were generated via genome skimming following methods described in Köhler et al. (2020a, b). Then, a reference mapping pipeline was conducted using the Geneious mapper feature to assemble the same molecular markers downloaded from GenBank for the newly sequenced materials. To test for phylogenetic incongruences between chloroplast and nuclear genomes, and a putative hybrid signal, we also assembled complete sequences of the nuclear ribosomal repeat DNA (nrDNA; including the ETS, 18S, ITS-1, 5.8S, ITS-2, and 28S regions), using the same iterative mapping approach as described above (adapted from Ripma et al., 2014). The nrDNA of *Cylindropuntia bigelovii* (Engelm.) F.M. Knuth (Majure, unpublished data) was used as the reference to initiate the iterative mapping, and the sequences were then annotated by transferring the annotations from it to the sequences.

A multiple sequence alignment was performed across all samples for sequences of each marker using the MAFFT v. 7 (Kato & Standley, 2013) plugin in Geneious with default settings. The chloroplast markers were concatenated and treated as one sequence for downstream analyses (cpDNA), and the nuclear ribosomal repeat was treated as another independent sequence (nrDNA: ETS + 18S + ITS-1 + 5.8S + ITS-2 + 28S). We used PartitionFinder 2 (Lanfear et al., 2017) to evaluate the best scheme partition and molecular model of evolution for each of the sequences setting each independent marker as data blocks. We then explored three datasets for phylogeny reconstruction: (1) the chloroplast sequences only (cpDNA); (2) the nuclear ribosomal repeat sequences only (nrDNA); and (3) the combined/concatenated sequences (cpDNA + nrDNA). Each of these datasets was analysed using maximum likelihood (ML) and Bayesian inference (BI) approaches. The ML analyses were implemented in RAxML 8.2.4 (Stamatakis, 2014) using the best scheme partition and the GTR + G model of molecular evolution. Support values were estimated by implementing 1,000 bootstrap pseudoreplicates. The BI analyses were performed using MrBayes v.3.2.7a (Ronquist et al., 2012), with two independent runs of 10 million generations and four chains per run, sampling trees every 1,000 generations, and a chain temp of 0.2

and uniform priors. Chain convergence, stationarity, and estimated sample size (ESS) were assessed in Tracer 1.7 (Rambaut et al., 2018) by visually examining plots of parameter values and log-likelihood against the number of generations. Convergence and stationarity were accepted when the average standard deviation of split frequencies reached 0.01 or less, and ESS was considered satisfactory when > 200, discarding the first 20% of samples as burn-in. All analyses were carried out on CIPRES Science Gateway Web Portal (Miller et al., 2010).

## Cytogenetic analyses

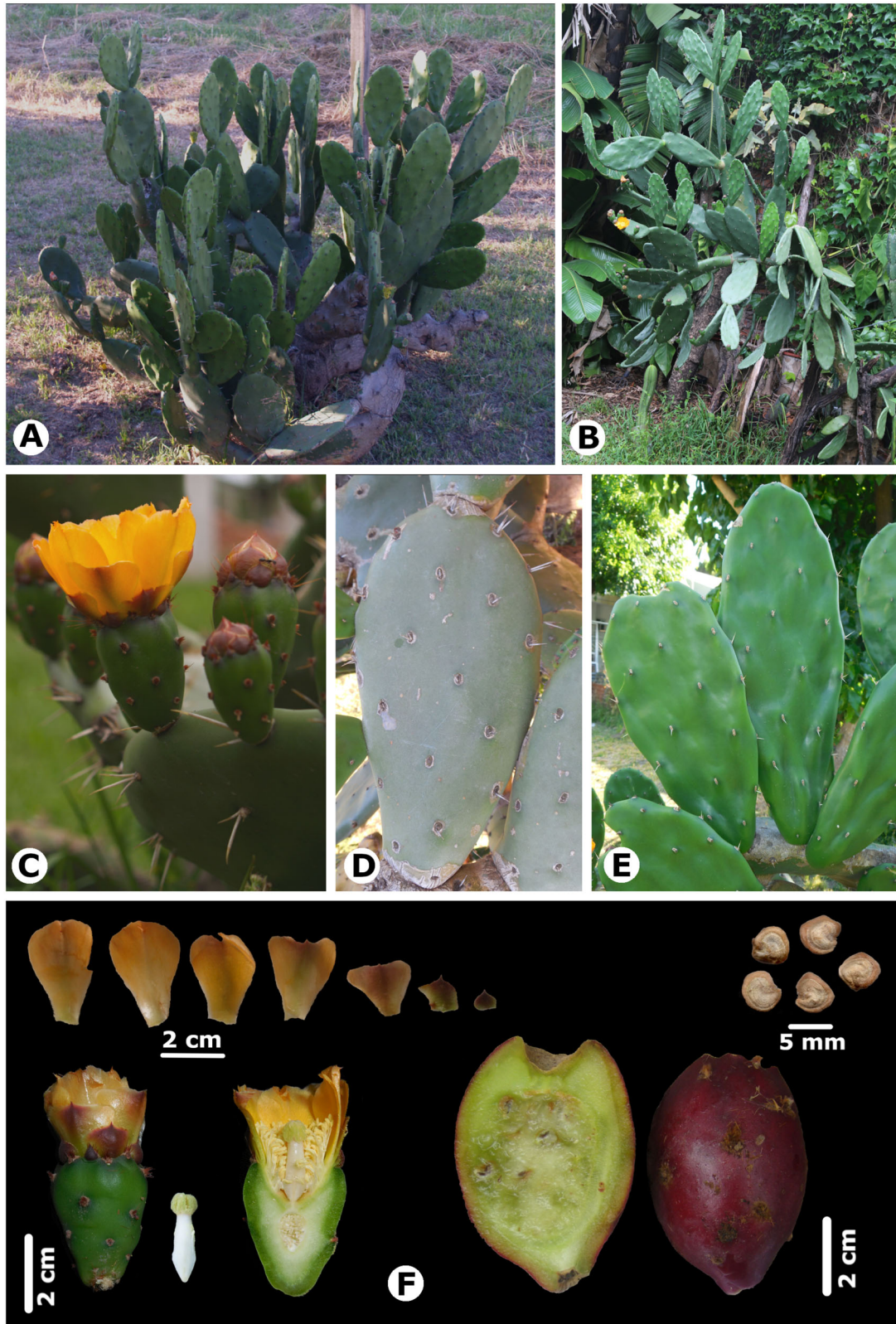
Adventitious root tips were collected from cultivated vegetative propagules of *Opuntia* aff. *ficus-indica*. The roots were pre-treated with 2 mM 8-hydroxyquinoline for 24 h at 4 °C and fixed in Carnoy solution (3:1, ethanol: acetic acid). The root tips were washed twice in distilled water (10 min), digested with Pectinex® (Novo Nordisk TM) at 37 °C for 30 min, and squashed in a drop of 45% acetic acid; after, the coverslip was removed in liquid nitrogen, and stored at –20 °C. For the CMA/DAPI banding, slides were prepared with adaptations of Schweizer and Ambros (1994): the slides were first stained with a drop of 0.5 mg/mL chromomycin A<sub>3</sub> (CMA) for 90 min, washed with distilled water, and then stained with 2 µg/mL 4'-6-diamidino-2-phenylindole (DAPI) for 30 min (both stains from Sigma-Aldrich), and mounted in McIlvaine's buffer–glycerol (1:1 v/v). For the fluorescent *in situ* hybridization (FISH), we followed the protocol described by Schwarzacher and Heslop-Harrison (2000). Briefly, we used the pTa71 as a probe to identify the rDNA 18S-5.8S-26S loci (Gerlach & Bedbrook, 1979), and a specific probe from *Pereskia aculeata* Mill. for analysis of the 5S rDNA loci (Las Peñas et al., 2011). The DNA fragments were labeled with biotin-14-dUTP (Bionick, Invitrogen, Carlsbad, California, USA) and digoxigenin-11-dUTP (DIG Nick Translation Mix, Roche Diagnostics, Mannheim, Germany) by nick translation, and subsequently detected with avidin-FITC (Sigma-Aldrich) and Anti-DIG-Rhodamine (Roche Diagnostics). Cytogenetic data of other species not generated in this study were gleaned from the literature (Ahumada et al., 2020; Las Peñas et al., 2017; Realini et al., 2014).

## Results and discussion

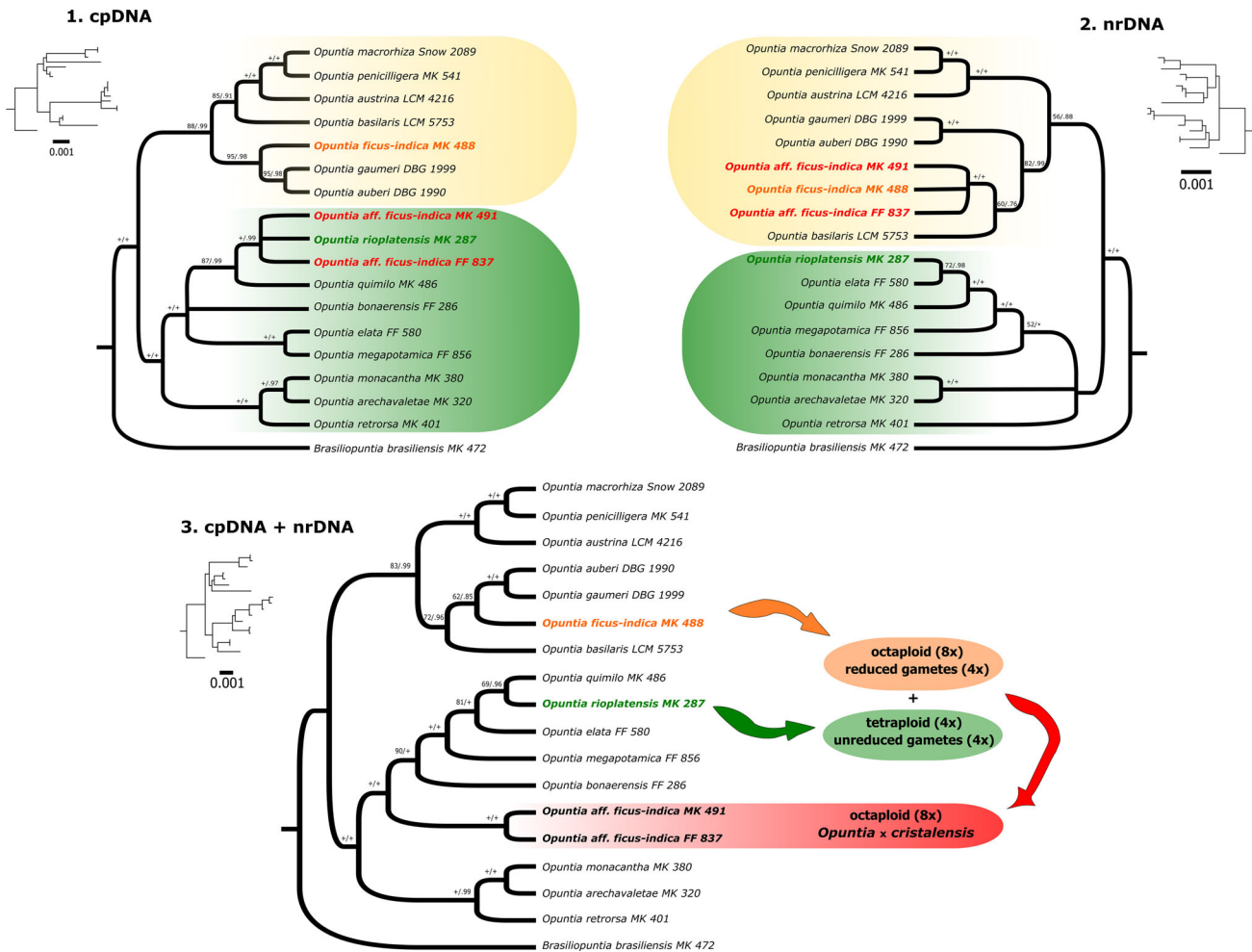
### Cytonuclear discordance and polyploidy

Our resulting alignments were 5,491 base pairs (bp) in length (including gaps) for the chloroplast sequence (cpDNA), 6,439 for the nuclear ribosomal repeat





**Fig. 1.** Morphological features of *Opuntia* aff. *ficus-indica* (*Opuntia* × *crystalensis*). (A, B) Habit. (C) Detailed pericarpel showing acute bud flower, and orange opened flower. (D) Old flattened stems (cladodes) with glaucous epidermis. (E) New bright dark-green cladodes with elongated elliptic cladodes and spines. (F) Reproductive features: on the left below, flower in longitudinal section and detailed pistil; on the upper-left, tepals; on the right below, fruits in longitudinal section; on the upper-right, seeds.



**Fig. 2.** Phylogenetic relationships of *Opuntia* aff. *ficus-indica* (*Opuntia* × *cristalensis*) evidencing cytonuclear discordance. On the upper-left, analysis based on chloroplast markers shows *O. aff. ficus-indica* closely related to *O. rioplatensis*; on the upper-right, nuclear ribosomal repeat DNA evidencing *O. aff. ficus-indica* embedded within *O. ficus-indica* clade; on the bottom, concatenated sequences supporting *O. aff. ficus-indica* as a distinct lineage, with the hybrid hypothesis highlighted. Nodes support are depicted above the branches (BS/PP, + means 100% or 1.0 p.p.; \* unavailable value due to incongruent topology).

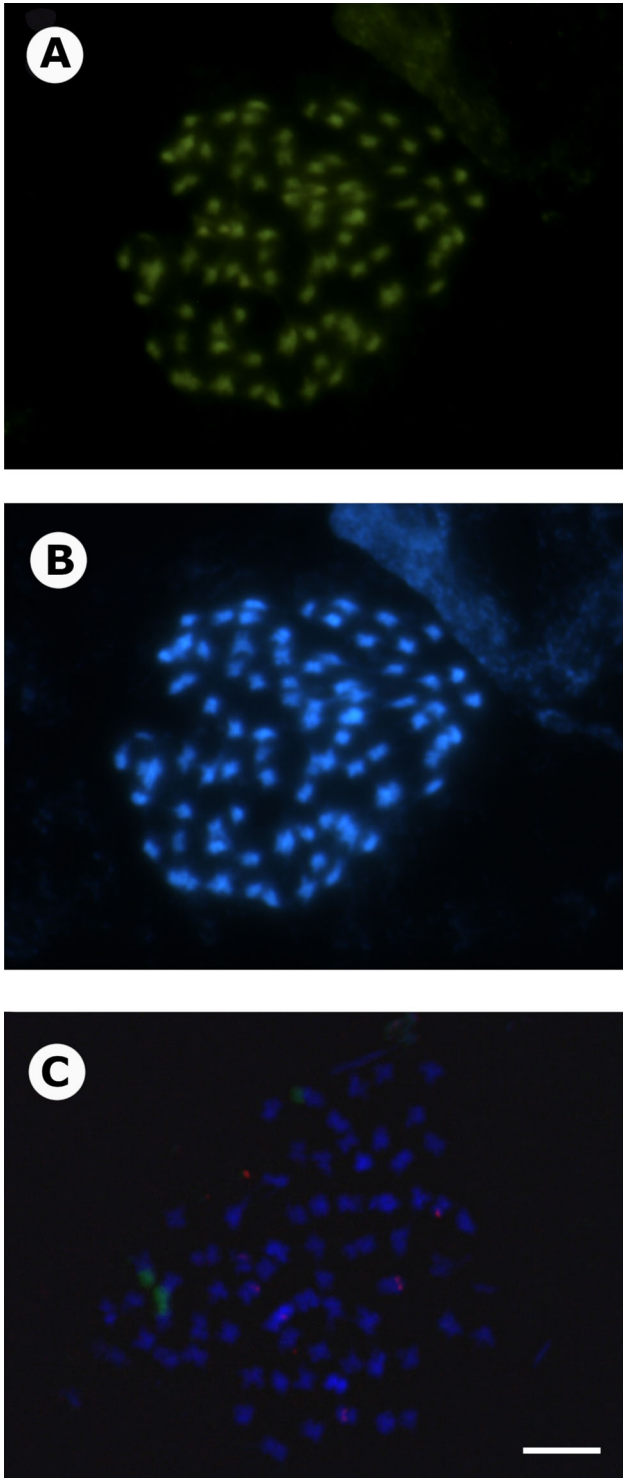
(nrDNA), and 11,930 for the combined (cpDNA + nrDNA) sequences. The cpDNA alignment had 96 distinct patterns, 52 parsimony-informative sites (PIS), and 5,385 constant sites; while the nrDNA alignment had 86 distinct patterns, 59 PIS and 6,339 constant sites. When combined (cpDNA + nrDNA), our alignment had 161 distinct patterns, 111 PIS and 11,724 constant sites.

The three datasets recovered the two geographically structured clades previously reported by Majure et al. (2012a), i.e. one comprising the North American species and the other the South American species (Fig. 2, yellow and green highlighted clades), except for the taxon formerly thought to be endemic to Argentina (*Opuntia penicilligera* Speg.), which was again resolved within the North American clade as closely related with *O. macrorhiza* Engelm., suggesting a North American origin (Majure et al., 2020). Overall, the analyses (ML and

BI) within each dataset were congruent in lineage relationships and statistical support (see Fig. 2 for detailed bootstrap and posterior probabilities values (BS/PP), as well as lineage relationships).

The cpDNA dataset resolved our accession of *Opuntia ficus-indica* as a sister lineage of the Nopalea clade (represented by *O. gaumeri* (Britton & Rose) R.Puente & Majure and *O. auberi* Pfeiff., Fig. 2.1, orange colour label), with which it forms a clade sister to the Basilares (represented by *O. basilaris* Engelm. & J.M. Bigelow) + Humifusa clade (*O. austrina* Small + *O. penicilligera* + *O. macrorhiza*) (sensu Majure et al., 2012a). This is congruent with previous analyses, which suggested that the putative parental taxa of the polyploid *O. ficus-indica* may likely be from the Basilares and the Nopalea clade (Majure et al., 2012a). In contrast, our two accessions of *O. aff. ficus-indica* were strongly supported



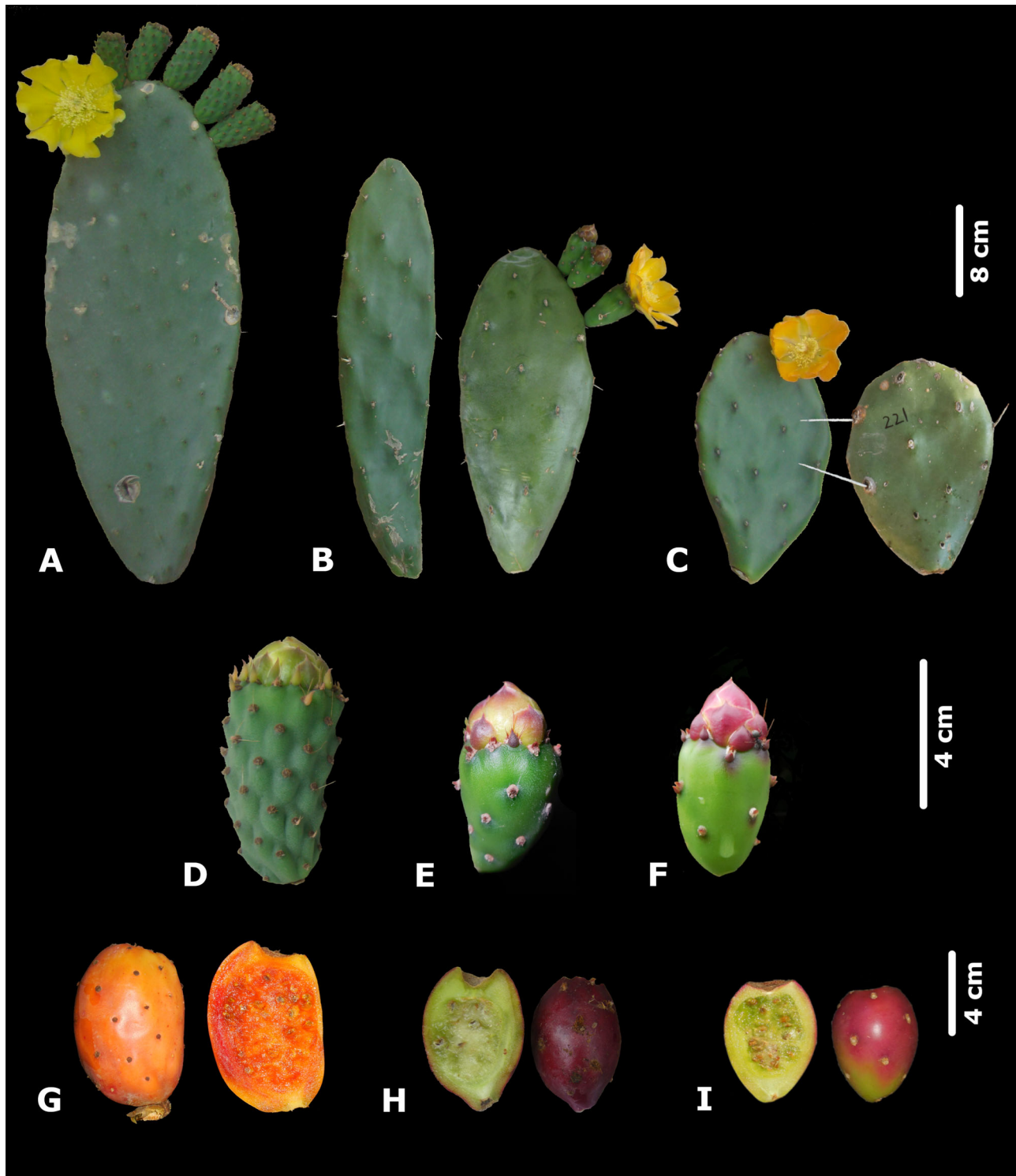


**Fig. 3.** Cytogenetic features of *Opuntia* × *cristalensis*. (A) Fluorochrome chromosome banding with CMA fluorescence. (B) Fluorochrome chromosome banding with DAPI fluorescence and CMA<sup>+</sup>/DAPI<sup>-</sup> NOR associated bands. (C) FISH using 18-5.8-26S (green) and 5S (red) rDNA probe to metaphase chromosomes. Scale bar = 5 μm (all images are to the same scale).

with the South American species forming a clade with *O. rioplatensis* under the cpDNA dataset (Fig. 2.1, red colour label). On the other hand, when exploring the nrDNA dataset, the two accessions of *O. aff. ficus-indica* were well-supported within the North American clade nested with *O. ficus-indica* (Fig. 2.2). Otherwise, the concatenated dataset (cpDNA + nrDNA) supported the accessions of *O. aff. ficus-indica* as a distinct lineage within the South American clade (Fig. 2.3, red highlighted).

Our cytogenetic analyses found *Opuntia* aff. *ficus-indica* as octoploid ( $2n = 88$ , Fig. 3). The chromosomes are small with a mean length (C) of 3.32 μm, and the total haploid karyotype length (TLH) of 36.46 μm. The CMA/DAPI banding technique revealed two chromosome pairs with CMA<sup>+</sup>/DAPI<sup>-</sup> bands associated with nuclear-organized regions (NORs) comprising a percentage of 5.51% of the TLH (Fig. 3A, B). The 18S-5.8S-26S rDNA sites were coincident with the CMA<sup>+</sup>/DAPI<sup>-</sup> signals associated with the NOR band of the two chromosome pairs described above (Fig. 3C). The 5S rDNA site banding was located on four chromosome pairs in a pericentromeric position (Fig. 3C). The size, number, and banding intensity of all nrDNA loci were similar among homologous chromosomes (Fig. 3C).

Previous studies have consistently reported counts for *Opuntia rioplatensis* as tetraploid ( $2n = 44$ , Majure *et al.*, 2012b, as *O. quitensis* F.A.C.Weber; Realini *et al.*, 2014, as *O. elata* Salm-Dyck; Las Peñas *et al.*, 2017, as *O. elata* var. *obovata* E.Walther; Köhler, unpublished data). On the other hand, *O. ficus-indica* has been recorded as diploid, pentaploid, hexaploid, and octoploid (see Majure *et al.*, 2012b, Ahumada *et al.*, 2020, and references therein). However, considering that the circumscription of *O. ficus-indica* may vary (Kiesling, 1998, 1999; Kiesling & Metzger, 2017; Reyes-Agüero *et al.*, 2005), most of those records may include other taxa instead of *O. ficus-indica*. Nevertheless, Ahumada *et al.* (2020) conducted the most comprehensive and taxonomically updated study comprising the two forms of *O. ficus-indica* in Argentina, invariably reporting octoploid ( $2n = 88$ ) counts for *O. ficus-indica* f. *ficus-indica* and hexaploid ( $2n = 66$ ) for *O. ficus-indica* f. *amyclaea*. All *Opuntia* species here studied had small chromosomes and symmetrical karyotypes mostly composed of metacentric and submetacentric chromosomes, and base number  $x = 11$ , which has been frequent across other members of Cactaceae (Las Peñas *et al.*, 2009, 2014, 2017, 2019, and references therein). This phenomenon has been related to karyotypic orthoselection, preserving relatively similar complements in Opuntioideae (Las Peñas *et al.*, 2009, 2017, and references therein).



**Fig. 4.** Morphological comparison between *Opuntia ficus-indica* (always on the left), the putative hybrid *O. × cristalensis* (in the middle), and *O. rioplatensis* (always on the right). *Opuntia ficus-indica* (A) cladodes and flower, (D) flower bud, (G) fruits. *Opuntia × cristalensis*: (B) cladodes and flower, (E) flower bud, (H) fruits. *Opuntia rioplatensis*: (C) cladodes and flower, (F) flower bud, (I) fruits.

Our analyses revealed a pattern of phylogenetic incongruence between the chloroplast (cpDNA) and the nuclear ribosomal DNA (nrDNA) markers involving the

accessions of *Opuntia* aff. *ficus-indica* (Fig. 2). Events like this – i.e. cytonuclear discordance – have become commonly reported in both plant and animal





**Fig. 5.** Distribution map of the studied taxa. *Opuntia ficus-indica* is widely introduced in many world regions and is here schematized only for the region of interest. The putative overall distribution of *Opuntia x cristalensis* is suggested with the known records highlighted.

phylogenetic studies (Bruun-Lund *et al.*, 2017; Huang *et al.*, 2014; Lee-Yaw *et al.*, 2019), but considering that different factors can lead to phylogenetic incongruence, detecting the processes underlying this phenomenon can be difficult (Som, 2015). In our dataset, we highlight two major biological factors that can be related to the observed pattern: incomplete lineage sorting (ILS) or hybridization. While ILS represents the extant retention of an ancestral polymorphism during successive speciation events, which failed to coalesce back in time in the most recent common ancestors (Maddison & Knowles, 2006; Som, 2015), hybridization is the mixing

of previously isolated gene pools (Rieseberg & Wendel, 1993). ILS is more likely to be observed between lineages with large effective population sizes relative to their time of divergences (Maddison & Knowles, 2006; Meng & Kubatko, 2009), which may not be the case observed here. *Opuntia ficus-indica* is a species domesticated for a long time (Kiesling, 1998; Kiesling & Metzger, 2017) with low sequence divergence among closely related species (Labra *et al.*, 2003; Majure *et al.*, 2012a), and a very narrow genetic base among the spineless accessions (Caruso *et al.*, 2010). Furthermore, *O. ficus-indica* and *O. rioplatensis* are greatly diverged

and resolved in disparate clades, with the former in the North American clade (Griffith, 2004; Majure et al., 2012a), whereas *O. rioplatensis* is well supported within the southern South American clade (Fig. 2; Köhler et al., 2020a, unpublished data).

### Putative hybrid speciation and taxonomy

Species are a fundamental pillar in biology and the foundation for an array of downstream analyses. However, the definition of a species in plants has been a constantly debated and controversial topic (Rieseberg & Willis, 2007; Soltis & Soltis, 2009), especially because of frequent reports of interspecific hybrids which violate the biological species concept prevailing for animal species (Mayr, 1999). In this context, hybrid speciation has been recognized as an important mechanism in plant speciation in general, as well as in angiosperm evolution (Hegarty & Hiscock, 2005; Mallet, 2007; Soltis & Soltis, 2009). Furthermore, in the age of climate change, auto- and allopolyploidy are expected to be the most dominant modes of speciation, overwhelming the classical lineage splitting mode (Gao, 2019; Levin, 2019).

Our analyses suggested that the taxon found in Santa Fe, Argentina, is a putative hybrid between *O. ficus-indica* and *O. rioplatensis*. Morphological observations conform with this hypothesis, both by the expectation of hybrids exhibiting morphological intermediate features of their putative parents, as well as the introduction of novel traits (Rieseberg et al., 1993; Soltis & Soltis, 2009). *Opuntia* aff. *ficus-indica* resembles *O. ficus-indica* by the elongated elliptic to obovate flattened stem (cladodes) (Figs 1 and 4). Older cladodes of *O. aff. ficus-indica* also exhibit glaucous epiderms (Fig. 1D), which is a common feature in *O. ficus-indica* (Fig. 4A) (Reyes-Agüero et al., 2005; Kiesling & Metzger, 2017). On the other hand, *O. aff. ficus-indica* resembles *O. rioplatensis* by the comparatively short non-tuberculate pericarpel, with acute flower buds, orange-coloured flowers (Fig. 4), and by the obovate fruits with the green inner-pericarp and pulp (Fig. 4).

*Opuntia* aff. *ficus-indica* is octoploid (8×), and assuming a hybrid origin, we hypothesize it could have formed by the merger of reduced parental gametes of *O. ficus-indica* (4×) with unreduced maternal gametes of *O. rioplatensis* (4×) (Fig. 2.3). Both putative parental species occur in the area where *O. aff. ficus-indica* has been found (Fig. 5). *Opuntia rioplatensis* is an endemic of the grasslands of southern South America in the Pampa biome or *Rio de la Plata* grasslands and savannas of southern South America in two biogeographic units of the Chaquénian domain ('Pampeana' and

'Espinal', but also with small populations in the south-east edge of 'Chaqueña' – locally called "*Cuña Boscosa Santafesina*", Lewis & Pire, 1981), occurring in central and eastern Argentina, central-western Uruguay and extreme south-western Brazil (Font, 2014; Las Peñas et al., 2017; Köhler et al., 2018; Fig. 5), but has already been introduced as an ornamental species outside of its native range, i.e. California and Arizona states of the USA (Walther, 1930; Köhler, pers. observations). On the other hand, *O. ficus-indica* has been introduced worldwide, such as in Argentina (Kiesling, 1998; Fig. 5), and is widely cultivated for fruit production and fodder for livestock.

The combined matrix (cpDNA + nrDNA, Fig. 2.3) increased bootstrap support values for some non-reticulate species relationships but obfuscated the relationship of *O. aff. ficus-indica* with the North American clade, which could obscure the placement of the putative hybrid. In addition, the combined analysis supported *O. aff. ficus-indica* as a distinct lineage in the South American clade, non-related with the putative closest maternal parent due to the signal from the ribosomal data. These are caveats frequently highlighted when inferring phylogenetic relationship of putative hybrids with ribosomal data (Álvarez & Wendel, 2003; Feliner & Rosselló, 2007; Soltis et al., 2008), reinforcing the importance of incorporating more data – such as cytological, geographic, and morphological – when including reticulate taxa in phylogenies (Majure, in press).

In *Opuntia*, hybridization and polyploidization is recurrently invoked as a crucial factor generating diversity (Granados-Aguilar et al., 2020; Grant & Grant, 1979; Griffith, 2001, 2003; Majure et al., 2012a, 2017; Majure & Puente, 2014; Pinkava, 2002; Puente & Hamann, 2005; Rebman & Pinkava, 2001). Roughly 60% of the known diversity in tribe Opuntieae is polyploid (Majure et al., 2012b). Many species can live in sympatry, overlapping in flowering and sharing pollinators which may lead to interspecific pollen flow and natural hybrid formation (Grant & Grant, 1979; Osborn et al., 1988; Schlindwein & Wittmann, 1997). Accompanied with the many environmental stress conditions in which *Opuntia* species may live (e.g. water limitation), events of polyploidization can be triggered by the formation of unreduced gametes, as well as several other genomic instabilities (Levin, 2019; Pinkava, 2002; Ramsey & Schemske, 1998; Van de Peer et al., 2021).

Whilst gene flow and interspecific hybridization are known and speculated in *Opuntia*, mechanisms of reproductive isolation are also reported between sympatric and closely related species (Fachardo & Sigrist, 2020). This is a key process in plant hybrid speciation, in which new hybrid lineages can establish pre- or post-



zygotic barriers contributing to genetic divergence and isolation. *Opuntia* aff. *ficus-indica* produces fertile fruits with apparently viable seeds (Fig. 1F), which provides evidence for the taxon not being a temporary infertile hybrid. Given that we are still lacking additional studies regarding the reproductive biology of this hybrid ensuring its species status, we describe the taxon as a new nothospecies: *Opuntia* × *crystalensis* Oakley, Font & M.Köhler (see “Taxonomic proposal” below).

As far as we know, this is the first report of hybridization between a southern South American lineage and a North American species of *Opuntia*. During our literature revision, we found one mention of a spineless cultivar of *O. ficus-indica* proceeding from La Plata, Argentina (Burbank, 1907; USDA, 1901; Wiersma, 2008). *Opuntia ficus-indica* cv. “Anacantha” (not *Opuntia anacantha* Speg.) is described in Luther Burbank’s catalogue of spineless *Opuntias* useful for cultivation (Burbank, 1907). During the early 20th century, Burbank was the most famous plant breeder of his day (Smith, 2010), introducing several new plant varieties, and cooperating in agricultural experiments from the US Department of Agriculture, which was introducing several seeds and plants in the country. In his catalogue (Burbank, 1907), Burbank made two mentions to the variety “Anacantha”: one referring to the US accession 3423 (“*Opuntia anacantha*”, USDA, 1901, from Argentina), and the other to the US 9352 (“*Opuntia ficus-indica*”, USDA, 1905, from Malta). Apparently, Burbank was confused about the materials under propagation, associating the spineless form of *O. ficus-indica* from Malta with “Anacantha”, which also means “without spines” (but refers to another taxon, *Opuntia anacantha* Speg., non-related to *O. ficus-indica*). Our analyses revealed that none of the materials described by Burbank (Pinkava, 2002; Wiersma, 2008) or by the USDA (USDA – U.S. Department of Agriculture, 1901, 1905) are related to our studied material, here described as *Opuntia* × *crystalensis* (previously *O. aff. ficus-indica*).

The role of human activities in species hybridization is well known (Anderson & Stebbins, 1954), and the introduction of exotic species may trigger scenarios leading to new cases (Guo, 2014; Vilà *et al.*, 2000). Our report of hybridization between a North American *Opuntia* species and a southern South American species reinforces the potential impacts of introducing exotic plants in new areas, producing hybrid zones, in which complex patterns of reticulation may underlie future evolutionary scenarios.

### Taxonomic proposal

*Opuntia* × *crystalensis* Oakley, Font & M.Köhler  
nothosp. nov. (Fig. 1; Fig. 4B, E, H).

(*Opuntia ficus-indica* (L.) Mill. × *Opuntia rioplatensis* Font)

**TYPE:** ARGENTINA. Prov. Santa Fe: Depto. Vera, 20 km E of the Nacional Road 11, towards the lagoon “El Cristal”, 30°01′00.45″S, 60°07′02.91″W, 33 m asl, collected in Zavalla (Santa Fe, Argentina) from cultivated material grown from the original locality, M. Köhler 491, 9 November 2018 (holotype ICN).

**Diagnosis:** It is morphologically similar to *Opuntia rioplatensis*, but can be distinguished from it by the usual presence of small spines in all areoles of the cladodes, and the cladodes elongated elliptic, spatulated to obovate. It is also morphologically similar to *O. ficus-indica*, but can be distinguished from it by the non-tuberculate pericarpels, and the shorter obovate fruits.

**Description:** *Shrub*, erect, 1.5–3 m tall; strongly branched, with the oldest stem segments subcylindrical, lignified, forming a well-developed greyish-green rhytidome with irregular longitudinal fissures. *Stem segments (cladodes)* 18–40 cm × 8–15 cm, 1.8–3 cm thick, spatulated, elliptic, or obovate to subrhomboid; the younger more elliptic to obovate, lustrous dark-green; the older ones more subrhomboid to long-elliptic, greyish-green, opaque. *Areoles* 18–22 per cladode face, 2–4(–5) mm in diameter, elliptic, covered by a grey-white indumentum up to 3 mm long; areoles with secondary growth, expanding up to 1.2 cm × 0.9 cm. *Leaves* conic to subulate, green to vinaceous, 3–7 mm long, caducous, not appressed, ascending. *Glochids* present, ferruginous, but not abundant in cladode areoles, more frequent in pericarpel. *Spines* 2–2.5(–3) cm long, acicular, white to light grey, 0–2(–3) per areole, antrorse, or retrorse. *Pericarpel* 4.5 cm × 2.5 cm, obconic to obpyriform, dark-green; 18–20 areoles around the surface, not tuberculated, covered with ferruginous glochids, brush-like, up to 2 mm long. *Flower bud apex* acute, mucronate. *Flowers* numerous, 6.5–7 cm in diameter at anthesis, developing along the edges from the upper third towards the apex of cladodes; external tepals mucronate, triangular, trapezoidal to deltoid, dark-red to vinaceous; inner tepals spatulate to elliptic-obovate, orange, up to 3 cm × 1.7 cm; external petaloid tepals mucronate, with a darker longitudinal line in its upper third; internal petaloid tepals obtuse and somewhat lacerate apex. *Stamens* numerous, 2.5 cm long, creamy coloured filaments and anthers. *Stigma* 7–8 lobed, connivent, light-green to eventually cream-coloured, 0.6 cm × 1 cm. *Style* obclaviform, 2–2.2 cm × 0.5–0.7 cm, white-cream. *Ovary* obovoid, 0.7–0.9 cm × 1–1.4 cm, in the upper part of the pericarpel, numerous ovules. *Fruit* obovate to elliptic, (4–)4.5–5(–7) cm × (3–)3.5–4.2(–4.5) cm,



semicircular umbilicate markedly concave, vinaceous to dark-red when ripe, inner pericarpel light greenish, pleasant aroma; ovarian cavity 2.3–3.6 cm × 1.7–2.7 cm, with abundant fleshy placenta inside. *Seed* glabrous, lenticular, light-brown to beige, few, 4–5 mm in diam., 1.5 mm thick.

**Distribution and habitat:** As far as is known, *Opuntia* × *cristalensis* is only observed in the north-east region of the Santa Fe Province (Argentina), where it grows on the edge of xerophytic forests. It is also recorded ~70 km NW of the type locality under cultivation for fruit consumption (Fig. 5). In both areas, *O. rioplatensis* is part of the native flora, whereas *O. ficus-indica* is occasionally cultivated in home gardens and small farms of the vicinity.

**Etymology:** The epithet derives from “*El Cristal*”, the locality around the lagoon of the same name, where the plant was collected for the first time.

**Chromosome number:**  $2n = 88$  (octaploid, Fig. 3).

**Additional examined material (Paratype):** ARGENTINA. Prov. Santa Fe, Dpto. Vera. La Gallareta, cultivado en el pueblo, 13 November 2009, F.Font 571 (BAF, BCWL). [Cultivated Accession F.Font (FF) 837, Castelar, Prov. Buenos Aires, Argentina].

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## Supplemental material

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## ORCID

Matias Köhler  <http://orcid.org/0000-0003-1863-6309>  
Luis J. Oakley  <http://orcid.org/0000-0001-5006-1053>  
Fabián Font  <http://orcid.org/0000-0003-0118-7092>  
M. Laura Las Peñas  <http://orcid.org/0000-0003-4244-7807>  
Lucas C. Majure  <http://orcid.org/0000-0002-0369-1906>

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