UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

POSGRADO EN CIENCIAS BIOLÓGICAS

INSTITUTO DE BIOLOGÍA
SISTEMÁTICA

Origen de *Opuntia tehuacana* (Cactaceae) y su relación filogenética con especies simpátricas

TESIS

QUE PARA OPTAR POR EL GRADO DE:

DOCTORA EN CIENCIAS

PRESENTA:

GRANADOS AGUILAR XOCHITL CITLALMINA

TUTOR PRINCIPAL DE TESIS: DR. ÁNGEL SALVADOR ARIAS MONTES
JARDÍN BOTÁNICO, INSTITUTO DE BIOLOGÍA, UNAM
COMITÉ TUTOR: DR. ULISES YUNUEN ROSAS LÓPEZ
JARDÍN BOTÁNICO, INSTITUTO DE BIOLOGÍA, UNAM
DR. ANTONIO GONZÁLEZ RODRÍGUEZ
INSTITUTO DE INVESTIGACIONES EN ECOSISTEMAS Y SUSTENTABILIDAD, UNAM

CIUDAD UNIVERSITARIA, CD. MX. 2022

UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

POSGRADO EN CIENCIAS BIOLÓGICAS

INSTITUTO DE BIOLOGÍA
SISTEMÁTICA

Origen de *Opuntia tehuacana* (Cactaceae) y su relación filogenética con especies simpátricas

TESIS

QUE PARA OPTAR POR EL GRADO DE:

DOCTORA EN CIENCIAS

PRESENTA:

GRANADOS AGUILAR XOCHITL CITLALMINA

TUTOR PRINCIPAL DE TESIS: DR. ÁNGEL SALVADOR ARIAS MONTES
JARDÍN BOTÁNICO, INSTITUTO DE BIOLOGÍA, UNAM
COMITÉ TUTOR: DR. ULISES YUNUEN ROSAS LÓPEZ
JARDÍN BOTÁNICO, INSTITUTO DE BIOLOGÍA, UNAM
DR. ANTONIO GONZÁLEZ RODRÍGUEZ
INSTITUTO DE INVESTIGACIONES EN ECOSISTEMAS Y SUSTENTABILIDAD, UNAM

CIUDAD UNIVERSITARIA, CD. MX. 2022

COORDINACIÓN DEL POSGRADO EN CIENCIAS BIOLÓGICAS

INSTITUTO DE BIOLOGÍA

OFICIO CPCB/1192/2021

ASUNTO: Oficio de Jurado

M. en C. Ivonne Ramírez Wence
Directora General de Administración Escolar, UNAM
P r e s e n t e

Me permito informar a usted que en la reunión ordinaria del Comité Académico del Posgrado en Ciencias Biológicas, celebrada el día **27 de septiembre de 2021** se aprobó el siguiente jurado para el examen de grado de **DOCTORA EN CIENCIAS** de la estudiante **GRANADOS AGUILAR XOCHITL CITLALMINA** con número de cuenta **102002285** con la tesis titulada **"Origen de *Opuntia tehuacana* (Cactaceae) y su relación filogenética con especies simpátricas",** realizada bajo la dirección del **DR. ÁNGEL SALVADOR ARIAS MONTES,** quedando integrado de la siguiente manera:

Presidente:	DRA. SONIA VÁZQUEZ SANTANA
Vocal:	DR. PEDRO MERCADO RUARO
Vocal:	DRA. SOFÍA SOLÓRZANO LUJANO
Vocal:	DRA. TERESA MARGARITA TERRAZAS SALGADO
Secretario:	DR. ANTONIO GONZÁLEZ RODRÍGUEZ

Sin otro particular, me es grato enviarle un cordial saludo.

A T E N T A M E N T E
"POR MI RAZA HABLARÁ EL ESPÍRITU"
Ciudad Universitaria, Cd. Mx., a 07 de diciembre de 2021

COORDINADOR DEL PROGRAMA

DR. ADOLFO GERARDO NAVARRO SIGÜENZA

POSGRADO EN CIENCIAS BIOLOGICAS
COORDINACIÓN

Agradecimientos

Al posgrado en Ciencias Biológicas, Universidad Nacional Autónoma de México, así como al Jardín Botánico e Instituto de Biología, gracias a los cuales he podido llevar a cabo mi formación académica durante la tesis de Licenciatura y Doctorado. También agradezco el apoyo del CONACyT por la beca No. 453987 otorgada para mi manutención durante los años de estudio del Doctorado y a la beca PAPIIT No. IN208619, gracias a la cual fue posible realizar la investigación para mi proyecto.

Agradezco a la UNAM, por educarme desde Iniciación Universitaria hasta el Doctorado, así como a toda su infraestructura de Laboratorios que me permitieron realizar una muy buena investigación.

A los miembros de comité tutor, por todo su apoyo, comentarios, sugerencias e información compartida para enriquecer mi conocimiento y poderme formar como científica.

Dr. Salvador Arias Montes, por tener una visión amplia y nunca dejarse influenciar por comentarios ajenos a la academia y así permitirme continuar con mi Doctorado. Agradezco que me haya dejado conocer un grupo de plantas tan genial como las Cactáceas, también que me haya permitido formar parte de su grupo de trabajo durante estos años, que fueron muy gratificantes.

Dr. Ulises Rosas, agradezco por sus valiosos comentarios y preguntas que me retaron a investigar y ampliar mi panorama. También por su ayuda en el laboratorio, así como la corrección y mejora de los títulos de los capítulos que integran este trabajo.

Dr. Antonio González, agradezco sus comentarios y visión diferente de la perspectiva filogenética en la que siempre me enfocaba, también por prestarme su libro el cual he leído completo más de dos veces, muchas gracias.

Agradecimientos a título personal

Al jurado para examen de grado, Dra. Sonia Vázquez Santana, Dr. Pedro Mercado Ruaro, Dra. Teresa Margarita Terrazas Salgado, por sus correcciones para mejorar esta tesis, muchas gracias.

A la Dra. Carolina Granados, por todo su apoyo para la publicación del artículo de redes filogenéticas, todas sus recomendaciones y consejos, muchas gracias.

A la Maestra Miriam Ladd Otero, por su apoyo en el uso y análisis de datos del citómetro de flujo.

Al personal del Laboratorio Nacional de Citometría de Flujo, por su apoyo y por hacerme sentir parte de su comunidad, muchas gracias.

A la M. en C. Andrea R. Jiménez, M. en C. Laura Márquez Valdelamar y M. en C. Nelly María López Ortiz por su ayuda en los Laboratorios Nacionales de Biodiversidad del Instituto de Biología de la UNAM.

Al M. en C. Javier Ramírez Ramón por su invaluable apoyo en la técnica de conteo de cromosomas, sin la cual este trabajo no podría haberse llevado a cabo.

Agradezco todo el apoyo de mi mamá durante toda mi vida para que nunca me faltara nada, pudiera estudiar y dedicarme a algo que me gusta tanto como la Biología, te agradezco todo el apoyo que me diste incluso cuando parecía que no iba a estudiar nada o que no iba a hacer un doctorado, gracias por entender y apoyarme.

También agradezco el invaluable apoyo de mi tía Ofelia, gracias por hacerme mi fruta todos los días, por escucharme, por prestarme tu auto, por hacerme deliciosa comida todos los días después de un largo día en el Instituto, en verdad muchas gracias.

A mi tía Lety y mis primos, por alegrarme y distraerme del estrés que causa el posgrado, gracias por las horas de risa y debate.

A mi amiga Gabriela Espino, muchas gracias por escuchar todos mis problemas personales y del doctorado, en verdad me da mucho gusto haberte conocido y que me siguieras hablando, aunque te lo prohibieron, jejeje. Recuerdo lo felices que éramos en el laboratorio, en verdad la pasamos muy bien.

Agradezco a Yolanda Morales su apoyo para poder realizar todas mis actividades en el Laboratorio de Cactáceas e Invernadero, gracias también por escucharme y permitirme ser tu amiga. Agradezco a mis compañeros del Laboratorio de Cactáceas, Cristian Cervantes, Daniel Franco, David Aquino e Isaura Rosas por su apoyo en cuanto a dudas y materiales de consulta sobre Cactáceas.

Finalmente, agradezco a aquellos que me pusieron el pie para verme caer pero que, como el ave fénix resurgí de las cenizas y con más fuerza, gracias.

Índice

Lista de figuras y cuadros

Resumen

La hibridación natural es un proceso común en plantas. Particularmente, Cactaceae presenta numerosos ejemplos de este proceso, siendo la subfamilia Opuntioideae la más conocida por presentar hibridación en muchos de sus géneros. Un miembro de esta subfamilia conocido por sus problemas taxonómicos debido a poliploidía e hibridación es *Opuntia*. Dentro de este género existen múltiples especies silvestres, las cuales son un laboratorio natural para estudiar procesos de hibridación y evolución reticulada. El objetivo del presente trabajo es conocer el marco de referencia actual sobre hibridación natural en la familia Cactaceae y en particular analizar el probable origen híbrido de *Opuntia tehuacana* utilizando evidencias moleculares y citológicas. Realizamos una revisión de artículos científicos sobre hibridación en Cactaceae en Web of Science y Google Scholar para conocer el estado actual de la hibridación natural en la familia. Utilizamos tres marcadores de cloroplasto (*matK, psbJ-petA* y *ycf1*) para ubicar a *O. tehuacana* en un contexto filogenético amplio, adicionalmente mediante minado de genoma y transcriptoma identificamos dos nuevas regiones nucleares (*AT3G48380* y *AT1G18270*). Con los marcadores de cloroplasto y nucleares se realizaron análisis de Inferencia Bayesiana, redes filogenéticas y análisis de invariantes bajo el modelo coalescente. Adicionalmente realizamos conteo de cromosomas para tres especies de *Opuntia* y análisis de citometría de flujo en cinco localidades de *O. tehuacana*. Los reportes de hibridación natural en Cactaceae se presentan únicamente en dos de las cuatro subfamilias. En Cactoideae ocurren principalmente entre especies de distintos géneros y estos son principalmente descriptivos, mientras que en Opuntioideae la hibridación ocurre entre especies del mismo género y hay evidencia de su impacto en la evolución de esta subfamilia. En un marco filogenético amplio *O. tehuacana* se encuentra en el clado *Basilares*, todas sus terminales forman un clado monofilético, su ploidía es de 11x y 12x y existen diferencias significativas en el tamaño de genoma al interior de la especie. Además, se encontró introgresión en algunos individuos de *O. tehuacana* proveniente de las especies *O. decumbens* y *O. huajuapensis*. Se concluye que *O. tehuacana* es una especie con poliploide, cuyas barreras al intercambio genético son semi-permeables, ya que se encontró evidencia genética y citológica de que existe intercambio genético con sus especies simpátricas, pero

se descarta que sea un híbrido entre las especies *O. pilifera* y *O. huajuapensis* como había sido sugerido previamente.

Palabras clave: análisis filogenéticos, hibridación natural, Opuntioideae, *Opuntia*, poliploidía, redes filogenéticas.

Abstract

Natural hybridization is a common process in plants. Particularly, Cactaceae has numerous examples of this process, being subfamily Opuntioideae the best known to present hybridization in many genera. *Opuntia* is a member of this subfamily known for its taxonomic problems due to polyploidy and hybridization. There are multiple wild species within this genus, which are a natural laboratory to study hybridization processes and reticulated evolution. This work aimed to know the current status of natural hybridization in the family Cactaceae and to analyze the probable hybrid origin of *Opuntia tehuacana* using molecular and cytological evidence. We performed a review of papers about Cactaceae hybridization in the Web of Science and Google Scholar to know the current state of natural hybridization in the family. We used three chloroplast markers (*matK, psbJ-petA* and, *ycf1*) to place *O. tehuacana* in a broad phylogenetic context, additionally by genome and transcriptome mining we identified two new nuclear regions (*AT3G48380* and *AT1G18270*). With the chloroplast and nuclear markers, Bayesian inference, phylogenetic networks and invariant analysis under the coalescent model were performed. Additionally, we made chromosome count for three species of *Opuntia* and flow cytometry analysis in five localities of *O. tehuacana*. Reports of natural hybridization in Cactaceae are present only in two of the four subfamilies. In Cactoideae reports are mainly descriptive and occur primarily between species of different genera, while in Opuntioideae, hybridization occurs between species of the same genus, and there is evidence of its impact on the evolution of the subfamily. In a broad phylogenetic framework, *O. tehuacana* is in the *Basilares* clade; all its terminals form a monophyletic clade, its ploidy is 11x and 12x, and there are significant differences within the species in its genome size. Furthermore, introgression was found into *O. tehuacana*

individuals from the species *O. decumbens* and *O. huajuapensis*. It is concluded that *O. tehuacana* is a polyploid species, whose barriers to gene exchange are semi-permeable because we found genetic and cytologic evidence of gene exchange with its sympatric species, but it is not a hybrid between *O. pilifera* and *O. huajuapensis* as suggested before.

Key words: Opuntioideae, *Opuntia*, natural hybridization, phylogenetic analysis phylogenetic network, polyploidy.

Introducción general

A lo largo de la historia del estudio de la biología, la hibridación ha captado la atención desde naturalistas como Linneo y Darwin, hasta biólogos evolutivos del periodo de la síntesis moderna de la teoría de la evolución (Arnold, 2006; Mallet, 2007). Botánicos de esa época consideraron a este proceso como relevante para la evolución, mientras que los zoólogos consideraron a la hibridación como una herramienta importante en la comprensión de la especiación (Arnold, 1997). En plantas, a diferencia de los animales, la hibridación ocurre de manera natural y es común en algunas familias. Se estima que se presenta en un 40% de las plantas vasculares (Whitney et al., 2010).

La hibridación puede definirse como la mezcla exitosa de dos linajes diagnosticablemente distintos, la cual ocurre entre individuos de dos poblaciones o de grupos de poblaciones (Arnold 1997). En un sentido amplio podría decirse que la hibridación ocurre entre especies distintas (pudiendo ser entre especies del mismo género o diferentes géneros) pero el concepto anteriormente descrito evita la polémica dentro de los conceptos de especie (Mayr, 1942; Arnold, 1997; Harrison and Larson, 2014). La definición de hibridación suele contener a dos procesos, por una parte, la especiación por hibridación, en la cual una población híbrida (proveniente de la mezcla de dos poblaciones parentales) forma un nuevo linaje; por otra parte, incluye a la introgresión, proceso mediante el cual se incorpora material genético en el genoma de una población por entrecruza o retrocruza, sin aún llegar al punto de diferenciarse de las poblaciones parentales (Elworth et al., 2018).

El intercambio genético puede ocurrir de manera artificial por medio de intervención antropogénica, o de manera espontánea en la naturaleza siendo conocida como hibridación natural (Arnold 1997). Para que ocurra hibridación natural es necesaria una unión exitosa en la naturaleza de gametos entre individuos provenientes de poblaciones distintas, las cuales pueden distinguirse unas de otras con base en uno o más caracteres heredables (Harrison, 1993; Arnold, 1997). Esta unión de gametos puede ocurrir entre individuos con diferentes números cromosómicos llevando a eventos de alopoliploidización. La hibridación y la poliploidía están muy relacionados y cuando se presentan en conjunto existe evidencia de su impacto en la evolución y especiación (Soltis, 2013).

Existe una gran cantidad de familias de plantas en las que se reporta hibridación algunos ejemplos incluyen a las familias Pinaceae Spreng. ex Rudolphi (*Pinus*), Iridaceae Juss. (*Iris*), Fagaceae Dumort. (*Quercus)*, Cactaceae Juss. (*Opuntia*, *Cylindropuntia*), Rosaceae Juss. (*Fragaria, Rosa, Lachemilla*), Asteraceae Bercht. & J. Presl (*Helianthus*, *Tragopogon*) y Violaceae Batsch. (*Viola*). En Cactaceae existen numerosos reportes de hibridación natural en las subfamilias Cactoideae Eaton. y Opuntioideae Burnett. (Anderson, 2001; Pinkava, 2002; Hunt et al., 2006; Machado, 2008) y se estima que el 26% de especies en la familia son poliploides (Pinkava, 2002).

La hibridación en Cactaceae, aunque ampliamente estudiada, no ha sido abordada con el mismo método para todos los taxa. En particular en la subfamilia Cactoideae existen géneros de interés comercial u ornamental como *Selenicereus* (A. Berger) Britton & Rose o *Astrophytum* Lem. en los cuales se realizan cruzas experimentales para mejorar ciertas

características, permitiendo conocer posibles escenarios de hibridación, que no ocurren en la naturaleza (Tel-Zur et al., 2004; Montanucci, 2015). Mientras que en la subfamilia Opuntioideae la hibridación natural ha sido ampliamente documentada e involucra procesos como evolución reticulada y poliploidía. Por ello conocer los procesos mediante los cuales ocurre la hibridación en la naturaleza a partir del intercambio genético entre individuos de distintas especies permite un mejor entendimiento de procesos como la especiación por hibridación, haciendo a los miembros de Opuntioideae un excelente modelo de estudio.

El género *Opuntia* Mill. agrupa el mayor número de especies (ca. 150 especies) en la subfamilia Opuntioideae, 93 de las cuales se distribuyen en México (Hunt et al., 2006). Este género muestra un patrón de distribución natural amplio en América, desde Canadá hasta Argentina (Barthlott et al., 2015). Este género se caracteriza por tener hojas vestigiales deciduas, tallos planos llamados cladodios, aréolas con glóquidas, espinas y en algunos casos cerdas, flores generalmente hermafroditas con tépalos de colores amarillo, anaranjado, rosa o rojo y frutos dulces conocidos como tunas o xoconostles (Bravo-Hollis, 1978; Arias et al., 2012). Las especies de *Opuntia,* que reciben el nombre común de nopales, tienen una gran importancia económica por sus tallos y frutos comestibles, ecológica al fungir como plantas nodrizas, proveer refugio a insectos, aves y mamíferos, así como medicinal y cultural (Bravo-Hollis, 1991). Los nopales son conocidos por presentar múltiples problemas taxonómicos debido a la amplia variación en sus caracteres morfológicos. Esta variación se puede explicar en parte por factores como la poliploidía, la hibridación y en algunos casos por plasticidad en caracteres morfológicos (Majure et al., 2012b).

La hibridación en *Opuntia* se presenta debido a la ausencia de límites reproductivos entre especies cercanamente emparentadas, cuya descendencia suele mantenerse por propagación vegetativa, posteriormente estabiliza su número cromosómico y puede llegar a producir gametos viables, dando origen a probables retrocruzas (Griffith, 2001; Pinkava, 2002; Scheinvar et al., 2015). En este contexto, diversos estudios se han enfocado en entender el origen híbrido tanto en taxa silvestres como cultivados, utilizando diversos métodos, entre los que destacan, cruzas experimentales, conteos cromosómicos, análisis filogenéticos y utilización de marcadores moleculares altamente variables para encontrar a las probables especies parentales así como diferencias entre especies cercanamente emparentadas (Griffith, 2001, 2003; Pinkava, 2002; Segura et al., 2007; Griffith y Porter, 2009; Majure et al., 2012a).

Las especies silvestres son un laboratorio natural para estudiar procesos de hibridación y evolución reticulada (Rieseberg et al., 1993). El proceso de reticulación involucra la mezcla de dos o más linajes evolutivos independientes, en tres niveles diferentes: cromosomas, genomas y especies. A nivel de especie, procesos como la especiación por hibridación y la transferencia horizontal de genes (proceso mediante el cual los genes se transfieren entre especies) son las principales causas de la evolución reticulada (Linder et al., 2004) (Figura 1).

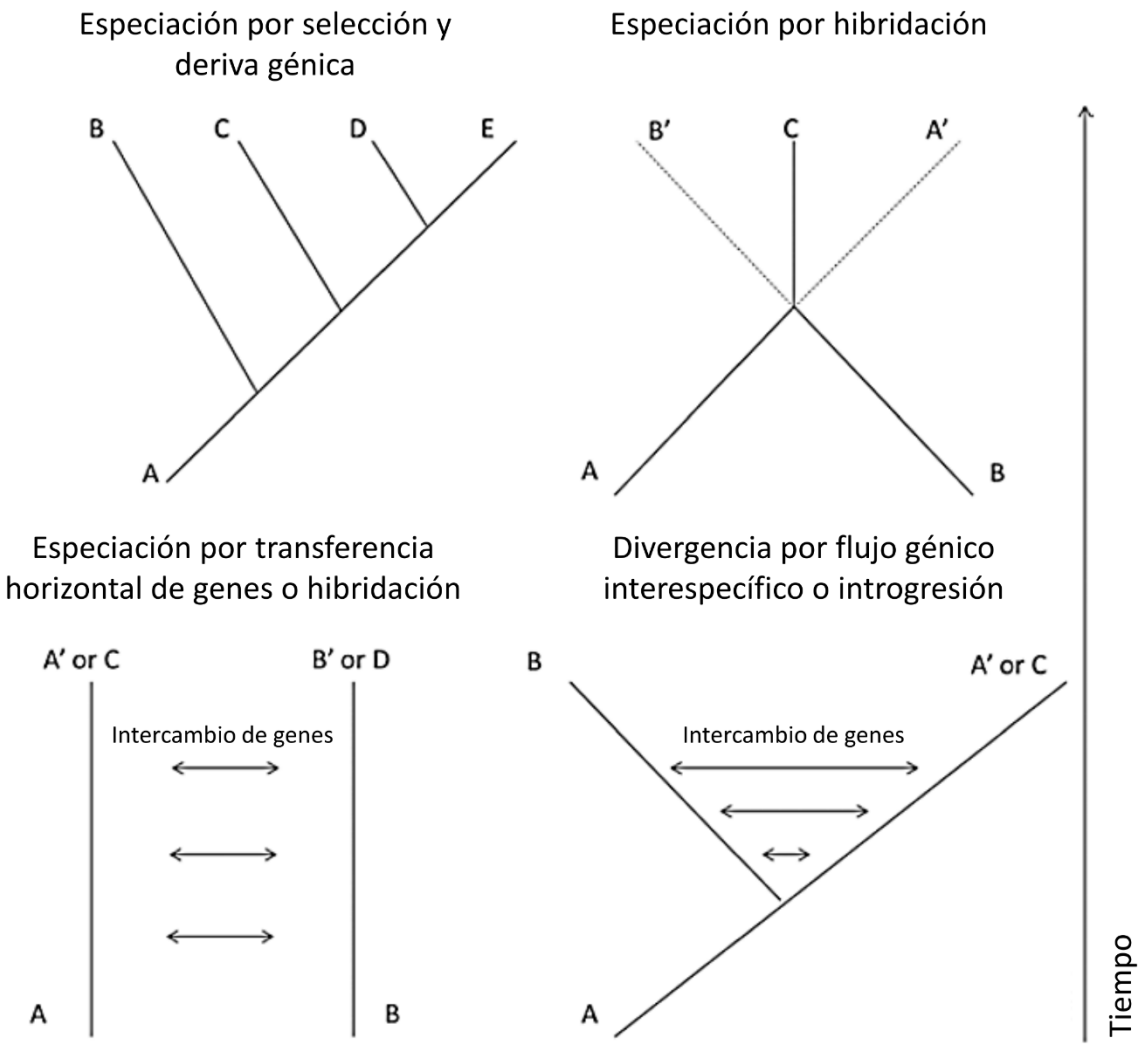


Figura 1. Tipos de especiación y su visualización grafica. Modificado de (Gontier, 2015).

El presente trabajo se enfoca en *Opuntia tehuacana* S. Arias & U. Guzmán (Fig. 1.3 A), que es una especie silvestre endémica del Valle de Tehuacán-Cuicatlán, y que no ha sido manejada ni cultivada. Esta especie se caracteriza por su hábito arbustivo, flores amarillas a rojo-anaranjadas con frutos verde-amarillos, los cuales a veces no producen semillas, por lo que desde su descripción se sugiere que podría ser un híbrido (Arias et al., 2012). Los individuos de esta especie son morfológicamente parecidos a los de *Opuntia pilifera* F.A.C.

Weber (Fig. 1.3 E) y *Opuntia huajuapensis* Bravo (Fig. 1.3 C). Estas especies presentan una distribución geográfica simpátrica con *O. tehuacana* y con las cuales se postula una probable relación parental debido a que algunos caracteres morfológicos como la presencia de tricomas en las aréolas, la forma del cladodio y del fruto son potencialmente intermedios entre las especies anteriormente mencionadas (Arias et al., 2012). Con base en este marco teórico el objetivo general del presente trabajo fue hacer una revisión del estatus actual de la hibridación natural en la familia Cactaceae y explorar mediante evidencias moleculares y citológicas si *O. tehuacana* es de origen híbrido entre las especies *O. huajuapensis* y *O. pilifera*. Los objetivos particulares de esta tesis se centran en analizar los patrones de hibridación natural reportada en Cactaceae, sus probables causas genéticas y reproductivas, así como las implicaciones filogenéticas y evolutivas, enfatizando en la subfamilia Opuntioideae; conocer la posición filogenética de *O. tehuacana* y su relación con especies simpátricas utilizando marcadores del cloroplasto (*matK*, *ycf1* y *psbJ-petA*) y nucleares de copia simple (*AT3G48380* y *AT1G18270*), así como conocer la ploidía de *O. tehuacana* y sus supuestas especies parentales, así como la cantidad de ADN 2C en cinco localidades de *O. tehuacana* e inferir si presenta hibridación de primera generación. Finalmente identificar a las probables especies parentales *O. tehuacana*, mediante un análisis de redes filogenéticas e invariantes bajo el modelo coalescente (marcadores nucleares; *AT3G48380* y *AT1G18270*).

Capítulo 1. The prickly problem of interwoven lineages: hybridization processes in Cactaceae

Xochitl Granados-Aguilar, Ulises Rosas, Antonio González-Rodríguez and Salvador Arias

Botanical Sciences (sometido)

The prickly problem of interwoven lineages: hybridization processes in Cactaceae

Xochitl Granados-Aguilar[1, 2*], Ulises Rosas[2], Antonio González-Rodríguez[3] and Salvador Arias[2]

[1]Posgrado en Ciencias Biológicas, Instituto de Biología, Universidad Nacional Autónoma de México, Mexico City, Mexico.

[2]Jardín Botánico, Instituto de Biología, Universidad Nacional Autónoma de México, Mexico City, Mexico.

[3]Instituto de Investigaciones en Ecosistemas y Sustentabilidad, Universidad Nacional Autónoma de México, Morelia, Michoacán, Mexico.

* Author for correspondence: xochitl.granados@st.ib.unam.mx

Running title: Hybridization processes in Cactaceae

Author contributions: Xochitl Granados-Aguilar (https://orcid.org/0000-0001-7570-4211), hybridization investigation, methodology conceptualization, writing the first draft, text revision, and editing; Ulises Rosas (https://orcid.org/0000-0001-5088-2679) text revision, and editing; Antonio González-Rodríguez (https://orcid.org/0000-0002-6196-7288) text revision, and editing; Salvador Arias (https://orcid.org/0000-0002-7674-7050) researcher leading of this study and obtained the financial support.

Supporting Agencies: UNAM-DGAPA-PAPIIT <IN208619>

Abstract

Background: Hybridization in nature occurs in numerous botanical families. Particularly, the Cactaceae family displays a large number of genera in which hybridization is reported. **Questions:** Which are the patterns of reported natural hybridization in Cactaceae and their probable causes? Are there phylogenetic and evolutionary implications related to hybridization, particularly in Opuntioideae? **Data description:** A total of 62 articles about natural hybridization and classical Cactaceae literature was reviewed. **Study site and dates:** Since 1900 to June 2021 **Methods:** A search in Web of Science and Google Scholar with the keywords "Cactaceae hybridization" and in time span we selected "all years" as well as information from classic family monographs by Anderson (2001) and Hunt *et al*. (2006). **Results:** Natural hybrids in Cactaceae are included in two of the four recognized subfamilies, Cactoideae and Opuntioideae. Mechanisms of reproductive isolation are often inferred as non-selective, but there is no evidence for all taxa. In thirty-six articles analyzed of Cactoideae members, the main approach used was morphological description, and the tribe with the highest number of natural hybrids was Trichocereeae. In Opuntioideae the eighteen articles reviewed performed mostly chromosome counts, morphometric and phylogenetic analysis, and the tribe Opuntieae showed the highest number of natural hybrids. **Conclusions:** It has been suggested that hybridization impacts the evolution of Cactoideae and Opuntioideae, but only few studies formally tested this hypothesis. For the postulation

that hybridization impacts the evolution of Opuntioideae, there is evidence of the importance of this process in their evolution unlike what occurs in Cactoideae, on which hybrid reports are mainly descriptive.

Keywords: Discordant phylogenies, natural hybridization, Opuntioideae, reticulate evolution, speciation.

Resumen

Antecedentes: La hibridación natural se presenta en numerosas familias botánicas. Particularmente Cactaceae presenta un gran número de géneros en los que se reporta hibridación.

Preguntas: ¿Cuáles son los patrones reportados de hibridación natural en Cactaceae y sus probables causas? ¿Existen implicaciones filogenéticas y evolutivas relacionadas a la hibridación, particularmente en Opuntioideae?

Descripción de datos: Se revisó un total de 62 artículos sobre hibridación natural y literatura clásica de Cactaceae.

Sitio y años de estudio: Desde 1900 a junio de 2021

Métodos: Una búsqueda en Web of Science y Google Scholar con las palabras clave "Cactaceae hybridization" y un lapso de tiempo de " all years ", así como información de las monografías clásicas de Anderson (2001) y Hunt *et al*. (2006).

Resultados: Los híbridos naturales en Cactaceae están incluidos en dos de las cuatro subfamilias reconocidas, Cactoideae y Opuntioideae. Los mecanismos de aislamiento reproductivo a menudo se infieren como no selectivos, pero no hay evidencia para todos los taxones. En treinta y seis artículos analizados para miembros de Cactoideae, el enfoque principal fue descripción morfológica, y la tribu con el mayor número de híbridos naturales fue Trichocereeae. Para Opuntioideae en los dieciocho artículos revisados se realizaron

principalmente recuentos cromosómicos, análisis morfométricos y filogenéticos, y la tribu Opuntieae mostró el mayor número de híbridos naturales.

Conclusiones: Se sugiere que la hibridación impacta en la evolución de Cactoideae y Opuntioideae, pero pocos estudios probaron formalmente esta hipótesis. Para la postulación de que la hibridación impacta en la evolución de Opuntioideae, hay evidencia de la importancia de este proceso en su evolución, a diferencia de lo que ocurre en Cactoideae, con informes de híbridos principalmente descriptivos.

Palabras clave: Filogenias discordantes, especiación, evolución reticulada, hibridación natural, Opuntioideae.

Hybridization is a process that occurs in plants from ferns to angiosperms (Whitney *et al*. 2010) and is considered a process that impacts the speciation and evolution of the groups where it is present (Anderson 1953, Arnold 2004, Soltis & Soltis 2009, Soltis 2013). It is a natural and common process in numerous families, with some classic examples of hybridization occurring in Asteraceae (*Helianthus*), Cactaceae (*Opuntia*), Fagaceae (*Quercus*), Iridaceae (*Iris*), Pinaceae (*Pinus*) and Rosaceae (*Lachemilla, Rosa*) (Critchfield 1986, Rieseberg 1991, Jensen *et al*. 1993, Pinkava 2002, Arnold 2006; Meng *et al.* 2011, Morales-Briones *et al*. 2018). Although numerous studies have been conducted in these families, it is still difficult to fully understand the dynamics and outcomes of hybridization in these groups.

Hybridization has been defined as the mixture of two diagnostically distinct lineages, which can occur between different species or within a species (Arnold 1997). The genetic exchange can occur between individuals from different populations, or lineages belonging to any other taxonomic category; the result of this crossing is known as a hybrid (Arnold

1997, Soltis 2013). The genetic exchange can occur by artificial or natural crossing; when spontaneous hybridization occurs in nature without anthropogenic intervention it is known as natural hybridization (Arnold 1997).

Cactaceae has approximately 1,438 to 1,851 species distributed in four subfamilies, Pereskioideae, Maihuenioideae, Cactoideae, and Opuntioideae, the last two being the most diverse with about 1,221 and 176 species, respectively (Hunt *et al*. 2006, Korotkova *et al*. 2021). Members of Cactaceae exhibit a high number of evolutionary novelties to arid environments like crassulacean acid metabolism (CAM), presence of waxes and trichomes (Anderson 2001), as well as a wide range of growth forms like globular, globose-depressed, cylindrical, columnar, and articulated (Vázquez-Sánchez *et al*. 2012).

In particular, the family Cactaceae has numerous genera in which natural hybridization has been reported, according to classical taxonomic reviews and some recent studies (Anderson 2001, Pinkava 2002, Hunt *et al*. 2006, Machado 2008), mainly occurring in subfamilies Cactoideae and Opuntioideae. The Cactoideae (Figure 1 A and B) has the most charismatic genera in the family, being widely studied in terms of description of new genera and species based mainly on morphological characters. The hybrids found among members of this subfamily have been considered infrequent, and their impact on evolution has been poorly studied; furthermore, there are also few published works focused on demonstrating the existence of those hybrids (Machado 2008).

Opuntioideae is known by its areoles with glochids, an articulated growth pattern related to vegetative dispersion, and orbicular seeds covered by a bony aril (Anderson 2001)

111 (Figure 1 B). The morphological characteristics that have allowed this group to adapt to
112 extreme environmental conditions also make its members underrepresented in biological
113 collections due to the difficulty of collecting specimens (Pinkava 2002, Majure *et al*.
114 2012b). Despite this, hybridization has been comparatively better understood in this group,
115 which, along with polyploidy, have been pointed out to play a role in the evolution of the
116 species (Pinkava 2002).

117 Phylogenetic analyses carried out in the Cactaceae subfamilies have revealed that some
118 traditional classifications at the generic level are not representative of true phylogenetic
119 relationships between species considered as belonging to certain genera. Some examples
120 include phylogenetic analysis carried out in the tribes Cacteae (Vázquez-Sánchez *et al*.
121 2013) and Hylocereeae (Korotkova *et al*. 2017), as well as the genus *Astrophytum*
122 (Vázquez-Lobo *et al*. 2015), *Cephalocereus* (Tapia *et al*. 2017), *Opuntia* (Majure *et al*.
123 2012b) and *Pereskia* (Butterworth & Wallace 2005). Although for the tribes and genera
124 mentioned above, their phylogenetic relationships have been better understood, yet there
125 are still other groups in this family in which relationships at the level of genus or species
126 have not been resolved due to processes such as incomplete lineage sorting (ILS) or
127 hybridization (Majure *et al*. 2012b, Copetti *et al*. 2017, Granados-Aguilar *et al*. 2021).

128 It has been proposed that hybridization is a fundamental part of the evolutionary process
129 in Cactaceae. Hence our objective was to analyze the genera reported with natural
130 hybridization, its probable causes, as well as the phylogenetic and evolutionary
131 implications on the groups that present it, emphasizing the case of Opuntioideae. A

previous hybridization review (Machado 2008) summarizes examples of putative hybrids in Cactoideae, but more than a decade after, an update is necessary to review recent evidence allowing us to move forward in the study of this process. The particular objectives of the current review are: I) to carry out a literature review of natural hybrids reported for Cactaceae in classical monographs and scientific articles, II) to briefly summarize examples of prezygotic and postzygotic barriers in Cactaceae, III) to evaluate the evidence of the role that natural hybridization plays in the evolution of Cactaceae subfamilies, and IV) to identify if there is evidence of unresolved or discordant phylogenetic histories due to hybridization in Opuntioideae.

Materials and methods

We used as information sources the classic family monographs by Anderson (2001) and Hunt *et al*. (2006) as well as a search in the Web of Science with the keywords "Cactaceae hybridization", and in the time period we selected "all years". Of the 72 articles obtained in the search, 18 were excluded because they only used some of the keywords, but their main topic was not hybridization in Cactaceae. In addition, a search was conducted in Google scholar with the same search criteria and eight other articles were found. Appendix 1 was integrated with the information collected, following the taxonomic classification reported by Hunt (2006).

Discussion

Reproductive isolation in Cactaceae. Interspecific gene flow in Cactaceae, as in other angiosperms, is mediated by prezygotic barriers, which include asynchronous flowering

periods, different pollination syndromes, incompatible crosses, and postzygotic barriers, which prevent hybridization avoiding the formation of seeds and reducing the quantity and quality of the progeny (Kay 2006, Baack *et al*. 2015). Very few studies have focused on knowing the reproductive system of cacti, with less than 5 % of the species having such evidence (Mandujano *et al*. 2010). Furthermore, there are very few studies on the existence of barriers to genetic exchange, so the knowledge about pollen-pistil interactions and hybrid formation is mainly based on inferences and extrapolations about what may be occurring in nature.

Pollination in Cactaceae is carried out mainly by bees, birds, bats and sphingid moths; other insects such as butterflies, beetles, wasps, and ants have been reported as relevant in some groups (Anderson 2001, Schlumpberger 2011). The pollinators are responsible for transporting the pollen to the gynoecium, which accepts or rejects it through an incompatibility system (Guzmán *et al*. 2013). In the case of this family, there is evidence that in some genera this system is not so selective, allowing genetic exchange between individuals from different species or even different genera (Rowley 1994, Anderson 2001). If this occurs, the postzygotic barriers come into action, as in the case of abortion of the hybrid embryo through the death of the endosperm, preventing seed formation (Marks 1966, Nishiyama & Yabuno 1979, Cisneros & Tel-Zur 2012). Another significant postzygotic barrier is the ploidy of the embryo, because hybridization between diploid and tetraploid species can give rise to triploid zygotes, which have no stability in their chromosomal number and are aborted (Baack *et al*. 2015, Tel-Zur *et al*. 2020). Despite those mentioned above, in Cactaceae, there is evidence of triploid hybrids in the genus

175 *Cylindropuntia* (Pinkava 1999), while in *Selenicereus* these hybrids do not survive or
176 require a doubling of their chromosomes to survive (Tel-Zur *et al*. 2020).

177 Reproductive isolation in *Opuntia* has been proposed as low or non-existent among
178 certain members of this genus, mainly in North American species (Majure *et al*. 2012b),
179 while there are fewer reports of hybridization among South American species, as well as
180 fewer species (Hunt 2014). A study between two South American species of *Opuntia*
181 demonstrated prezygotic reproductive isolation between *O. elata* and *O. retrorsa* which are
182 sympatric in Brazil, supporting the general idea that hybridization occurs less frequently
183 among species in this part of the continent (Anderson 2001, Hunt *et al*. 2006). Potential
184 evidence on the importance of hybridization in the evolution of *Opuntia* is the species
185 diversity, which is higher in the northern part of America than in the south of the continent,
186 thus where more hybridization events are presented, there is a higher species diversity
187 (Reyes-Agüero *et al*. 2006, Hernández *et al*. 2014, Hunt 2014).

188 The idea of hybrids in nature has attracted the attention of botanists, often looking for
189 individuals with intermediate characteristics; however, these types of individuals are often
190 not found or do not exist. In other words, the hybrid might instead resemble one of the
191 putative parental species, or it is not found in nature despite being viable when artificially
192 obtained. For instance, this dilemma arises in *Astrophytum* due to common hybridization
193 among cultivated species, but even when the species are sympatric in the wild, the
194 corresponding hybrids are not seen in nature. About this subject, Montanucci (2015)
195 investigated the reasons why there were no reports of natural hybrids between *A.*

196 *coahuilense* and *A. capricorne* var. *senile*, although both are sympatric in northern Mexico
197 and their flowering periods overlap. The author made experimental crosses, and in some
198 cases, there was no formation of fruits (prezygotic barriers), while in other cases, the
199 germination of hybridized seeds was very low or led to the death of the seedling
200 (postzygotic barriers). Finally, the outcome of hybridization often does not give offspring
201 intermediate phenotypes between the parental lineages. The complexity of the interactions
202 such as dominance and epistasis, making the phenotype prediction far from a simple
203 average between two parentals (Rosas *et al*. 2010).

204 As discussed earlier, little is known about all mechanisms which prevent or allow hybrid
205 formation in Cactaceae. It is necessary to develop studies to improve the current
206 understanding of the processes involved in the hybridization and reproductive isolation of
207 species. In classic examples such as *Iris* (Iridaceae) and *Helianthus* (Asteraceae),
208 experimental crosses have been performed to better understand how the barriers to gene
209 exchange act in the hybrid formation, allowing crosses between certain species. In *Iris* the
210 prezygotic barriers are decisive for the hybrid formation while in *Helianthus* the
211 postzygotic barriers could be reducing the expected number of hybrids (Anderson 1953,
212 Heiser *et al*. 1969, Arnold 1994, Rieseberg 1995); therefore, it is necessary to know
213 whether there are patterns on the barriers to gene exchange in cacti.

214 *Natural hybridization and its impact on Cactaceae evolution.* Some studies have
215 highlighted that evolution in Cactaceae involves complex processes such as polyploidy,
216 hybridization, and ILS, as well as morphological adaptations to extreme environments, but

217 demonstrating that the occurrence of these processes and their impact on evolution is quite
218 complex (Pinkava 2002, Machado 2008, Majure *et al*. 2012b, Hernández-Hernández *et al*.
219 2014, Copetti *et al*. 2017). For example, inferences have been performed using
220 chromosomic numbers to know the ploidy of putative parentals and hybrids, and given
221 these results, it was found that the formation of polyploids has been of significant
222 importance in the speciation and adaptation of cytotypes to specific environmental
223 conditions (Pinkava 2002, Majure *et al*. 2012a). A phylogenomic approach found
224 incongruence between gene and species trees in Echinocereeae, probably due to long
225 generation times and ILS, highlighting the importance of using multiple evidence to
226 understand phylogenetic relationships among morphologically similar species (Copetti *et*
227 *al*. 2017). Cactaceae diversification involves multiple factors, such as adaptations to arid
228 environments, diverse pollination syndromes and growth forms related to water foraging
229 and storage. Therefore, environmental factors as well as those intrinsic to cacti species have
230 promoted the large number of species that exist in this family (Hernández-Hernández *et al*.
231 2014).

232 In Cactaceae, one of the main criteria for an individual plant found in nature to be
233 considered a hybrid is through intermediate morphological characteristics concerning two
234 morphologically differentiable species. A smaller proportion of hybrids have been reported
235 by chromosome comparisons, experimental crossings, or the use of molecular markers
236 (Anderson 2001, Hunt *et al*. 2006). The reports of natural hybrids in Cactaceae comprise
237 two of the four recognized subfamilies, Cactoideae and Opuntioideae (Appendix 1). Hybrid
238 formation in Cactoideae has been reported for six of the seven tribes, and this process

239 occurs more frequently among species belonging to different genera than among members
240 of the same genus (Appendix 1). On the other hand, in Opuntioideae, hybridization occurs
241 more frequently between species from the same genera. The most diverse genus is *Opuntia*
242 which has highly variable morphological traits and is known for presenting multiple
243 hybridization events, mainly due to weak barriers to genetic exchange and successful
244 vegetative dispersion in hybrid lineages (Pinkava 2002, Hunt *et al*. 2006, Reyes-Agüero *et*
245 *al*. 2006).

246 *Cactoideae subfamily*.- In Cactoideae, the natural hybridization may occur between diploid
247 or polyploid species (Figure 1 A and B), and there are more reports of intergeneric hybrids,
248 which have even been classified as nothogenera. It is essential to highlight that, although
249 hybridization is reported between different genera, it could also be due to an unresolved
250 classification at the genus level, or that does not reflect the phylogenetic relationships of
251 analyzed species.

252 A large number of studies have focused on reporting and testing hybridization using
253 different approaches. In the literature, we found 54 articles about hybridization, of which 36
254 analyze Cactoideae species; the main approach used was morphological descriptions. The
255 tribe with the highest number of natural hybrids is Trichocereeae, one of the most diverse,
256 and hybrids reports were mainly inferred based only on morphological descriptions
257 (Appendix 1). The phylogenetic relationships within this group have not been resolved and
258 their limits based on morphological characters are not clear (Guerrero *et al*. 2019). In this
259 subfamily, it is necessary to reconstruct phylogenetic relationships and clarify generic
260 boundaries. Its highly variable morphological traits might give an erroneous idea of

hybridization when genuine genera relationships remain unknown, as in groups like ferns (He & Zhang 2012).

Evolutionary implications of hybridization have been identified as important for Cactaceae, but very few hybridization cases have been tested. An interesting example occurs in hybrid zones of *Melocactus* in Brazil (Khan *et al*. 2020). This particular case tested the impact of hybrids on parental genetic integrity but did not analyze the role of hybrids on population dynamics. Authors inferred reproductive barriers, but they did not conduct a formal test for this assertion. In *Iris* (Iridaceae), a gradual loss of reproductive isolation after the first hybridization event was reported (Arnold 2006). This could occur in *Melocactus* hybrid zones, where the backcrosses are common, but because of natural selection and genetic drift, introgressed loci are lost. Their population structure analyses show hybrids F_1 as well as hybrids from subsequent backcrosses, so although they infer the existence of barriers to genetic exchange, the results indicate that these barriers in *Melocactus* are permeable; thus, formal analyses must be performed to know the dynamics of gene exchange barriers in the studied species.

Among columnar cacti, a recent study addressed the hybridization between *Polaskia chichipe* and *Escontria chiotilla* (Cruz-Zamora *et al*. 2017), both inhabiting the Tehuacán-Cuicatlán biosphere reserve and sharing pollinators. Using morphological and genetic evidence, these species were identified as parentals of hybrid individuals. It is relevant to mention that the authors also performed artificial crosses between parentals, whose hybrids were similar to the natural ones. This demonstrates that, despite the slow development in

most Cactaceae members it is possible to carry out experimental crosses to obtain hybridized progeny as has been done in other plant groups such as *Helianthus* (Asteraceae) (Rieseberg 1991). Therefore, studies of experimental crosses should be carried out between putative parents of hybrids, to validate the hybrids observed in the wild experimentally.

In Cactoideae, there are many species with small populations which are threatened (Goettsch *et al*. 2015). Hence the integrity of endemic or rare species is of great relevance for their conservation. One example is *Sclerocactus*, whose members inhabit western North America. There is evidence of hybridization between *S. wetlandicus* and *S. brevispinus* (Tepedino *et al*. 2010) and *S. glaucus* and *S. parviflorus* (Schwabe *et al*. 2015). Hybridization can be an issue when the populations are small, by the interspecific gene flow, which might facilitate the loss of alleles in threatened species. One such case was studied in *S. glaucus*, endemic to western Colorado (United States of America). Introgression was found into *S. glaucus* from *S. parviflorus*, a species with wider distribution; despite this, the genetic integrity of *S. glaucus* seems to be intact thus, the real threat to this species are the anthropogenic pressures (Goettsch *et al*. 2015, Schwabe *et al*. 2015). It is also interesting that most *Sclerocactus* species are diploids (Rice *et al*. 2015); therefore, it would be relevant to know the hybrids ploidy because if they have the same ploidy as their parentals, homoploid hybridization would be occurring. This type of hybridization has been poorly documented in Cactoideae, and its study would be worth addressing to understand speciation on this subfamily better (Rieseberg 1991, Arnold 1997).

Although most of the articles focus on Cactoideae, there are still many genera for which there are no reports of natural hybridization, even though there is evidence of hybridization in cultivation. One interesting case is *Mammillaria*, for which little is known about natural hybridization; only some reports exist, but no formal studies have been performed (Hunt 1977), yet hybridization in cultivation is common. Other cases are anecdotally reported by breeders, where hybridization in cultivation is possible between *Turbinicarpus* and *Thelocactus*, as well as other cultivated species from *Epiphyllum*, *Selenicereus* and *Aporocactus*, among other species (Cullmann *et al*. 1987). Although these reports are not part of the scientific work, they allow a better understanding of the presence or absence of barriers to gene exchange.

After analyzing many papers about hybridization in this subfamily, we can conclude that the reports of hybrids in this group are mainly made by describing strange individuals with intermediate morphological traits. However, we presume that given the artificial hybrids that breeders have developed, hybridization in the wild is probably underestimated. Thus, it is necessary to perform phylogenetic and reticulation analysis and experimental crosses to test hybridization hypotheses and determine the impact of this process on the evolution of Cactoideae.

Opuntioideae subfamily.-Although Opuntioideae is a group with frequent natural hybridization, we only found 18 articles on this subject, which could be related to their not so fascinating characteristics as in Cactoideae members. During the 1990s and the first decade of the 21st century, the natural hybridization of Opuntioideae in North American

species was studied for several years by the group of Pinkava and collaborators. Their compilation of studies in *Opuntia* and *Cylindropuntia* contributed to understanding the evolutionary implications of this process in the subfamily (Baker & Pinkava 1987, Pinkava 1999). Their analyses include data from taxonomic treatments, chromosomal counts, morphometric and biogeographic analysis, which led to the postulation that the members of this subfamily tend to have rapid speciation due to the small and isolated populations that they frequently exhibit, their perennial habit, vegetative reproduction (Figure 1), apomixis, and allopolyploidization allowing accumulation of heritable mutations on their descendants. Vegetative reproduction impacts the evolutionary processes because it helps the maintenance of hybrid genotypes, which later can also reproduce sexually with parental genotypes or other species (Arnold 2006). The same pattern has been reported in *Opuntia* due to high levels of clonal reproduction and the presence of putative hybrids (Pinkava 2002, Reyes-Agüero *et al*. 2006). A prominent example using three of the above-mentioned evidences was described in the *Opuntia polyacantha* complex. The authors found differences in chromosome numbers on the boundaries of species distribution, allowing them to infer the process of peripatric speciation (Mayr 1954, Pinkava 2002).

In Opuntioideae, hybridization events have been documented using chromosome numbers; for example, within *Opuntia* populations, there is variation in chromosome numbers, sometimes associated with hybridization events and subsequent chromosome duplication, which occurs to ensure the correct pairing of chromosomes, allowing the subsequent reproduction of the hybrids (Soltis *et al*. 2003, Tel-Zur *et al*. 2020). Chromosome doubling does not always occur, and putative hybrids may have originated by

346 the union of unreduced gametes (Pinkava 2002). To circumscribe species complexes in
347 *Opuntia* is necessary to understand their ploidy throughout its distribution, and therefore to
348 know which species are related, if they form hybrids and understand the boundaries
349 between species. One such example was performed in the *O. humifusa* complex (Majure *et*
350 *al*. 2017). Another remarkable example of the use of chromosome counts was performed in
351 *Opuntia s.l.* from South America; it was notable that ploidy numbers ranged from diploid
352 (2x) to pentaploid (5x), and important cytological characteristics to understand
353 hybridization patterns like chromosomal terminal satellites were found in *O. aurantiaca*
354 (3x) and *O. salmiana* (3x) (Realini *et al*. 2014).

355 Interestingly few studies have tested the hybridization hypothesis using reciprocal crosses
356 and breeding systems, probably due to slow growth in most cacti. However, experiments
357 performed in prickly-pear from the Chihuahuan Desert found five combinations of
358 interfertile taxa, involving seven species and four varieties (Griffith 2001). These
359 experiments are essential to support putative natural hybridization events and the use of
360 more evidence like intermediate morphological traits, ploidy levels, and shared biparentally
361 inherited markers are conclusive evidence for hybridization cases in North American
362 *Opuntia* (Griffith 2003). Species in this part of the continent are abundant, and more
363 hybridization cases have been reported, being this genetic exchange as well as polyploidy
364 the probable causes of more events of speciation (Soltis & Soltis 2009, Soltis 2013).

365 Another widely studied genus is *Cylindropuntia*, in which, from the 33 species
366 recognized by Hunt *et al*. (2006), there are 10 natural hybrids, most of them are triploids

(3x) and have vegetative reproduction. These triploid individuals were helpful in better understanding the genetic exchange between species with different ploidies. Through these analyses, it was shown that triploids formation was generally via non-reduced gametes and not because of crossing between diploid (2x) and tetraploid (4x) individuals (Baker & Pinkava 1987, Pinkava, 2002). Other authors collected information about hybrid fertility, showing that triploid individuals were not entirely infertile (Grant & Grant 1971). The hybrid formation was inferred in *Cylindropuntia* and it was demonstrated that hybrids with odd ploidy are still capable of breeding; thus, backcrosses may occur, giving rise to new lineages (Arnold 1997).

To characterize the ploidy in Opuntioideae and other Cactaceae species is crucial because it allows one to understand their speciation patterns. In Cactoideae, homoploid hybridization could frequently be occurring undetected because most of the species are diploid (Baker *et al*. 2009, Rice *et al*. 2015, Baker & Pinkava 2018), whereas in polyploid Opuntioideae allopolyploid speciation has been detected through chromosome counts and molecular markers (Majure *et al*. 2012a, b). Despite its relevance, the study of ploidy levels is in disuse in Cactoideae, whereas in Opuntioideae, it is still relevant due to many polyploids and their reticulate evolution (Soltis & Soltis 2009, Majure *et al*. 2012a). It has been proposed that polyploidy is a condition that allows rapid speciation and evolution in plants, being highly frequent in Opuntioideae allowing the great diversity of species currently observed (Stebbins 1971, Hunt 2014). The origin of polyploidy in plants may be due to somatic duplication in meristems, unreduced gametes, or by hybridization, the last

two being the most frequent in Opuntioideae (Otto & Whitton, 2000, Pinkava 2002, Majure *et al*. 2012a, b).

Different from what might be expected, not all species in Opuntioideae present hybridization. We searched explicitly for hybridization papers in Andean species from *Grusonia*, *Maihueniopsis*, *Austrocylindropuntia* and *Cumulopuntia* but there were no reports or molecular evidence of hybridization (Anderson 2001, Ritz *et al*. 2012). Phylogenetic analysis performed on Tephrocacteae and the previously mentioned genera using plastid and nuclear markers yielded almost the same trees, so no evidence of hybridization was found (Ritz *et al*. 2012). In *Tephrocactus*, no discordant phylogenetic histories were found, although in previous karyotypic analysis hybridization evidence was found for *T. recurvatus* (Las Peñas *et al*. 2009), so more studies should be conducted using multiple sources of evidence to understand the evolutionary history of South American species.

Although in Opuntioideae there is more evidence about hybridization among its members, more analysis focused on reticulated evolution should be performed on species poorly studied to better understand the impact of hybridization on their speciation processes, as well as to know why hybridization did not occur in certain lineages whereas on their sister lineages is very common. We can infer that the process of hybrid speciation plays a role of great importance in the most hybridizing genera *Opuntia* and *Cylindropuntia*, because they are also the most diverse of Opuntioideae. However, a more comprehensive analysis is still needed to test this hypothesis formally.

Discordant phylogenetic stories in Opuntioideae. Through phylogenetic analysis, we can understand the ancestral-descendant relationship from focal taxa and, although is not their purpose, some patterns on phylogenetic trees have been used to infer hybridization. One of the most used patterns is discordance between phylogenetic trees obtained from molecular markers of uniparental versus biparental inheritance (Arnold 1997, Sang *et al*. 1997, Russell *et al*. 2010, Meng *et al*. 2011).

The phylogenetic history of Opuntioideae is complicated, and although different groups of botanists have studied it over time, its phylogenetic relationships have not yet been fully understood. Traditional species recognition involves morphological characters, but in Opuntioideae, the significant variability of morphological traits within species makes necessary molecular, cytological, biogeographic, and reproductive evidences necessary to circumscribe species from this subfamily (Pinkava 2002, Majure *et al*. 2014, 2017).

Phylogenetic analysis has been performed on most subfamily members, mainly using chloroplast markers because the use of nuclear markers has led to discordant positions in some cases. One interesting example involves the genus *Consolea*, whose origin has been suggested as a hybrid due to intermediate morphological characters with *Brasiliopuntia*, *Opuntia*, and *Tacinga*, and because it has not been possible to know with certainty its phylogenetic position. In a first approach, this genus was recovered outside *Opuntia sensu stricto* (*s.s.*) (Wallace & Dickie 2002). Subsequently, *Consolea* was found inside *Opuntia s.s.* (Griffith & Porter 2009). Moreover, in a combined analysis (with plastid and nuclear markers) *Consolea* was found outside *Opuntia s.s*. The analysis includes only diploid

430 species (Majure *et al*. 2012b). Most *Consolea* species are polyploids (Negrón-Ortiz 2007),
431 and their autopolyploid or allopolyploid origin should be studied under approaches like
432 high-throughput sequencing.

433 Another remarkable case involves *Nopalea*, previously considered outside *Opuntia* due to
434 its flowers and pollination syndrome, which is phylogenetically nested within *Opuntia s.s.*;
435 therefore, now it is considered part of this genus, providing a remarkable example of
436 morphological characteristics in Opuntioideae leading to an artificial classification.
437 Additionally, there is evidence that members from this clade hybridize with members of the
438 clades Basilares, Scheerianae and *Opuntia s.l*. In *Opuntia* it has been observed that hybrids
439 survive only if the pollen donor has higher ploidy than the female receptor (Griffith 2001).
440 Most species previously known as *Nopalea* have diploid chromosome numbers, so they
441 may act as pollen receptors for species with higher chromosome numbers, explaining the
442 many hybridization events with species from other clades.

443 Phylogenetic analyses in *Opuntia* are complicated due to widespread polyploidy. For
444 species in this genus, being polyploid represents an important evolutionary adaptation for
445 survival (Majure *et al*. 2017). However, these highly relevant biological processes make it
446 difficult to interpret the evolutionary relationships between species. A clear example is
447 presented in the most complete phylogenetic analysis for the genus, in which it is possible
448 to define large groups within *Opuntia s.s*. but excluding hybrid and polyploid taxa. More
449 than 50% of the *Opuntia* species are polyploids, and this condition can occur due to the
450 union of unreduced gametes and/or hybridization (Pinkava 2002). This estimated and

451 extremely high number of polyploids could suggest a considerable number of
452 diversification events likely from hybrid origin, either between populations of the same
453 species or of different species. In addition, it is important to emphasize that it is necessary
454 to carry out phylogenetic analyses, perhaps using another kind of molecular information
455 like genomics, transcriptomics, proteomics, plastomes, or nuclear-targeted regions, and
456 include these polyploid species in order to know their relationship with the rest of the
457 diploid species.

458 We conclude that in Cactaceae is necessary to improve and accelerate the formal study of
459 isolation barriers between not cultivated species from the subfamilies with reports of
460 hybrids. Particularly, hybridization reports in tribe Trichocereeae should be revised because
461 of the complex relationships among their members leading to the inference of false
462 hybridization events between different genera. For Opuntioideae is a priority to know the
463 chromosome numbers in all species, their phylogenetic relationships, and the boundaries
464 between species, which are difficult to elucidate due to hybridization and polyploidy. The
465 analysis of a process as complex as hybridization must be comprehensive. It should include
466 cytological, ecological, reproductive, morphological, molecular, and evolutionary evidence
467 to better understand the dynamics of hybrids, since their primary formation, to the demise
468 of a hybrid lineage or until their establishment as a new species. Independently studied
469 processes such as pollination and interaction between pollen and pistil, polyploid
470 chromosome arrangements, how chromosomes and genes segregate in a newly formed
471 hybrid, the establishment of new hybrid populations, and their separation from parental
472 species must be studied in an integrative context. In the case of reproductive biology, it is

473 necessary to carry out studies in those species whose pollen-pistil interactions are unknown.
474 For the cytogenetic part, it is necessary to know the chromosomal numbers of studied
475 species, for hybridization cases perform GISH and FISH, and to know the different
476 cytotypes that coexist within a species. From the phylogenetic and population genetics
477 perspective, it is necessary to make analyses that include a greater number of individuals,
478 variable markers, coalescence analysis, and phylogenetic networks to understand the
479 complex processes that occur when two distinct lineages are mixed. The taxonomy studies
480 should analyze the species as permeable entities in which the identity can be modified by
481 processes such as hybridization, using all possible evidence and formal morphological
482 analysis that includes statistical analysis, slowly leaving the species description using only
483 the taxonomist criterion and describing Cactaceae species as a whole and not just one
484 individual. All above mentioned are recommendations to improve the understanding of
485 hybridization and evolution process in the interesting and beautiful group of plants
486 popularly known as cacti.

487 **Acknowledgements**

488 This paper is part of the doctoral research of X. Granados, who thanks the graduate
489 program Doctorado en Ciencias Biológicas, UNAM and the scholarships provided by
490 CONACYT. We thank C. Granados and I. Cacho (Instituto de Biología, UNAM) for their
491 advice in conceptualization of methods. This contribution was financially supported by
492 UNAM-DGAPA-PAPIIT <IN208619> granted to S. Arias.

493

Literature cited

Anderson E. 1953. Introgressive Hybridization. *Biological Reviews* **28**: 280–307. DOI: https://doi.org/10.1111/j.1469-185X.1953.tb01379.x

Anderson EF. 2001. *The Cactus Family*. Portland, Oregon: Timber Press.

Arias S, Gama S, Vázquez B, and Guzmán LU. (2012). *Flora del Valle de Tehuacán-Cuicatlán*. Fascículo 95, Cactaceae. Instituto de Biología, Universidad Nacional Autónoma de México 95.

Arnold ML. 2006. *Evolution Through Genetic Exchange*. Oxford University Press Available at: https://books.google.com.mx/books?id=R5QUDAAAQBAJ.

Arnold ML. 2004. Transfer and Origin of Adaptations through Natural Hybridization: Were Anderson and Stebbins Right? *The Plant Cell* **16**: 562–570. DOI: https://doi.org/10.1105/tpc.160370

Arnold ML. 1997. *Natural Hybridization and Evolution*. Nueva York: Oxford University Press. ISBN 10: 0-88192-498-9

Arnold ML. 1994. Natural Hybridization and Louisiana Irises. *BioScience* **44**: 141–147. DOI: https://doi.org/10.2307/1312250

Baack E, Melo MC, Rieseberg LH, Ortiz-Barrientos D. 2015. The origins of reproductive isolation in plants. *New Phytologist* **207**: 968–984. DOI: https://doi.org/10.1111/nph.13424

Baker MA, Johnson RA. 2000. Morphometric Analysis of *Escobaria sneedii* var. *sneedii*, *E. sneedii* var. *leei*, and *E. guadalupensis* (Cactaceae). *Systematic Botany* **25**: 577–587. DOI: https://doi.org/10.2307/2666722

Baker MA, and Pinkava, DJ. 2018. Chromosome Numbers in Some Cacti of Western North America — IX. *Haseltonia* **25**: 2–29. DOI: https://doi.org/10.2985/026.025.0103

Baker MA, Pinkava DJ 1987. A Cytological and Morphometric Analysis of a Triploid Apomict, *Opuntia* ×*kelvinensis* (Subgenus Cylindropuntia, Cactaceae). *Brittonia* **39**: 387–401. DOI: https://doi.org/10.2307/2807141

Baker MA, Rebman JP, Parfitt BD, Pinkava DJ, Zimmerman AD. 2009. Chromosome Numbers in Some Cacti of Western North America— VIII. *Haseltonia*, **15**: 117–134. DOI: https://doi.org/10.2985/026.015.0112

Barthlott W, Burstedde K, Geffert J, Ibisch P, Korotkova N, Miebach A, *et al*. 2015. Distribution maps of Cactaceae. *In*: Metzing D. ed. *Biogeography and Biodiversity of Cacti*. Alemania: Schumannia, pp. 1–205.

Britton, NL, and Rose, J. N. (1919). *The Cactaceae: descriptions and illustrations of plants of the cactus family*. Washington: Carnegie Institution of Washington, doi:10.5962/bhl.title.46288.

Britton, NL, and Rose, J. N. (1920). *The Cactaceae: descriptions and illustrations of plants of the cactus family*. Washington: Carnegie Institution of Washington, Available at: https://www.biodiversitylibrary.org/item/100134.

Britton, NL, and Rose, J. N. (1922). *The Cactaceae: descriptions and illustrations of plants of the cactus family*. Washington: Carnegie Institution of Washington, Available at: https://www.biodiversitylibrary.org/item/100215.

Britton, NL, and Rose, J. N. (1923). *The Cactaceae: descriptions and illustrations of plants of the cactus family*. Washington: Carnegie Institution of Washington, Available at: https://www.biodiversitylibrary.org/item/100134.

Butterworth CA, Wallace RS. 2005. Molecular Phylogenetics of the Leafy Cactus Genus *Pereskia* (Cactaceae). *Systematic Botany* **30**: 800–808.

Cisneros A, Tel-Zur N. 2012. Evaluation of Interspecific-Interploid Hybrids (F1) and Back Crosses (BC1) in *Hylocereus* species (Cactaceae). *In*: Swan A. ed. *Meiosis*. Rijeka: IntechOpen, pp.20. DOI: https://doi.org/10.5772/32435

Copetti D, Búrquez A, Bustamante E, Charboneau JL, Childs K L, Eguiarte LE, *et al*. 2017. Extensive gene tree discordance and hemiplasy shaped the genomes of North American columnar cacti. *PNAS* **114**: 12003–12008. DOI: https://doi.org/10.1073/pnas.1706367114

Critchfield WB. 1986. Hybridization and Classification of the White Pines (*Pinus* Section Strobus). *Taxon* **35**: 647–656. DOI: https://doi.org/10.2307/1221606

Cruz-Zamora Y, Cruz-García F, Orozco-Arroyo G, Reyes-Santiago J, Martínez-Castilla LP, Wegier A, *et al*. 2017. × *Polascontria* (Cactaceae), a nothogenus that represents the gene flow between *Escontria* and *Polaskia*. *Phytotaxa* **295**: 141–158. DOI: https://doi.org/10.11646/phytotaxa.295.2.3

Cullmann W, Goetz E, Gröner G. 1987. *The Encyclopedia of Cacti*. Timber Press.

Eggli U, Giorgetta M. 2018. A Putative Intercladal *Echinopsis s.l.* (Cactaceae) Hybrid from Andean Chile. *Haseltonia* 2018, 7–16. DOI: https://doi.org/10.2985/026.024.0103

557 Font F, Picca P. 2001. Natural hybridization between *Trichocereus atacamensis* (Philippi)
558 Marshall and *Denmoza rhodacantha* (Salm-Dyck) Britton & Rose (Cactaceae). *Bradleya*
559 **19**: 59–66. DOI: https://doi.org/10.25223/brad.n19.2001.a7
560 Goettsch B, Hilton-Taylor C, Cruz-Piñón G, Duffy JP, Frances A, Hernández HM, *et al*.
561 2015. High proportion of cactus species threatened with extinction. *Nature Plants* **1**: 1–
562 7. DOI: https://doi.org/10.1038/nplants.2015.142
563 Granados-Aguilar X, Granados Mendoza C, Cervantes CR, Montes JR, Arias S. 2021.
564 Unraveling reticulate evolution in *Opuntia* (Cactaceae) from Southern Mexico.
565 *Frontiers in Plant Science* **11**: 606809. DOI: https://doi.org/10.3389/fpls.2020.606809
566 Grant V, Grant KA 1971. Natural Hybridization between the Cholla Cactus Species
567 *Opuntia spinosior* and *Opuntia versicolor*. *PNAS* **68**: 1993–1995.
568 Griffith PM. 2003. Using molecular evidence to elucidate reticulate evolution in *Opuntia*
569 (Cactaceae). *Madroño* **50**: 162–169.
570 Griffith PM. 2001. Experimental Hybridization of Northern Chihuahuan Desert Region
571 *Opuntia* (Cactaceae). *A Journal of Systematic and Evolutionary Botany* **20**: 37–42.
572 Griffith PM., Porter M. 2009. Phylogeny of Opuntioideae (Cactaceae). *International*
573 *Journal of Plant Sciences* **170**: 107–116.
574 Guerrero PC, Majure LC, Cornejo-Romero A, Hernández-Hernández T. 2019. Phylogenetic
575 relationships and evolutionary trends in the cactus family. *Journal of Heredity* **110**: 4–
576 21. DOI: https://doi.org/10.1093/jhered/esy064
577 Guzmán JM, Ortega MC, Gordillo MM, Segovia AO, Santana SV. 2013. *Biología de*
578 *angiospermas*. UNAM, Facultad de Ciencias. ISBN: 97 8-607-02-2705-9

He LJ, Zhang XC. 2012. Exploring generic delimitation within the fern family Thelypteridaceae. *Molecular Phylogenetics and Evolution* **65**: 757–764. DOI: https://doi.org/10.1016/j.ympev.2012.07.021

Heiser CB, Smith DM, Clevenger SB, Martin WC. 1969. The North American Sunflowers (*Helianthus*). *Memoirs of the Torrey Botanical Club* **22**: 1–218.

Hernández H, Gómez-Hinostrosa C, Bárcenas R, Puente-Martinez R, Reyes-Agüero J. 2014. A checklist of the subfamily Opuntioideae (Cactaceae) in North and Central America. *In*: Hunt D. ed. *Further Studies in the Opuntioideae (Cactaceae).* Succulent Plant Research. England: DH Books, pp. 185–200.

Hernández-Hernández T, Brown JW, Schlumpberger BO, Eguiarte LE, Magallón S. 2014. Beyond aridification: multiple explanations for the elevated diversification of cacti in the New World Succulent Biome. *New Phytologist*. **202**: 1382–1397. DOI: https://doi.org/10.1111/nph.12752

Hunt DR. 2014. An Interim Checklist of the Subfamily Opuntioideae (Cactaceae) in the Caribbean Region and South America. *In:* Hunt D. ed. *Further Studies in the Opuntioideae (Cactaceae).* Succulent Plant Research. England: DH Books, pp. 201–223.

Hunt DR, Taylor NP, Charles G. 2006. *The New Cactus Lexicon: Text.* Inglaterra: DH books Available at: https://books.google.com.mx/books?id=OHyZAQAACAAJ.

Hunt DR. 1977. The Classification of *Mammillaria*. *The National Cactus and Succulent Journal* **32**: 75–81.

Jensen R J, Hokanson SC, Isebrands JG, Hancock JF. 1993. Morphometric variation in oaks of the apostle islands in Wisconsin: evidence of hybridization between *Quercus*

rubra and *Q. ellipsoidalis* (Fagaceae). *American Journal of Botany* **80**: 1358–1366. DOI: https://doi.org/10.2307/2445721

Kay KM. (2006). Reproductive Isolation Between Two Closely Related Hummingbird Pollinated Neotropical Gingers. *Evolution* **60**: 538–552. DOI: https://doi.org/10.1111/j.0014-3820.2006.tb01135.x

Khan G, Franco FF, Silva GAR, Bombonato JR, Machado M, Alonso DP, *et al*. 2020. Maintaining genetic integrity with high promiscuity: Frequent hybridization with low introgression in multiple hybrid zones of *Melocactus* (Cactaceae). *Molecular Phylogenetics and Evolution* **142**: 106642. DOI: https://doi.org/10.1016/j.ympev.2019.106642

Korotkova N, Aquino D, Arias S, Eggli U, Franck A, Gómez-Hinostrosa C, *et al*. 2021. Cactaceae at Caryophyllales.org – a dynamic online species-level taxonomic backbone for the family. *Willdenowia* **51**: 251–270. DOI: https://doi.org/10.3372/wi.51.51208

Korotkova N, Borsch T, Arias S. 2017. A phylogenetic framework for the Hylocereeae (Cactaceae) and implications for the circumscription of the genera. *Phytotaxa* **327**: 1–46. DOI: https://doi.org/10.11646/phytotaxa.327.1.1

Lambert SM, Borba EL, Machado MC, Andrade DS. 2006a. Allozyme Diversity and Morphometrics of *Melocactus paucispinus* (Cactaceae) and Evidence for Hybridization with *M. concinnus* in the Chapada Diamantina, North-eastern Brazil. *Annals of Botany* **97**: 389–403. DOI: https://doi.org/10.1093/aob/mcj052

621 Las Peñas ML, Urdampilleta JD, Bernardello G, Forni-Martins ER. 2009. Karyotypes,
622 heterochromatin, and physical mapping of 18S-26S rDNA in Cactaceae. *Cytogenetic*
623 *and Genome Research* **124**: 72–80. DOI: https://doi.org/10.1159/000200090
624 Machado MC. 2008. What is the role of hybridization in the evolution of the Cactaceae?
625 *Bradleya*, **26**: 1–18. DOI: https://doi.org/10.25223/brad.n26.2008.a1
626 Majure LC, Judd WS, Soltis PS, Soltis DE. 2017. Taxonomic revision of the *Opuntia*
627 *humifusa* complex (Opuntieae: Cactaceae) of the eastern United States. *Phytotaxa* **290**:
628 1–65. DOI: https://doi.org/10.11646/phytotaxa.290.1.1
629 Majure LC, Judd WS, Soltis PS, Soltis DE. 2012a. Cytogeography of the Humifusa clade
630 of *Opuntia s.s.* Mill. 1754 (Cactaceae, Opuntioideae, Opuntieae): correlations with
631 pleistocene refugia and morphological traits in a polyploid complex. *Comparative*
632 *Cytogenetics* **6**: 53–77. DOI: https://doi.org/10.3897/CompCytogen.v6i1.2523
633 Majure LC, Puente R, Griffith MP, Judd WS, Soltis PS, Soltis DE. 2012b. Phylogeny of
634 *Opuntia s.s.* (Cactaceae): Clade delineation, geographic origins, and reticulate evolution.
635 *American Journal of Botany* **99**: 847–864. DOI: https://doi.org/10.3732/ajb.1100375
636 Majure LC, Soltis DE, Soltis PS, Judd WS. 2014. A case of mistaken identity, *Opuntia*
637 *abjecta*, long-lost in synonymy under the Caribbean species, *O. triacantha*, and a
638 reassessment of the enigmatic *O. cubensis*. *Brittonia* **66**: 118–130. DOI:
639 https://doi.org/10.1007/s12228-013-9307-z
640 Mandujano MC, Carrillo-Angeles I, Martínez-Peralta C, Golubov J. 2010. Reproductive
641 Biology of Cactaceae. *In*: Ramawat KG. ed. *Desert Plants: Biology and Biotechnology*.
642 Berlin: Springer, 197–230. DOI: https://doi.org/10.1007/978-3-642-02550-1_10

643 Marks GE. 1966. The Origin and Significance of Intraspecific Polyploidy: Experimental
644 Evidence from *Solanum chacoense*. *Evolution* **20**: 552–557. DOI:
645 https://doi.org/10.2307/2406589
646 Mayr E. 1954. Change of genetic environment and evolution. *In*: Huxley J, Har-
647 dy AC. eds. *Evolution as a Process* (London: Unwin Brothers), pp. 157–180.
648 Meng J, Fougere-Danezan M, Zhang LB, Li DZ, and Yi TS. 2011. Untangling the hybrid
649 origin of the Chinese tea roses: Evidence from DNA sequences of single-copy nuclear
650 and chloroplast genes. *Plant Systematic and Evolution* **297**: 157–170. DOI:
651 https://doi.org/10.1007/s00606-011-0504-5
652 Montanucci RR. 2015. Experimental Evidence for Reproductive Isolation between
653 *Astrophytum coahuilense* (H. Möller) Kayser and *A. Capricorne* var. *senile* (Fric)
654 Okumura (Cactaceae). *Haseltonia* **20**: 13–21. DOI:
655 https://doi.org/10.2985/026.020.0104
656 Morales-Briones DF, Romoleroux K, Kolář F, Tank DC. 2018. Phylogeny and evolution of
657 the neotropical radiation of *Lachemilla* (Rosaceae): Uncovering a history of reticulate
658 evolution and implications for infrageneric classification. *Systematic Botany* **43**: 17–34.
659 DOI: https://doi.org/10.1600/036364418X696897
660 Negrón-Ortiz V. 2007. Chromosome numbers, nuclear DNA content, and polyploidy in
661 *Consolea* (Cactaceae), an endemic cactus of the Caribbean Islands. *American Journal of*
662 *Botany* **94**: 1360–1370. DOI: https://doi.org/10.3732/ajb.94.8.1360

663 Nishiyama I, Yabuno T. 1979. Triple fusion of the primary endosperm nucleus as a cause
664 of interspecific cross-incompatibility in Avena. *Euphytica* **28**: 57–65. DOI:
665 https://doi.org/10.1007/BF00029173
666 Otto SP, Whitton J. 2000. Polyploid incidence and evolution. *Annual Review of Genetics*
667 **34**: 401–437. DOI: https://doi.org/10.1146/annurev.genet.34.1.401
668 Pinkava DJ. 2000. On the evolution of the North American Opuntioideae. *In*: Hunt D. ed.
669 *Studies in the Opuntioideae (Cactaceae).* England: Succulent Plant Research. DH
670 Books, pp. 59–98. Available at:
671 https://books.google.com.mx/books?id=lTAmAQAAMAAJ.
672 Pinkava DJ. 1999. Cactaceae Cactus Family: Part Three: *Cylindropuntia* (Engelm.) Knuth
673 Chollas. *Journal of the Arizona-Nevada Academy of Science* **32**: 32–47.
674 Powell AM, Zimmerman AD, Hilsenbeck RA. 1991. Experimental documentation of
675 natural hybridization in Cactaceae: origin of Lloyd's Hedgehog Cactus, *Echinocereus* ×
676 *lloydii*. *Plant Systematics and Evolution* **178**: 107–122.
677 Realini M, Gottlieb A, Font F, Picca P, Poggio L, González G. 2014. Cytogenetic
678 characterization of southern South American species of *Opuntia s.l.* (Cactaceae-
679 Opuntioideae). *In*: Hunt D. ed. *Further Studies in the Opuntioideae*. England: Succulent
680 Plant Research. DH Books, pp. 31–50.
681 Řepka R, Mráček, J. 2012. *Gymnocalycium esperanzae*: A Nothospecies? *Haseltonia* **18**:
682 105–115. DOI: https://doi.org/10.2985/026.018.0113

683 Reyes-Agüero JA, Aguirre R, Valiente-Banuet A. 2006. Reproductive biology of *Opuntia*:
684 A review. *Journal of Arid Environments* **64**: 549–585. DOI:
685 https://doi.org/10.1016/j.jaridenv.2005.06.018
686 Rice A, Glick L, Abadi S, Einhorn M, Kopelman NM, Salman-Minkov A, *et al*. 2015. The
687 Chromosome Counts Database (CCDB) – a community resource of plant chromosome
688 numbers. *New Phytologist* **206**: 19–26. DOI: https://doi.org/10.1111/nph.13191.
689 Rieseberg LH. 1995. The Role of Hybridization in Evolution: Old wine in new skins.
690 *American Journal of Botany* **82**: 944–953. DOI: https://doi.org/10.2307/2445981
691 Rieseberg LH. 1991. Homoploid Reticulate Evolution in *Helianthus* (Asteraceae):
692 Evidence from ribosomal genes. *American Journal of Botany* **78**: 1218–1237. DOI:
693 https://doi.org/10.2307/2444926
694 Ritz CM, Reiker J, Charles G, Hoxey P, Hunt D, Lowry M, *et al*. (2012). Molecular
695 phylogeny and character evolution in terete-stemmed Andean opuntias
696 (Cactaceae−Opuntioideae). *Molecular Phylogenetics and Evolution* **65**: 668–681. DOI:
697 https://doi.org/10.1016/j.ympev.2012.07.027
698 Rosas U, Barton NH, Copsey L, Reuille PB, Coen E. 2010. Cryptic Variation between
699 Species and the Basis of Hybrid Performance. *PLOS Biology* 8, e1000429. DOI:
700 https://doi.org/10.1371/journal.pbio.1000429
701 Rowley GD. 1994. Spontaneous Bigeneric Hybrids in Cactaceae. *Bradleya* **12**: 2–7. DOI:
702 https://doi.org/10.25223/brad.n12.1994.a2
703 Russell A, Samuel R, Klejna V, Barfuss MHJ, Rupp B, Chase MW. 2010. Reticulate
704 evolution in diploid and tetraploid species of *Polystachya* (Orchidaceae) as shown by

plastid DNA sequences and low-copy nuclear genes. *Annals of Botany* **106**: 37–56. DOI: https://doi.org/10.1093/aob/mcq092

Sang T, Crawford DJ, Stuessy TF. 1997. Chloroplast DNA phylogeny, reticulate evolution, and biogeography of *Paeonia* (Paeoniaceae). *American Journal of Botany* **84**: 1120–1136. DOI: https://doi.org/10.2307/2446155

Schlumpberger BO. 2011. A survey on pollination modes in cacti and a potential key innovation. *In*: Patiny S. ed. *Evolution of Plant-Pollinator Relationships* (Systematics Association Special Volume Series). Cambridge: Cambridge University Press, pp. 301–319. DOI: https://doi.org/10.1017/CBO9781139014113.011

Schwabe AL, Neale J R, McGlaughlin ME. 2015. Examining the genetic integrity of a rare endemic Colorado cactus *Sclerocactus glaucus* in the face of hybridization threats from a close and widespread congener *Sclerocactus parviflorus*. *Conservation Genetics* **16**: 443–457. DOI: https://doi.org/10.1007/s10592-014-0671-3

Soltis DE, Soltis PS, Tate, JA. 2003. Advances in the study of polyploidy since Plant speciation. *New Phytologist* **161**: 173–191. DOI: https://doi.org/10.1046/j.1469-8137.2003.00948.x

Soltis PS. 2013. Hybridization in Plants. *In*: Levin SA. ed. *Encyclopedia of Biodiversity* (Second Edition). Waltham: Academic Press, pp. 166–176. DOI: https://doi.org/10.1016/B978-0-12-384719-5.00202-1

Soltis PS, Soltis DE. 2009. The role of hybridization in plant speciation. *Annual Review of Plant Biology* **60**: 561–588. DOI: https://doi.org/10.1146/annurev.arplant.043008.092039

727 Stebbins GL. 1971. *Chromosomal evolution in higher plants*. 1st ed. Londres, UK: Edward
728 Arnold Publishers Available at: http://wellcomelibrary.org/item/b18036594
729 Tapia HJ, Bárcenas-Argüello ML, Terrazas T, Arias S. 2017. Phylogeny and
730 Circumscription of *Cephalocereus* (Cactaceae) Based on Molecular and Morphological
731 Evidence. *Systematic Botany* **42**: 709–723. DOI:
732 https://doi.org/10.1600/036364417X696546
733 Tel-Zur N, Abbo S, Bar-Ziv D, Mizrahi Y. 2004. Genetic relationships among *Hylocereus*
734 and *Selenicereus* vine cacti (Cactaceae): Evidence from hybridization and cytological
735 Studies. *Annals of Botany* **94**: 527–534. DOI: https://doi.org/10.1093/aob/mch183
736 Tel-Zur N, Mouyal J, Zurgil U, Mizrahi Y. 2020. In Support of Winge's Theory of
737 "Hybridization Followed by Chromosome Doubling." *Frontiers in Plant Science* **11**:
738 954 DOI: https://doi.org/10.3389/fpls.2020.00954
739 Tepedino VJ, Griswold TL, Bowlin WR. 2010. Reproductive biology, hybridization, and
740 flower visitors of rare *Sclerocactus* taxa in Utah's Uintah Basin. *Western North*
741 *American Naturalist* **70**: 377–386. DOI: https://doi.org/10.3398/064.070.0310
742 Vázquez-Lobo A, Morales GA, Arias S, Golubov J, Hernández-Hernández T, Mandujano
743 MC. 2015. Phylogeny and Biogeographic History of *Astrophytum* (Cactaceae).
744 *Systematic Botany* **40**: 1022–1030. DOI: https://doi.org/10.1600/036364415X690094
745 Vázquez-Sánchez M, Terrazas T, Arias S, Ochoterena H. 2013. Molecular phylogeny,
746 origin and taxonomic implications of the tribe Cacteae (Cactaceae*). Systematics and*
747 *Biodiversity* **11**: 103–116. DOI: https://doi.org/10.1080/14772000.2013.775191

748 Vázquez-Sánchez M, Terrazas T, Arias S. 2012. El hábito y la forma de crecimiento en la
749 tribu Cacteae (Cactaceae, Cactoideae). *Botanical sciences* **90**: 97–108 Available at:
750 http://www.scielo.org.mx/scielo.php?script=sci_arttext&pid=S2007-
751 42982012000200001.
752 Vite F, Portilla E, Zavala-Hurtado JA, Valverde PL, Díaz-Solís A. 1996. A natural hybrid
753 population between *Neobuxbaumia tetetzo* and *Cephalocereus columna-trajani*
754 (Cactaceae). *Journal of Arid Environments* **32**: 395–405. DOI:
755 https://doi.org/10.1006/jare.1996.0033
756 Wallace RS, Dickie SL. 2002. Systematic implications of chloroplast DNA sequence
757 variation in the Opuntioideae. *In*: Hunt D. ed. *Studies in the Opuntioideae (Cactaceae)*
758 England: DH Books, pp. 9–24.
759 Whitney KD, Ahern JR, Campbell LG, Albert LP, King MS. 2010. Patterns of
760 hybridization in plants. *Perspectives in Plant Ecology, Evolution and Systematics* **12**:
761 175–182. DOI: https://doi.org/10.1016/j.ppees.2010.02.002
762 Yáñez-López ML, Barbosa-Martínez C, Serrato-Díaz A, Balderas-Morales A, Campos-
763 Muñiz C, Fernández FJ, *et al*. 2016. Genetic relationships between varieties of
764 *Stenocereus pruinosus* (Cactaceae) in the states of Puebla and Oaxaca, Mexico, by
765 means of AFLP markers. *Agrociencia* **50**: 595–601.
766

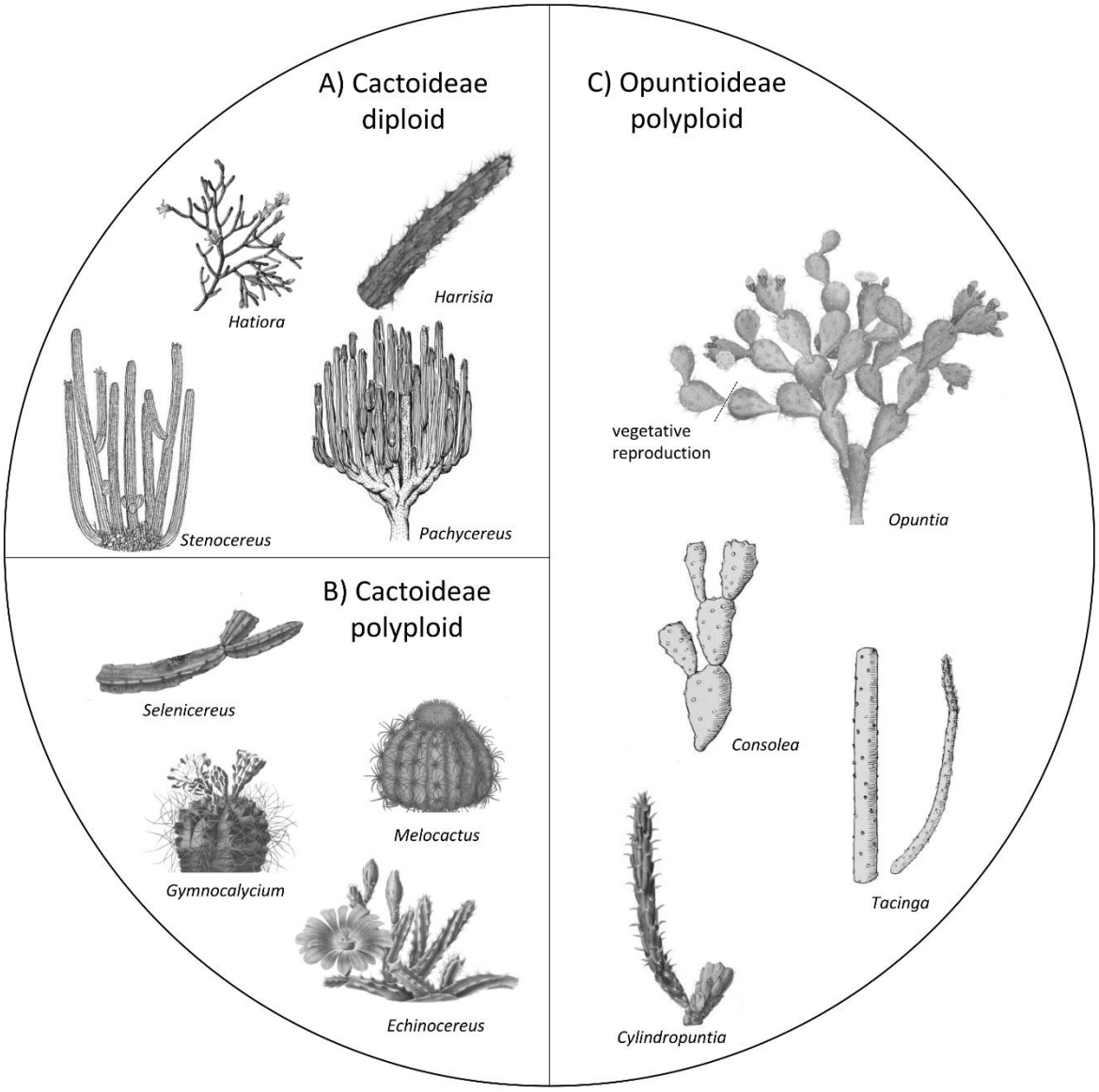


Figure 1. Representative examples of genera with hybridization in Cactaceae. A) Diploid genera from Cactoideae. B) Polyploid genera from Cactoideae. C) Opuntioideae representatives of the genus in the subfamily with reported hybridization. Images modified from Britton and Rose (1919, 1920, 1922, 1923) and Arias *et al.* 2012.

Appendix 1. Natural hybrids reported in Cactaceae based on classic monographs and scientific articles following the taxonomic classification reported by Hunt *et al*. (2006). Information about ploidy level was consulted in The Chromosome Counts Database (CCDB).

Subfamily	Tribe	Genus	Hybridization Interspecific=a Intergeneric=b	Reported ploidy	Evidence analyzed	Reference
Cactoideae	**Echinocereeae**	*Pachycereus*	b	2n	Morphological description / Molecular markers	(Hunt et al., 2006; Copetti et al., 2017)
		Cephalocereus	a	-	Morphometric analysis / Molecular markers	(Vite et al., 1996; Tapia et al., 2017)
		Echinocereus	a	2n, 3n, 4n	Experimental crosses and chromosome counting	(Powell et al., 1991)
		Stenocereus	b	2n	Molecular markers	(Yáñez-López et al., 2016)
		Bergerocactus	b	4n	**Morphological description**	(Hunt et al., 2006)
		Myrtillocactus	b	2n	**Morphological description**	(Hunt et al., 2006)
		Escontria	b	-	Morphometric analysis, molecular markers and experimental crosses	(Cruz-Zamora et al., 2017)

	Polaskia	b	-	Morphometric analysis, molecular markers and experimental crosses	(Cruz-Zamora et al., 2017)
Hylocereeae	*Disocactus*	a	2n	**Morphological description**	(Anderson, 2001)
	Selenicereus	a	2n, 4n	Experimental crosses and cytogenetic analyses	(Tel-Zur et al., 2004)
Cereeae	*Arrojadoa*	a	2n	**Morphological description**	(Anderson, 2001)
	Browningia	b	-	**Morphological description**	(Hunt et al., 2006)
	Cereus	b	2n	**Morphological description**	(Hunt et al., 2006)
	Coleocephalocereus	b	-	**Morphological description**	(Hunt et al., 2006)
	Melocactus	a	2n, 4n	Molecular markers and morphometric analysis / Molecular markers	(Lambert et al., 2006a, 2006b; Khan et al., 2020)
	Micranthocereus	b	2n	**Morphological description**	(Hunt et al., 2006)
	Pilosocereus	a, b	2n, 4n	**Morphological description**	(Anderson, 2001)
Trichocereeae	*Arthrocereus*	b	-	**Morphological description**	(Font and Picca, 2001)

	Cleistocactus	b	2n	**Morphological description**	(Anderson, 2001)
	Denmoza	b	-	**Morphological description**	(Font and Picca, 2001; Hunt et al., 2006)
	Echinopsis	a, b	2n	**Morphological description / Morphological description**	(Hunt et al., 2006; Eggli and Giorgetta, 2018)
	Espostoa	b	2n	**Morphological description**	(Hunt et al., 2006)
	Gymnocalycium	a	2n 4n	Molecular markers	(Řepka and Mráĉek, 2012)
	Haageocereus	b	-	**Morphological description**	(Hunt et al., 2006)
	Harrisia	b	2n	**Morphological description**	(Hunt et al., 2006)
	Matucana	b	2n	**Morphological description**	(Hunt et al., 2006)
	Oroya	b	-	**Morphological description**	(Hunt et al., 2006)
	Oreocereus	b	-	**Morphological description**	(Hunt et al., 2006)
	Weberbauerocereus	b	4n, 8n	**Morphological description**	(Hunt et al., 2006)
	Yungasocereus	b	-	**Morphological description**	(Hunt et al., 2006)

	Notocacteae	-	-			-
	Cacteae	*Escobaria*	a	2n	Morphometric analysis	(Baker and Johnson, 2000)
		Ferocactus	b	2n	**Morphological description**	(Anderson, 2001)
		Geohintonia	b	-	**Morphological description**	(Anderson, 2001)
		Leuchtenbergia	b	-	**Morphological description**	(Anderson, 2001)
		Sclerocactus	a	2n	Experimental crosses / Molecular markers	(Tepedino et al., 2010; Schwabe et al., 2015)
		Turbinicarpus	a	2n	**Morphological description**	(Hunt et al., 2006)
Opuntioideae	**Cylindropuntieae**	*Cylindropuntia*	a	2n, 3n, 4n, 8n	Morphometric, chromosome counting and pollen viability	(Baker and Pinkava, 1987)
	Opuntieae	*Opuntia*	a	2n, 3n, 4n, 5n, 6n, 8n, 9n	Chromosome counting / Molecular markers	(Pinkava, 2002; Majure et al., 2012a)
		Tacinga	a	2n, 4n	**Morphological description**	(Anderson, 2001)
		Consolea	b	2n, 6n, 8n, 12n	Chromosome counting	(Negrón-Ortiz, 2007)
Pereskioideae	-	-	-			-
Maihuenioideae	-	-	-			-

Capítulo 2. Genome evolution and phylogenetic relationships in *Opuntia tehuacana* (Cactaceae, Opuntioideae)

Xochitl Granados-Aguilar, G. Palomino, J. Martínez-Ramón and Salvador Arias

Phytotaxa (Sometido)

Genome evolution and phylogenetic relationships in *Opuntia tehuacana* (Cactaceae, Opuntioideae)

XOCHITL GRANADOS-AGUILAR [1, 2*], G. PALOMINO[2], J. MARTÍNEZ-RAMÓN[2] AND SALVADOR ARIAS[2*]

[1]*Posgrado en Ciencias Biológicas, Instituto de Biología, Universidad Nacional Autónoma de México, Mexico City, Mexico.*
xochitl.granados@st.ib.unam.mx; https://orcid.org/0000-0001-7570-4211

[2]*Jardín Botánico, Instituto de Biología, Universidad Nacional Autónoma de México, Mexico City, Mexico.*

palomino@ib.unam.mx; https://orcid.org/0000-0002-8150-9946
mramon@ib.unam.mx; https://orcid.org/0000-0002-7282-8734
sarias@ib.unam.mx; https://orcid.org/0000-0002-7674-7050
[*]*Authors for correspondence*

Abstract

This study is focused in the Tehuacán-Cuicatlán Valley (Oaxaca, Mexico), with a high diversity of *Opuntia* species whose phylogenetic relationships and chromosome numbers are unknown. We aim to know the phylogenetic position of *O. tehuacana* and their sympatric species, and to analyze the ploidy levels in five *O. tehuacana* localities. We performed Bayesian phylogenetic analysis using three chloroplast markers and two nuclear introns, as well as chromosome counts for three *Opuntia* species and flow cytometry analysis in *O. tehuacana*. Our phylogenetic trees shown *O. tehuacana* as member of Basilares clade as well as most of their sympatric species, except for *O. decumbens*, *O. lasiacantha* and *O. huajuapensis* which are in Nopalea clade. The ploidy level of *O. tehuacana* is 11x and 12x the highest reported so far for the genus, for *O. pilifera* is 8x and for *O. huajuapensis* is 2x. Finally, we found significant differences among *O. tehuacana* genome sizes and the high ploidy level might be due to multiple polyploidization events occurred between individuals from the same specie as well as from other *Opuntia* species.

Keywords: Aneuploidy, interspecific gene flow, phylogenetic analysis, polyploidy.

Introduction

In Cactaceae, one of the genera with the highest number of species is *Opuntia* Miller (1754: no pagination) with approximately 150 species, which are naturally distributed in the Americas from Canada to Argentina (Hunt *et al*., 2006; Barthlott *et al*., 2015). In Mexico are approximately 93 species, being one of the countries with the highest diversity of species of this genus (Hunt *et al*., 2006). *Opuntia* is characterized by deciduous vestigial leaves, flat stems called cladodes, areoles with glochids, flowers usually bisexual with tepals of yellow, orange, pink or red colors and fruits mostly fleshy, sweet or acidic. The prickly pears have a great cultural and economic importance of their edible stems and fruits as well as medicinal uses (Bravo-Hollis, 1978; Anderson, 2001).

There are multiple taxonomic problems in *Opuntia* mainly due to wide variation in morphological characters, hybridization, homoplasy and the fact that these species are mostly polyploids further complicating their study (Pinkava, 2002; Majure *et al*., 2012c). Being polyploid allows to survive in adverse environments like deserts where the weather is very hot in the day and the temperature drops at night, and also plays an important role in the speciation and evolution (Soltis *et al*., 2003; De Storme & Mason, 2014). Despite the great relevance of knowing the ploidy in *Opuntia* there are still species whose ploidy is unknown, or with wide distribution and their ploidy is only known from one locality (Baker & Pinkava, 2018). Sometimes opuntias have different levels of ploidy throughout their distribution (Majure *et al*., 2012a), thus carrying out cytogenetic analysis in different populations of the same species is of great relevance.

Phylogenetic analysis performed in *Opuntia sensu stricto* (*s.s.*) (Majure *et al*., 2012b) have shown that North American species are divided into two major clades which in turn are divided into the subclades Basilares, Nopalea, Scheerianae, Macrocentra and Humifusa. That

phylogenetic approach also revealed that polyploid species are interclade hybrids involving most of the North American subclades. Perform phylogenetic analysis using plastid and nuclear molecular markers is of great relevance for inferring maternal and biparental inheritance in genera with previous evidence of hybridization (Sang & Zhang, 1999; Russell *et al*., 2010; Meng *et al*., 2011; Majure *et al*., 2012b; Wang *et al*., 2014).

Studying areas with high species diversity is relevant, the biosphere reserve Tehuacán-Cuicatlán Valley is a natural area with a high number of *Opuntia* species, is located in the southeast part of Mexico and comprises the states of Puebla and Oaxaca. It has 15 *Opuntia* species, and although four of them have been included in phylogenetic analysis the rest of them have not. The species *Opuntia cochenillifera* Miller (1768: no pagination) and *O. decumbens* Salm-Reifferscheid (1834: 361) are in the Nopalea clade and *O. pilifera* Bois (1898: 894) and *O. tomentosa* Salm-Reifferscheid (1822: 8) were identified as interclade hybrids, but for the other 11 species there is no information about their phylogenetic position (Arias *et al*., 2012; Majure *et al*., 2012b).

One interesting species endemic to Tehuacán-Cuicatlán Valley is *Opuntia tehuacana* Arias & Guzmán (1997: 131), this species is characterized by shrubby habit, stem segments suborbicular to obovate, glabrous, areoles sometimes having scarce hairs, flowers yellow to red-orange and fruits greenish to yellowish which sometimes do not produce seeds. Their restricted distribution and morphological characteristics had led some taxonomist to consider it as a hybrid between *O. pilifera* and *O. huajuapensis* Bravo (1954: 484), or a variety of *O. lasiacantha* Pfeiffer (1837: 160) (Hunt *et al*., 2006; Arias *et al*., 2012). Another hypothesis is that some *O. tehuacana* individuals have introgression from *O. decumbens* and *O. huajuapensis*. *O. tehuacana* also has variable characteristics throughout its distribution, such as the color of the flowers, the shape of the stem segments, the presence of hairs and the length and number of spines, because of this certain populations were described as new species but later considered as a synonym and circumscribed as a single species (Arias *et al*., 2012; Martínez-González *et al*., 2021). Inhabits the same area with *O. decumbens*, *O. depressa* Britton and Rose (1908: 517), *O. huajuapensis*, *O. lasiacantha*, *O. pilifera*, and *O.*

velutina Weber (1904: 389) (Arias *et al*., 2012; Granados-Aguilar *et al*., 2021), and as many wild opuntias from this biosphere reserve their phylogenetic relations and ploidy remain unknown. In this study, we aimed to infer the phylogenetic position of *O. tehuacana* in the context of its sympatric species and analyze the genome evolution by estimating ploidy levels and genome size in five *O. tehuacana* populations.

Materials and methods

Plant material was collected through a series of field trips to Tehuacán-Cuicatlán Valley in October 2017, December 2017 and June 2018. We collected three to five individuals along *O. tehuacana* distribution from the localities Ajalpan and Coxcatlán in Puebla and Tecomavaca, Cuicatlán and Nochixtlán in Oaxaca (Supplementary Table 1). In addition, one individual per species was collected from the sympatric species of *O. tehuacana* (Supplementary Table 2). Additional plant material of *O. tehuantepecana* Bravo (1972: 118) and *Grusonia invicta* Anderson (1999: 325) as outgroup were taken from the cacti collection of the Botanical Garden of Instituto de Biología, Universidad Nacional Autónoma de México (JB-IBUNAM). Collected material was introduced to the living collection of cacti at JB-IBUNAM and voucher specimens were deposited in the MEXU herbarium. To place species in study in a broad phylogenetic context we download from GenBank sequences from plastid markers *matK*, *ycf1,* and *psbJ-petA* for 79 Opuntioideae species from main clades reported by Majure *et al.* (2012b).

Molecular methods

We extracted from dried tissue the total genomic DNA using the CTAB protocol (Doyle and Doyle, 1987). To avoid excess of mucilage we follow the modifications reported by Bustamante *et al.* (2016). We amplified one individual from seven *Opuntia* species (Fig. 1; Supplementary Table 2) and 12 individuals from *Opuntia tehuacana* for the plastid genes *matK*, *ycf1* and the intergenic spacer *psbJ-petA* using the primers reported by Majure *et al.*

(2012b), with some modifications for amplification in *ycf1* and *psbJ-petA*, and the nuclear introns *AT3G48380* and *AT1G18270* as reported by Granados-Aguilar *et al*. (2021). All PCR reactions were performed in 15 µL with the commercial mix "Platinum Taq" (Invitrogen). Reactions included 1.5 µL (1x) of 10x PCR buffer, 0.3 µL of dNTP mix, 0.3 µL of each primer (10 pmol/µL), 0.6 µL $MgCl_2$, and 0.075 µL (0.375 units) of Taq DNA polymerase. Only for nuclear markers we add 0.3 µL BSA (0.4%). Modified PCR conditions for *ycf1* were initial denaturalization at 94 °C for 2 min followed by 11 cycles of denaturalization at 94 °C for 30 s, then annealing decreasing temperature from 63 °C to 51 °C per minute, extension at 72 °C for 1.5 min and a final extension of 72 °C for 5 min, then 30 cycles of denaturalization at 94 °C for 30 s, annealing at 50 °C for 1 min, extension at 72 °C for 2.5 min, and a final extension of 72 °C for 5 min. For *psbJ-petA* PCR conditions were denaturalization at 94 °C for 2 min, followed by 32 cycles of denaturalization at 94 °C for 1 min, annealing at 55°C for 1 min using a 0.3 °C ramp per cycle, extension at 72 °C for 2.5 min, and a final extension at 72 °C for 5 min. Amplifications were made for all individuals of *O. tehuacana*, one individual from each collected *Opuntia* species and the outgroup. The sequencing and cleaning of PCR products was carried out at Laboratorio de Secuenciación Genómica de la Biodiversidad y la Salud del Instituto de Biología, UNAM. The sequences generated for this work were edited in Sequencher v.5.4.6 and aligned with the sequences downloaded from GenBank using the program Muscle v.3.8.31 (Edgar, 2004), then the alignments were manually revised in PhyDE v.0.9971 (Müller et al., 2005). Non-alignable zones containing poly T/A were excluded for *ycf1*, *psbJ-petA* and *AT1G18270*.

Reconstruction of phylogenetic trees

Chloroplast aligned matrix consisted of 100 terminals of which 79 were downloaded from GeneBank and 21 were generated for this study. Phylogenetic analyses were performed under Bayesian inference (BI) in MrBayes v.3.2.6 (Ronquist & Huelsenbeck, 2003) using two matrices, one with the newly generated sequences and the downloaded GenBank terminals from the chloroplast markers *matK*, *ycf1* and *psbJ-petA*, and another matrix only with the newly sequenced terminals from the three plastid markers and the nuclear introns

AT3G48380 and *AT1G18270*. Molecular substitution models that best fit each molecular marker, were identified using the Bayesian information criterion (BIC) implemented in jModelTest v.2.1.7 (Darriba *et al*., 2012), for *matK* was HKY, in *psbJ-petA* HKY+I+G, for *ycf1* HKY+G, and for nuclear introns *AT3G48380* and *AT1G18270* the model was F81+G. The BI analyses consisted of two runs of four parallel chains for 10 million generations, each run starting with a random tree and sampled every 1000 generations. A burn-in of 25% was selected and with the remaining trees a 50% majority-rule consensus tree was generated with its posterior probabilities (PP). The obtained trees were edited using the online tool iTOL (Letunic & Bork, 2007).

Haploid and diploid chromosome counts

Meiotic chromosome analysis was observed only for *O. huajuapensis* (Table 1; GAX 118) and *O. tehuacana* from three localities (Table 1; GAX 100, GAX 162, GAX 107), because after two years of cultivation were the only species that blooming. The analysis consisted in collect immature flowers which were cut in cross-section with a scalpel and fixed in Farmer solution (3:1 ethanol: glacial acetic acid), and stored for 24 hours in refrigeration at 4°C. Afterwards anthers were dissected, and 3 to 5 anthers were placed on a slide and a drop of propionic-orcein dye was added (1.8 %), then anthers were squashed, frozen in nitrogen, subsequently dried and fixed in Entellan resin for their posterior observation. Subsequently, all Metaphase I cells were observed and photographed with a 100x objective in the Axioskop (Carl-Zeiss) photomicroscope. Pollen mother cells (PMC) in Metaphase I were analyzed with the following data collected: type of bivalents (IIs), type of quadrivalents (IVs), haploid number of chromosomes and for each analyzed cell the chiasma frequency and recombination index (RI) to obtain the average value of the species.

Diploid chromosome counts were performed in *O. huajuapensis*, *O. tehuacana* and *O. pilifera* (Supplementary Table 1). Root meristems were obtained from individuals collected in the field and prepared according to the protocol described by Palomino & Vázquez (1991). The counts were performed by observing 20 mitotic cells per individual in

an Axioskop photomicroscope (Carl-Zeiss) and the best three cells per individual were photographed.

Estimation of 2C nuclear DNA content

To perform flow cytometry analysis, we used seedlings from the five sampled *O. tehuacana* localities (Supplementary Table 1). Young plants were obtained by germination of seeds collected in the field which were washed, mechanically scarified, and sown in sterilized substrate, with an approximate germination time of three months. We used young plants from more than three centimeters, to measure the amount of 2C DNA content by flow cytometry. For each of the five analyzed localities ten to twelve repetitions were made to obtain 2C DNA measurements. After preliminary analysis, *Zea mays* Linné (1753: 971) CE–777 2C DNA = 5.43 pg was selected as the internal standard (Doležel *et al*., 2018). We follow the protocol described by Otto (1990) to prepare the nuclei suspension with some modifications according to Doležel *et al*. (2007) and Palomino *et al*. (2016). For each sample we used 500 to 800 mg of young *O. tehuacana* stem and 50 mg of *Zea mays* leaf tissue, both were chopped with a razor blade and according with the cytometer used we followed two procedures. For the analyses performed on CyFlow (Partec), they were added 1 ml of Otto I (0.1 M citric acid and 1 % Tween 20) afterwards the material was filtered through a 42 μm nylon mesh. Next, we centrifuge the sample at 90 g for three minutes, the supernatant was discarded, we add 1 ml of Otto I and incubated for 15 min at room temperature. Thereafter, we add 2 ml of Otto II (0.4 M Na_2HPO_4), 125 μl of propidium iodide and 125 μl of RNAse (50 μg/ml). The analyses performed on CytoFLEX S (Beckman Coulter) were realized adding to each sample ice-cold buffer LB01 with 1 % of Triton (Doležel *et al*., 2007), subsequently samples were homogenized and filtered through a 42 μm nylon mesh. Afterwards we add 125 μl of propidium iodide and 125 μl of RNAse and incubated for 15 min on ice. Analyses were performed at Laboratorio de Citogenética JB-IBUNAM and Laboratorio Nacional de Citometría de Flujo UNAM, using the cytometers CyFlow (Partec) and CytoFLEX S (Beckman Coulter), respectively. For each sample run ten thousand nuclei were measured. The analyses of peak means, areas and coefficient of variation (lower than 5 %) were

performed using the software DPAC software (Partec) and FlowJo software v.10 on a Windows workstation.

Statistical analyses

We evaluated if there were differences of 2C nuclear content between the *O. tehuacana* analyzed localities through an unbalanced one-way ANOVA. Afterwards, if the ANOVA results were significant a Tukey's HSD test was performed. Analyses were performed in R (Team R. Core, 2013) using the packages WRS2, psych, reshape2, and agricolae.

Results

Phylogenetic analysis

Concatenated plastid matrix consisted of 2539 aligned characters and the resulting BI tree (Fig. 2) shown *Opuntia s.s.* as monophyletic with high support (Fig. 2, 1 PP). From the previously reported ten clades inside *Opuntia s.s*. (Majure et al., 2012a) our analysis recovered Basilares, Humifusa, Macrocentra, Nopalea Sheerianae, and Xerocarpa with low to high support (Fig. 2). Our analysis could not recover the relationships among major clades of *Opuntia s.s*. Species from Tehuacán-Cuicatlán Valley are in the clades Nopalea and Basilares. *O. decumbens*, *O. huajuapensis* and *O. lasiacantha* are in Nopalea and form a highly supported clade (Fig. 2, 0.9 PP). The Basilares clade is recovered with low support (Fig. 2, 0.6 PP) and from the previously reported subclades only Xerocarpa was recovered. Another subclade inside Basilares (Fig. 2, 0.5 PP) includes *O. megarhiza*, *O. pachyrrhiza* and *O. chaffeyi* from Rhizomatosa, *O. stenopetala* and *O. rufida* from *Microdasys* and the species from Tehuacán-Cuicatlán Valley *O. depressa*, *O. pilifera*, *O. streptacantha*, and *O. tehuantepecana*. Finally, inside Basilares all *O. tehuacana* terminals integrate a clade with high support (Fig. 2, 1 PP).

Nuclear plus plastid matrix consisted of 21 terminals, 4245 aligned characters and the majority-rule consensus tree (Fig. 3) shown *O. tehuantepecana* as sister of all *Opuntia* species from Tehuacán-Cuicatlán Valley (Fig. 3, 0.9 PP), subsequently *O. streptacantha* (0.6

PP) and *O. depressa* (Fig. 3, 0.5 PP) are sisters to the rest of sampled opuntias. *O. velutina* is sister to a highly supported clade (Fig. 3, 1 PP) which is integrated by two subclades, one with the species *O. decumbens*, *O. huajuapensis* and *O. lasiacantha* (1 PP), and the other consisting of all sampled terminals from *O. tehuacana* (1 PP) with a defined geographical structure and integrated by two subclades, the first (Fig. 3, highlighted in purple;1 PP) has terminals from southern distribution (Nochixtlán and Cuicatlán) and the second (Fig. 3, 0.9 PP) integrated by terminals from the northern part of its distribution (Ajalpan, Coxcatlán and Tecomavaca).

Chromosome numbers and genome size in opuntias from Tehuacán-Cuicatlán Valley

Meiotic and somatic chromosome counts (Table 1; Fig. 4 A and 5 A) confirm *O. huajuapensis* as diploid (x = 11 = n = 11; 2x = 2n = 22), *O. tehuacana* in Ajalpan as x = 11 = n = 63, 2n = 11x + 5 = 126 (Fig. 4 D and 5 C), *O. tehuacana* from Coxcatlán as x= 11= n= 66, 2n = 12x =132 (Fig. 4 B and 5D), and *O. tehuacana* from Cuicatlán as x = 11 = n = 63, 2n = 11x + 5 = 126 (Fig. 4 C and 5 E). Whereas somatic chromosome counts found *O. pilifera* as octoploid (2n = 8x = 88; Fig. 5 B) and *O. tehuacana* in Nochixtlán and Tecomavaca as 11x (x= 11 = 11x = 121) (Fig. 5 F and G).

Flow cytometry analyses for *O. tehuacana* (Supplementary Figure 1) are consistent with chromosome counts, the higher ploidy the larger genome size (Table 2) and the standard error ranged from 0.03 to 0.07 %. The highest ploidy was found in Coxcatlán 2n = 12x = 132 with a genome size of 2C DNA = 10.54 pg. In Ajalpan and Cuicatlán the chromosome number was 2n = 11x+5 = 126 and genome size was 2C DNA = 9.02 pg and 9.44 pg respectively, while in Tecomavaca and Nochixtlán we found the smallest ploidy 2n = 11x = 121 and their 2C DNA content was 8.40 pg and 8.81 pg respectively. We found significant differences between genome sizes of *O. tehuacana* localities according to a post-hoc Tukey honest significant differences test (Table 2).

Discussion

Phylogenetic relationships among opuntias from Tehuacán-Cuicatlán Valley

Previous phylogenetic analyses in *Opuntia* have been carried out excluding polyploids to better circumscribe larger clades, although it is also important to include polyploids and even more if their phylogenetic relationships are unknown, in order to know their maternal inheritance (Rieseberg & Soltis, 1991; Arnold, 1997). In a broad phylogenetic context with chloroplast markers (Fig. 2) *O. tehuacana* is nested within the Basilares clade, all of its terminals form a monophyletic group integrated by two cytotypes 11x and 12x (Table 2). Polyploid members of Basilares are involved in interclade hybridization (Majure *et al*., 2012b) and it might be the case for *O. tehuacana*. Phylogenetic analysis using plastid plus nuclear markers shown *O. tehuacana* (Fig. 3) as sister to *O. huajuapensis*, *O. decumbens*, and *O. lasiacantha*, these species were recovered in Nopalea clade in our plastid analysis, thus these incongruities can be indicative of hybridization or introgression (Rieseberg, 1991; Arnold, 1997). Interspecific gene flow between individuals of *O. huajuapensis*, *O. decumbens* and *O. tehuacana* has been previously indicated, therefore our results are consistent with former analyses (Granados-Aguilar *et al*., 2021).

Another polyploid from Tehuacán-Cuicatlán Valley, *O. pilifera*, was proposed in previous phylogenetic analyses as an interclade hybrid between Basilares and Nopalea and by means of phylogenetic network analysis, it was proposed as involved in hybridization with *O. decumbens*, although the phylogenetic position of the latter was still unknown (Majure *et al*., 2012b; Granados-Aguilar *et al*., 2021). Our plastid phylogeny (Fig. 2) shown that *O. decumbens* is in Nopalea clade, so both the first proposal of interclade hybridization as well as the proposal of this species as putative donor of genetic material are congruent to their phylogenetic position (Majure *et al*., 2012b; Granados-Aguilar *et al*., 2021). The ploidy for *O. pilifera* is 8x, according to our results and previous counts (Majure *et al*., 2012c), and for *O. decumbens* is 6x (Baker & Pinkava, 2018), thus the species with the lowest ploidy, *O. decumbens*, could be acting as pollen donor, reinforcing the possibility of genetic exchange between these species as has been reported in other *Opuntia* (Griffith, 2001).

Our analyses could not recover the relations between major clades of *Opuntia s.s*, probably due to a lower number of markers used in our analysis, three (*matK*, *ycf1*, and *psbJ-petA*) versus six in previous analysis (*atpB-rbcL*, *ndhF-rpl32*, *psbJ-petA*, *trnL-trnF*, *matK*, and *ycf1*) (Majure *et al*., 2012b). Despite this, most of the clades were recovered and we were able to know the phylogenetic position of the interest species. Subclades inside Basilares were not recovered probably due to the inclusion of polyploids. It is important to highlight those discrepancies between the chloroplast and the chloroplast plus nuclear phylogenetic trees could be indicating genetic exchange between members of Nopalea and Basilares, as has been regarded in other studies (Majure *et al*., 2012b; Majure & Puente, 2014).

Remarkable variation of genome size and ploidy level in *O. tehuacana*

Our results have shown significant differences between genome sizes in *O. tehuacana* localities (Table 2). These differences do not appear to be related to geographic distribution; for example, the nearest localities Ajalpan and Coxcatlán (Fig. 1) have a difference between them of 1.52 pg (approximately 1486.5 Mbp), while the most distant localities Ajalpan and Nochixtlán only differ in 0.21 pg (approximately 205.38 Mbp). Therefore, multiple polyploidization events inside each locality could be related to the evolution of genome size, as well as different cytotypes promoting variations within the species (Soltis & Soltis, 1999; Walker *et al*., 2005; Palomino *et al*., 2016; Nodal-Moreno *et al*., 2019). Polyploidy is considered a relevant evolutionary force, thus the fact that *O. tehuacana* is polyploid and has differences in both its chromosome number and the amount of 2C DNA content allows that a wide range of polyploid genotypes can coexist and originate new lineages whose survival will be determined by the action of natural selection (Soltis & Soltis, 1999).

The results are reliable although two different flow cytometers were used to measure the amount of 2C DNA content in *O. tehuacana*, because of the use of the same internal standard (*Zea mays* CE–777), the same number of measured nuclei as well as a coefficient of variation of G0/G1 peaks lower than 5 % for all samples. Furthermore, significant differences of 2C DNA content among *O. tehuacana* localities are in agreement with chromosome numbers (Table 2). The differences between Ajalpan (9.02 pg) and Cuicatlán

(9.44 pg) could be related to aneuploidies and between Tecomavaca (8.40 pg) and Nochixtlán (8.81 pg) due to the loss of DNA sequences after polyploidization (Leitch & Bennett, 2004; Chen *et al*., 2007; Palomino *et al*., 2016). Although genome size, downsizing is common among polyploids compared to their closely related diploids (Leitch & Bennett, 2004), the amount of DNA within-species usually varies very little and is stable (Leitch & Bennett, 2004; Del Angel *et al*., 2006; Eilam *et al*., 2010), unlike our results which show variation between the localities of *O. tehuacana*, which could be due to allopolyploidy, satellite DNA, transposable elements or ribosomal genes in different genomes (Biémont, 2008), furthermore, this condition must be studied more deeply through chromosome counts and the use of in situ hybridization techniques to understand the relevance of intraspecies variation as well as their causes.

According to our chromosome counts the ploidies for *O. tehuacana* are 11x and 12x, for other *Opuntia* species ploidy reports range from 2x to 9x, thus the chromosome number of *O. tehuacana* is the highest reported so far in *Opuntia* (Powell & Weedin, 2001; Baker *et al*., 2009; Majure *et al*., 2012c; Baker & Pinkava, 2018). The two cytotypes found for *O. tehuacana* may represent a mature or declining polyploid complex because they are located in restricted areas and we did not find diploids or individuals with ploidy lower than 11x throughout its distribution (Stebbins, 1971). It is inferred that the mature polyploid complexes originated between the Pliocene and Pleistocene, so this approximation is consistent with the information on the estimates of divergence times for the Basilares clade in which *O. tehuacana* is found (Stebbins, 1971; Majure *et al*., 2012b).

Finally, although polyploidy could explain the differences between genome sizes in *O. tehuacana* localities, it would be of great relevance to analyze more deeply in other studies the chromosome pairing using tolls like fluorescence in situ hybridization (FISH) and genomic in situ hybridization (GISH) as well as high throughput sequencing of chloroplast and nuclear genomes to better understand the origin and organization of these differences within a species.

Chromosome pairing and their implications on interspecific gene flow

In *O. tehuacana* from Ajalpan and Cuicatlán occurs aneuploidy, which may be originated by unequal translocations between non-homologous chromosomes and this process may be occurring by gene exchange from other species in individuals from these localities (Stebbins, 1971; Wu et al., 2014). Previously, evidence of introgression on the same localities have been reported based on phylogenetic network analysis, so our chromosomal counts support the proposal of gene exchange between sympatric species (Granados-Aguilar *et al*., 2021). Due to the high ploidy level in *O. tehuacana* is likely that multiple polyploidization events occurred between individuals from the same specie as well as from other opuntias (Soltis & Soltis, 1999).

Pollen mother cells of *O. tehuacana* from Ajalpan have univalent, this kind of chromosome is not associated with any homologue or homoeologous, and have been observed in neo-allopolyploids (Mestiri *et al*., 2010), thus in this particular individual (GAX 100) we could be observing laggard chromosomes or non-pairing chromosomes in a F1 hybrid (Mestiri *et al*., 2010; Wu *et al*., 2014). As discussed above, there is evidence of hybridization on certain individuals of *O. tehuacana* thus, is of great relevance to perform experimental crosses between sympatric species to know if the same pattern is presented in artificial hybrids.

The presence of multivalents in pollen mother cells of *O. tehuacana* from Cuicatlán may be related to that during meiosis in polyploids the chromosomes have more than one possible pair causing a non-preferential chromosome pairing or due to translocation heterozygosity (Wu *et al*., 2014). These PMC could originate aneuploid pollen grains, which are still fertile, but in aneuploid female gametes from model plants fertility problems have been found, thus aneuploid localities (Ajalpan and Cuicatlán) could be at a disadvantage with respect to those with complete sets of chromosomes like Coxcatlán which PMC shown almost normal chromosome pairing (Henry *et al*., 2009; Barke *et al*., 2020).

Opuntia species with higher ploidies, like *O. tehuacana* (11x, 12x) tend to act as pollen receptors for species with lower ploidies (Griffith, 2001). So sympatric species like *O. huajuapensis* (2x) or *O. decumbens* (6x) with lower ploidies could be donating pollen, as previously proposed by reticulation analysis (Granados-Aguilar *et al*., 2021). For other sympatric species like *O. depressa* and *O. velutina* studies of chromosome numbers are necessary to make inferences about their interactions with *O. tehuacana* as well as with the rest of the Tehuacán-Cuicatlán Valley species.

Lastly, in *Opuntia* it is of great relevance to know the ploidy level, to better understand their biological story and thus shed a light on the relationships of target species. The phylogenetic relationships among opuntias are complicated because of multiple events of polyploidization which difficult the understanding of phylogenetic trees, therefore it is important to use multiple sources of evidence to better understand the evolutionary history of these plants.

Acknowledgements

This paper is part of the doctoral research of X. Granados, who thanks the graduate program Doctorado en Ciencias Biológicas, UNAM and the scholarships provided by CONACyT. We thank Yolanda Morales, Daniel Franco, Brenda Calderón and Cristian Cervantes (Jardín Botánico, UNAM) for supporting the field work, Ulises Rosas and Andrea R. Jiménez (Jardín Botánico and Instituto de Biología, UNAM) for their support in molecular laboratory work, Miriam Ladd Otero for their support in flow cytometry work at Jardín Botánico, UNAM, Andrea Bedoya López and Evelyn Alvarez (Laboratorio Nacional de Citometría de Flujo) for their support in flow cytometry work, Jaroslav Dolezel (Institute of Experimental Botany, Czech Republic) for kindly providing control seeds for flow cytometry studies, the Laboratorio Nacional de Citometría de Flujo (LABNALCIT) at Instituto de Investigaciones Biomédicas, UNAM, the LANABIO laboratories at Jardín Botánico and Instituto de Biología, UNAM. This contribution was financially supported by UNAM-DGAPA-PAPIIT <IN208619> granted to S. A.

References

Anderson, E.F. (1999). Cactus and succulent journal. *Cactus and Succulent Journal* 71(6): 325.

Anderson, E.F. (2001). *The cactus Family*. Timber Press, Portland, Oregon, 776 pp.

Arias, S., Gama, S., Vázquez, B., and Guzmán, L.U. (1997). Flora del Valle de Tehuacán-Cuicatlán. Fascículo 95, Cactaceae. Instituto de Biología, Universidad Nacional Autónoma de México 95.

Arias, S., Gama, S., Vázquez, B., & Guzmán, L.U. (2012). Flora del Valle de Tehuacán-Cuicatlán. Fascículo 95, Cactaceae. Instituto de Biología, Universidad Nacional Autónoma de México 95.

Arnold, M.L. (1997). *Natural hybridization and evolution*. Oxford University Press, Nueva York, 215 pp.

Baker, M.A., Rebman, J.P., Parfitt, B.D., Pinkava, D.J., & Zimmerman, A.D. (2009). Chromosome numbers in some cacti of western North America–VIII. *Haseltonia* 117–134. doi:10.2985/026.015.0112

Baker, M., & Pinkava, D. (2018). Chromosome numbers in some cacti of western North America–IX. *Haseltonia* 25: 2–29. doi:10.2985/026.025.0103

Barke, B.H., Karbstein, K., Daubert, M., & Hörandl, E. (2020). The relation of meiotic behaviour to hybridity, polyploidy and apomixis in the *Ranunculus auricomus* complex (Ranunculaceae). *BMC Plant Biology* 20: 523. doi:10.1186/s12870-020-02654-3

Barthlott, W., Burstedde, K., Geffert, J. L., Ibisch, P. L., Korotkova, N., & Miebach, A. (2015). *Biogeography and Biodiversity of Cacti*. Schumannia, Alemania.

Biémont, C. (2008). Within-species variation in genome size. *Heredity* 101: 297–8.

Bravo-Hollis H. (1954). Iconografía de las cactáceas mexicanas (tercera serie). Cactáceas de las Mixtecas Altas. *Anales Instituto de Biología, Universidad Nacional Autónoma de México* 25: 473–552.

Bravo-Hollis H. (1972). Cactáceas y suculentas mexicanas. *Cactáceas y Suculentas Mexicanas* 17(4): 118.

Bravo-Hollis, H. (1978). *Las cactáceas de México*. vol. 1. Universidad Nacional Autónoma de México, Ciudad de México, 743 pp.

Bois, D. (1898). *Dictionnaire d'horticulture illustré*. Paris: P. Klincksieck, Available at: https://www.biodiversitylibrary.org/item/150900.

Britton, N.L., & Rose, J.N. (1908). *Smithsonian miscellaneous collections*. Smithsonian Institution, Washington. Available at: https://www.biodiversitylibrary.org/item/95087.

Bustamante, E., Búrquez, A., Scheinvar, E., and Eguiarte, L.E. (2016). Population genetic structure of a widespread bat-pollinated columnar cactus. *PLOS ONE* 11(3): e0152329. doi:10.1371/journal.pone.0152329

Chen, Z.J., Ha, M., & Soltis, D. (2007). Polyploidy: genome obesity and its consequences. *New Phytologist* 174: 717–720. doi:10.1111/j.1469-8137.2007.02084.x

Darriba, D., Taboada, G.L., Doallo, R., & Posada, D. (2012). jModelTest 2: more models, new heuristics and parallel computing. *Nature Methods* 9: 772. doi:10.1038/nmeth.2109

De Storme, N., & Mason, A. (2014). Plant speciation through chromosome instability and ploidy change: Cellular mechanisms, molecular factors and evolutionary relevance. *Current Plant Biology* 1: 10–33. doi:10.1016/j.cpb.2014.09.002

Del Angel, C., Palomino, G., García, A., & Méndez, I. (2006). Nuclear genome size and karyotype analysis in *Mammillaria* species (Cactaceae). *Caryologia* 59: 177–186. doi:10.1080/00087114.2006.10797914

Doležel, J., Čížková, J., Šimková, H., & Bartoš, J. (2018). One major challenge of sequencing large plant genomes is to know how big they really are. *International Journal of Molecular Sciences* 19(11): 3554. doi:10.3390/ijms19113554

Doležel, J., Greilhuber, J., & Suda, J. (2007). Estimation of nuclear DNA content in plants using flow cytometry. *Nature Protocols* 2: 2233–2244. doi:10.1038/nprot.2007.310

Doyle, J.J., & Doyle, J.L. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* 19: 11–15.

Edgar, R.C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research* 32(5): 1792–1797. doi:10.1093/nar/gkh340

Eilam, T., Anikster, Y., Millet, E., Manisterski, J., & Feldman, M. (2010). Genome size in diploids, allopolyploids, and autopolyploids of mediterranean Triticeae. *Journal of Botany* 2010: e341380. doi:10.1155/2010/341380

Granados-Aguilar, X., Granados Mendoza, C., Cervantes, C.R., Montes, J.R., & Arias, S. (2021). Unraveling reticulate evolution in *Opuntia* (Cactaceae) from Southern Mexico. *Frontiers in Plant Science*. 11: 606809. doi:10.3389/fpls.2020.606809

Griffith, P.M. (2001). Experimental hybridization of Northern Chihuahuan Desert region *Opuntia* (Cactaceae). *Aliso* 20: 37–42.

Henry, I.M., Dilkes, B.P., Tyagi, A.P., Lin, H.-Y., & Comai, L. (2009). Dosage and parent-of-origin effects shaping aneuploid swarms in *A. thaliana*. *Heredity* 103: 458–468. doi:10.1038/hdy.2009.81

Hunt, D.R., Taylor, N.P., & Charles, G. (2006). *The New Cactus Lexicon: Text*. DH books, The Manse, Chapel Lane, Milborne Port. Available at: https://books.google.com.mx/books?id=OHyZAQAACAAJ

Leitch, I.J., & Bennett, M.D. (2004). Genome downsizing in polyploid plants. *Biological Journal of the Linnean Society* 82, 651–663. doi:10.1111/j.1095-8312.2004.00349.x

Letunic, I., & Bork, P. (2007). Interactive tree of life (iTOL): an online tool for phylogenetic tree display and annotation. *Bioinformatics* 23: 127–128. doi:10.1093/bioinformatics/btl529

Linné, C. von (1753). *Species plantarum*. Holmiae: impensis laurentii salvii, Available at: https://www.biodiversitylibrary.org/item/13830.

Majure, L.C., Judd, W.S., Soltis, P. S., & Soltis, D.E. (2012a). Cytogeography of the Humifusa clade of *Opuntia s.s.* Mill. 1754 (Cactaceae, Opuntioideae, Opuntieae): correlations with pleistocene refugia and morphological traits in a polyploid complex. *Comparative Cytogenetics* 6: 53–77. doi:10.3897/CompCytogen.v6i1.2523

Majure, L.C., Puente, R., Griffith, M.P., Judd, W.S., Soltis, P.S., & Soltis, D.E. (2012b). Phylogeny of *Opuntia s.s*. (Cactaceae): Clade delineation, geographic origins, and reticulate evolution. *American Journal of Botany* 99: 847–864. doi:10.3732/ajb.1100375

Majure, L.C., Puente, R., & Pinkava, D.J. (2012c). Miscellaneous chromosome numbers in Opuntieae Dc. (Cactaceae) with a compilation of counts for the group. *Haseltonia*, 18: 67–78. doi:10.2985/026.018.0109

Majure, L.C., & Puente, R. (2014). Phylogenetic relationships and morphological evolution in *Opuntia s. str.* and closely related members of tribe Opuntieae. *In*: Hunt D. (Ed.). *Further Studies in the Opuntioideae (Cactaceae)* Succulent Plant Research, The Manse, Chapel Lane, Milborne Port, pp. 9–30.

Martínez-González, C., Morales-Sandoval, J., González Martínez, C.A., Jiménez-Ramírez, J., & Gallegos-Vázquez, C. (2021). *Opuntia tehuacana* and *O. olmeca* (Cactaceae,

Opuntioideae) are to be considered as ascribed to a single species morphological and molecular evidences. *Phytotaxa* 479: 160–172. doi:10.11646/phytotaxa.479.2.2

Meng, J., Fougere-Danezan, M., Zhang, L.-. B., Li, D.-. Z., & Yi, T.-. S. (2011). Untangling the hybrid origin of the Chinese tea roses: Evidence from DNA sequences of single-copy nuclear and chloroplast genes. *Plant Systematic and Evolution* 297: 157–170. doi:10.1007/s00606-011-0504-5

Mestiri, I., Chagué, V., Tanguy, A.-M., Huneau, C., Huteau, V., Belcram, H., *et al*. (2010). Newly synthesized wheat allohexaploids display progenitor-dependent meiotic stability and aneuploidy but structural genomic additivity. *New Phytologist* 186: 86–101. doi:10.1111/j.1469-8137.2010.03186.x

Miller, P. (1754). *The gardeners dictionary: fourth edition*. The fourth edition, corrected and enlarged. London: Printed for the author and sold by John and James Rivington, Available at: https://www.biodiversitylibrary.org/item/150894.

Miller, P. (1768). *The Gardeners Dictionary: eighth edition*. London: Printed for the author and sold by John and James Rivington, doi:10.5962/bhl.title.541

Müller, J., Müller, K., Neinhuis, C., & Quandt, D. (2005). PhyDE® - Phylogenetic Data Editor. Available at: http://www.phyde.de/index.html [Accessed May 26, 2019].

Nodal-Moreno, S.A., Palomino, G., Almaguer-Sierra, P., Blanco-Macías, F., Barrientos-Lozano, L., Flores Gracia, J., *et al*. (2019). Análisis del tamaño del genoma, poliploidía y patrón endopoliploide en poblaciones de *Nopalea cochenillifera* (L.) Salm-Dyck (Cactaceae) en Tamaulipas, México. *Acta Universitaria* 29: e2238. doi:10.15174/au.2019.2238

Otto, F. (1990). Chapter 11 DAPI staining of fixed cells for high-resolution flow cytometry of nuclear DNA. *In*: Darzynkiewicz and Crissman H.A. (Eds.) *Methods in Cell Biology*. Academic Press, pp. 105–110. doi:10.1016/S0091-679X(08)60516-6

Palomino, G., Martínez, J., Méndez, I., Muñoz-Urías, A., Cepeda-Cornejo, V., & Pimienta-Barrios, E. (2016). Nuclear genome size, ploidy level and endopolyploidy pattern in six species of *Opuntia* (Cactaceae). *Caryologia* 69: 82–89. doi:10.1080/00087114.2015.1109956

Palomino, G., & Vázquez, R. (1991). Cytogenetic studies in Mexican populations of species of *Crotalaria* L. (Leguminosae-Papilionoideae). *Cytologia* 56: 343–351. doi:10.1508/cytologia.56.343

Pfeiffer, L. (1837). *Enumeratio diagnostica cactearum hucusque cognitarum*. Berolini: L. Oehmigke Available at: https://www.biodiversitylibrary.org/item/51983.

Pinkava, D.J. (2002). On the evolution of the North American Opuntioideae. *In*: Hunt D. (Ed.) *Studies in the Opuntioideae* (Cactaceae). Succulent Plant Research, The Manse, Chapel Lane, Milborne Port, pp. 59–98. Available at: https://books.google.com.mx/books?id=lTAmAQAAMAAJ.

Powell, A.M., & Weedin, J.F. (2001). Chromosome numbers in Chihuahuan Desert Cactaceae–III. Trans-Pecos Texas. *American Journal of Botany* 88: 481–485. doi:10.2307/2657113

Rieseberg, L.H. (1991). Homoploid reticulate evolution in *Helianthus* (Asteraceae): evidence from ribosomal genes. *American Journal of Botany* 78: 1218–1237. doi:10.2307/2444926

Rieseberg, L., & Soltis, D. (1991). Phylogenetic consequences of cytoplasmic gene flow in plants. *Evolutionary Trends in Plants* 5: 65–84.

Ronquist, F., & Huelsenbeck, J.P. (2003). MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19: 1572–1574. doi:10.1093/bioinformatics/btg180

Russell, A., Samuel, R., Klejna, V., Barfuss, M.H.J., Rupp, B., & Chase, M.W. (2010). Reticulate evolution in diploid and tetraploid species of *Polystachya* (Orchidaceae) as shown by plastid DNA sequences and low-copy nuclear genes. *Annals of Botany* 106: 37–56. doi:10.1093/aob/mcq092

Salm-Reifferscheidt, J. (1822). *Observationes botanicae in horto Dyckensi*. Typis T. F. Thiriart.

Salm-Reifferscheidt, J. (1834). *Hortus dyckensis: ou catalogue des plantes cultivées dans les jardins de Dyck*. Dusseldorf: Arnz doi:10.5962/bhl.title.110204

Sang, T., & Zhang, D. (1999). Reconstructing hybrid speciation using sequences of low copy nuclear genes: hybrid origins of five *Paeonia* species based on *Adh* gene phylogenies. *Systematic Botany* 24: 148–163. doi:10.2307/2419546

Soltis, D.E., & Soltis, P.S. (1999). Polyploidy: recurrent formation and genome evolution. *Trends in Ecology & Evolution* 14: 348–352. doi:10.1016/S0169-5347(99)01638-9

Soltis, D.E., Soltis, P.S., & Tate, J.A. (2003). Advances in the study of polyploidy since Plant speciation. *New Phytologist* 161: 173–191. doi:10.1046/j.1469-8137.2003.00948.x

Stebbins, G.L. (1971). *Chromosomal evolution in higher plants*. Edward Arnold Publishers, Londres, UK. Available at: http://wellcomelibrary.org/item/b18036594

Team R. Core (2013). R: A language and environment for statistical computing.

Walker, D.J., Moñino, I., González, E., Frayssinet, N., & Correal, E. (2005). Determination of ploidy and nuclear DNA content in populations of *Atriplex halimus* (Chenopodiaceae). *Botanical Journal of the Linnean Society* 147: 441–448. doi:10.1111/j.1095-8339.2004.00379.x

Wang, Z., Du, S., Dayanandan, S., Wang, D., Zeng, Y., & Zhang, J. (2014). Phylogeny reconstruction and hybrid analysis of *Populus* (Salicaceae) based on nucleotide sequences of multiple single-copy nuclear genes and plastid fragments. *PLOS ONE* 9: e103645. doi:10.1371/journal.pone.0103645

Weber, F.A.C. (1904). *Bulletin du Muséum national d'histoire naturelle*. Imprimerie Nationale, Paris. Available at: https://www.biodiversitylibrary.org/item/216893.

Wu, J.-H., Datson, P.M., Manako, K.I., & Murray, B.G. (2014). Meiotic chromosome pairing behaviour of natural tetraploids and induced autotetraploids of *Actinidia chinensis*. *Theoretical and Applied Genetics* 127: 549–557. doi:10.1007/s00122-013-2238-y

FIGURE 1. *Opuntia* species collected in Tehuacán- Cuicatlán valley and sampled *O. tehuacana* localities.

FIGURE 2. Bayesian inference majority-rule consensus tree for the chloroplast markers *matK*, *ycf1* and *psbJ-petA*.

FIGURE 3. Bayesian inference majority-rule consensus tree for plastid *matK*, *ycf1* and *psbJ-petA* plus nuclear markers *AT3G48380* and *AT1G18270*. Known ploidy per specie as well as those described in this work are shown in bold letters. *O. tehuacana* terminals are highlighted by color, in blue from Ajalpan and Tecomavaca, in purple from Nochixtlán and Cuicatlán and in green from Coxcatlán.

FIGURE 4. Meiotic chromosomes for (A) *O. huajuapensis* n = 11, with 11 bivalents (9 ring II + 2 rod II). (B) *O. tehuacana* from Coxcatlán n = 66, with 32 bivalents (29 ring II + 3 rod II) + 1 quadrivalent ring. (C) *O. tehuacana* from Cuicatlán n = 63, with 24 II (8 ring II + 16 rod II) + 1 III rod + 8 IV rod + 1 V rod + 1 VI ring + 2 VI rod + 1 VIII ring. D) *O. tehuacana* from Ajalpan n = 63, with 5 univalent + 23 bivalents (16 ring II + 7 rod II) + 2 III rod + 5 IV (3 IV ring + 2 IV rod) + 1 VII ring + 4 VIII (3 VIII ring + 1 VIII rod) + 1 X ring.

FIGURE 5. Somatic chromosomes in three *Opuntia* species. A) *O. huajuapensis* (2n = 2x = 22), B) (*O. pilifera* 2n = 8x = 88), C) *O. tehuacana* from Ajalpan (2n = 11x = 121+5 = 126), D) *O. tehuacana* from Coxcatlán (2n = 12x = 132), E) *O. tehuacana* from Cuicatlán (2n = 11x = 121+5 = 126), F) *O. tehuacana* from Nochixtlán (2n = 11x = 121), G) *O. tehuacana* from Tecomavaca (2n = 11x = 121).

SUPPLEMENTARY FIGURE 1. Flow cytometry histograms of nuclear DNA content in *O. tehuacana* and *Zea mays* CE–777 (internal standard); peak 1 correspond to G_1 nuclei of *Zea mays*, peaks 2 and 3 correspond to G_1 and G_2 of *O. tehuacana*, analysis performed on CyFlow (Partec).

TABLE 1. Meiotic (n) and somatic (2n) chromosome numbers for *Opuntia* species.

TABLE 2. Ploidy level and average amount of nuclear 2C DNA content per *O. tehuacana* sampled locality. *1 pg = 978 megabase pairs (mbp) (Doležel *et al.*, 2007).

SUPPLEMENTARY TABLE 1. Collection data of *Opuntia* species sampled for cytogenetic analysis.

SUPPLEMENTARY TABLE 2. Collection data of sampled species for phylogenetic analysis.

Table 1

Species	Locality	Collection number	n	2n
O. huajuapensis	Cuicatlán	GAX 118	11	22
O. pilifera	Ajalpan	GAX 97	-	88
O. pilifera	Nochixtlán	GAX 128	-	88
O. tehuacana	Ajalpan	GAX 100	63	126
O. tehuacana	Cuicatlán	GAX 162	63	126
O. tehuacana	Coxcatlán	GAX 107	66	132

Table 2

Species	Locality	Ploidy level	Number of individuals analyzed	2C nuclear DNA content (pg*) $\bar{X}$ ± SE	1Cx (pg*)	1Cx (Mbp)	Tukey test
O. tehuacana	Ajalpan	11x (+5)	4	9.02 pg ± 0.04	0.82	801.96	a
O. tehuacana	Coxcatlán	12x	5	10.54 pg ± 0.04	0.87	850.86	c
O. tehuacana	Tecomavaca	11x	5	8.40 pg ± 0. 04	0.76	743.28	d
O. tehuacana	Cuicatlán	11x (+5)	4	9.44 pg ± 0.07	0.85	831.3	e
O. tehuacana	Nochixtlán	11x	3	8.81 pg ± 0.03	0.8	782.4	b

Figure 1

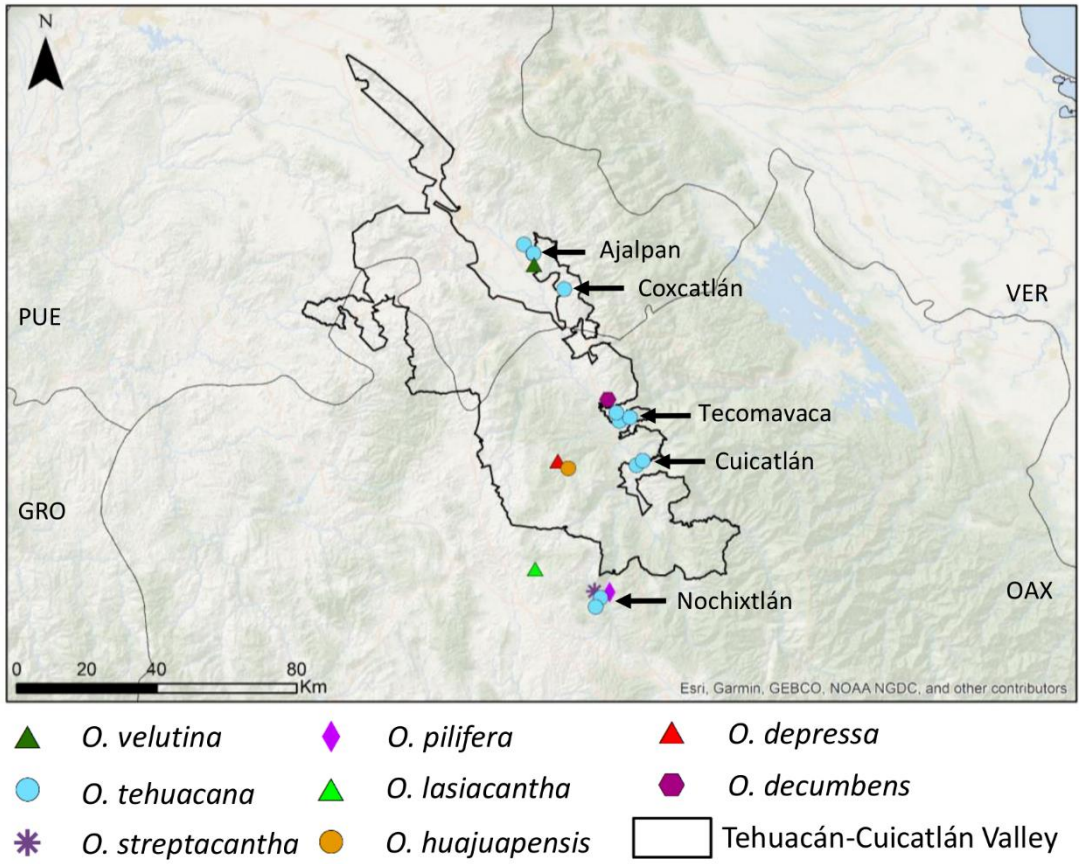


Figure 2

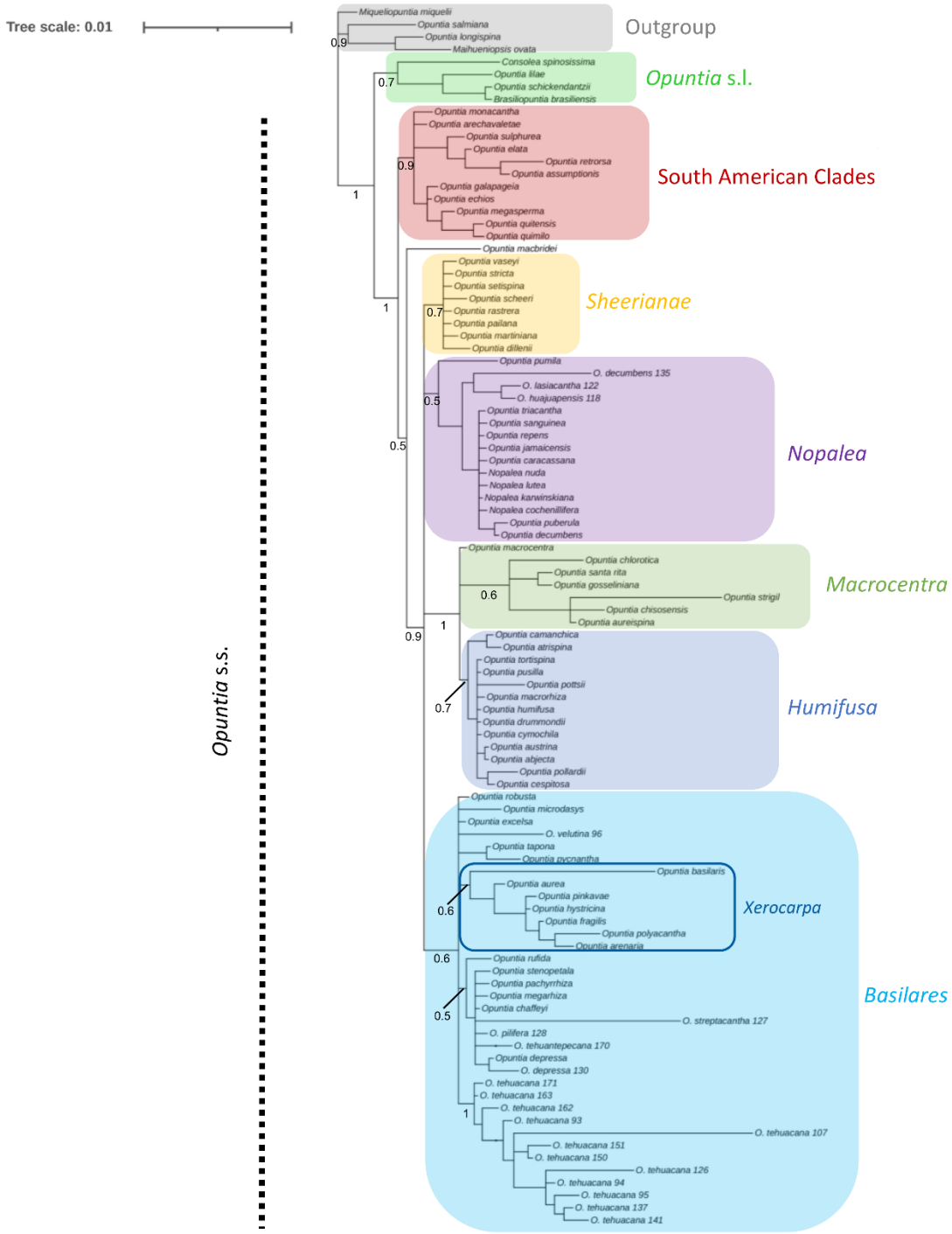

Tree scale: 0.01
Miqueliopuntia miquelii
Opuntia salmiana
Opuntia longispina
Maihueniopsis ovata
Outgroup
Consolea spinosissima
Opuntia lilae
Opuntia schickendantzii
Brasiliopuntia brasiliensis
Opuntia s.l.
Opuntia monacantha
Opuntia arechavaletae
Opuntia sulphurea
Opuntia elata
Opuntia retrorsa
Opuntia assumptionis
Opuntia galapageia
Opuntia echios
Opuntia megasperma
Opuntia quitensis
Opuntia quimilo
South American Clades
Opuntia macbridei
Opuntia vaseyi
Opuntia stricta
Opuntia setispina
Opuntia scheeri
Opuntia rastrera
Opuntia pailana
Opuntia martiniana
Opuntia dillenii
Sheerianae
Opuntia pumila
O. decumbens 135
O. lasiacantha 122
O. huajuapensis 118
Opuntia triacantha
Opuntia sanguinea
Opuntia repens
Opuntia jamaicensis
Opuntia caracassana
Nopalea nuda
Nopalea lutea
Nopalea karwinskiana
Nopalea cochenillifera
Opuntia puberula
Opuntia decumbens
Nopalea
Opuntia macrocentra
Opuntia chlorotica
Opuntia santa rita
Opuntia gosseliniana
Opuntia strigil
Opuntia chisosensis
Opuntia aureispina
Macrocentra
Opuntia camanchica
Opuntia atrispina
Opuntia tortispina
Opuntia pusilla
Opuntia pottsii
Opuntia macrorhiza
Opuntia humifusa
Opuntia drummondii
Opuntia cymochila
Opuntia austrina
Opuntia abjecta
Opuntia pollardii
Opuntia cespitosa
Humifusa
Opuntia robusta
Opuntia microdasys
Opuntia excelsa
O. velutina 96
Opuntia tapona
Opuntia pycnantha
Opuntia basilaris
Opuntia aurea
Opuntia pinkavae
Opuntia hystricina
Opuntia fragilis
Opuntia polyacantha
Opuntia arenaria
Xerocarpa
Opuntia rufida
Opuntia stenopetala
Opuntia pachyrrhiza
Opuntia megarhiza
Opuntia chaffeyi
O. streptacantha 127
O. pilifera 128
O. tehuantepecana 170
Opuntia depressa
O. depressa 130
O. tehuacana 171
O. tehuacana 163
O. tehuacana 162
O. tehuacana 93
O. tehuacana 107
O. tehuacana 151
O. tehuacana 150
O. tehuacana 126
O. tehuacana 94
O. tehuacana 95
O. tehuacana 137
O. tehuacana 141
Basilares
Opuntia s.s.

Figure 3

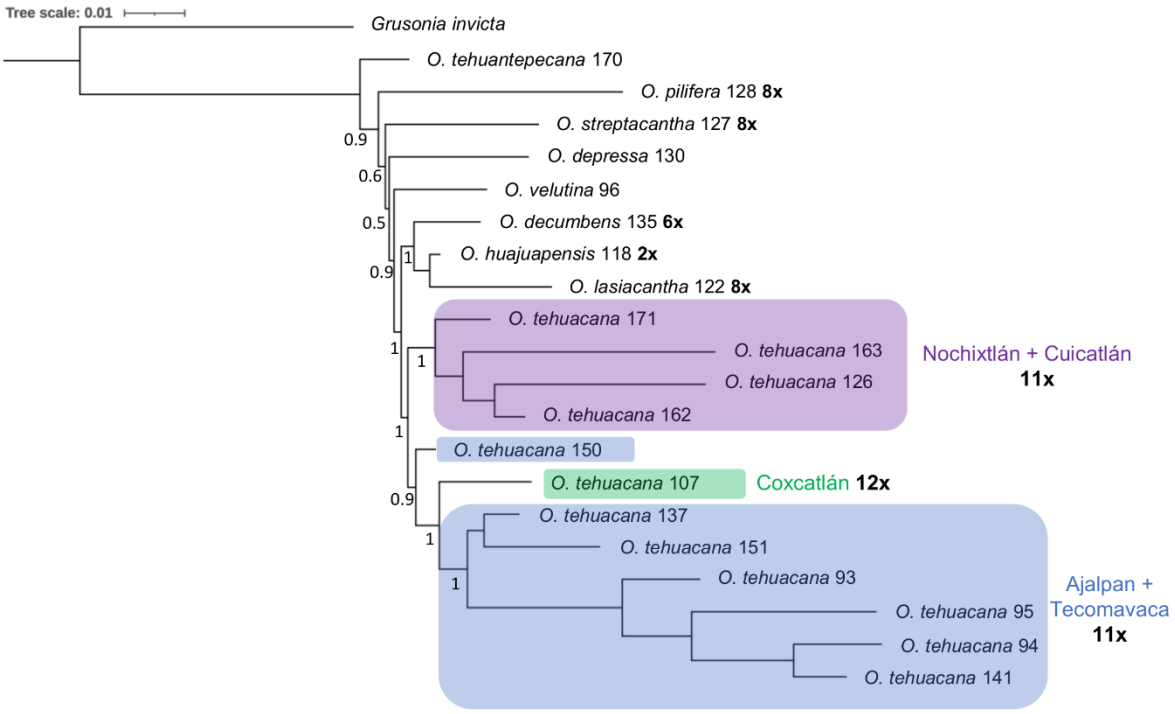

Tree scale: 0.01
Grusonia invicta
O. tehuantepecana 170
O. pilifera 128 8x
O. streptacantha 127 8x
O. depressa 130
O. velutina 96
O. decumbens 135 6x
O. huajuapensis 118 2x
O. lasiacantha 122 8x
O. tehuacana 171
O. tehuacana 163
O. tehuacana 126
O. tehuacana 162
Nochixtlán + Cuicatlán
11x
O. tehuacana 150
O. tehuacana 107
Coxcatlán 12x
O. tehuacana 137
O. tehuacana 151
O. tehuacana 93
O. tehuacana 95
O. tehuacana 94
O. tehuacana 141
Ajalpan +
Tecomavaca
11x
0.9
0.6
0.5
1
0.9
1
1
1
0.9
1
1

Figure 4

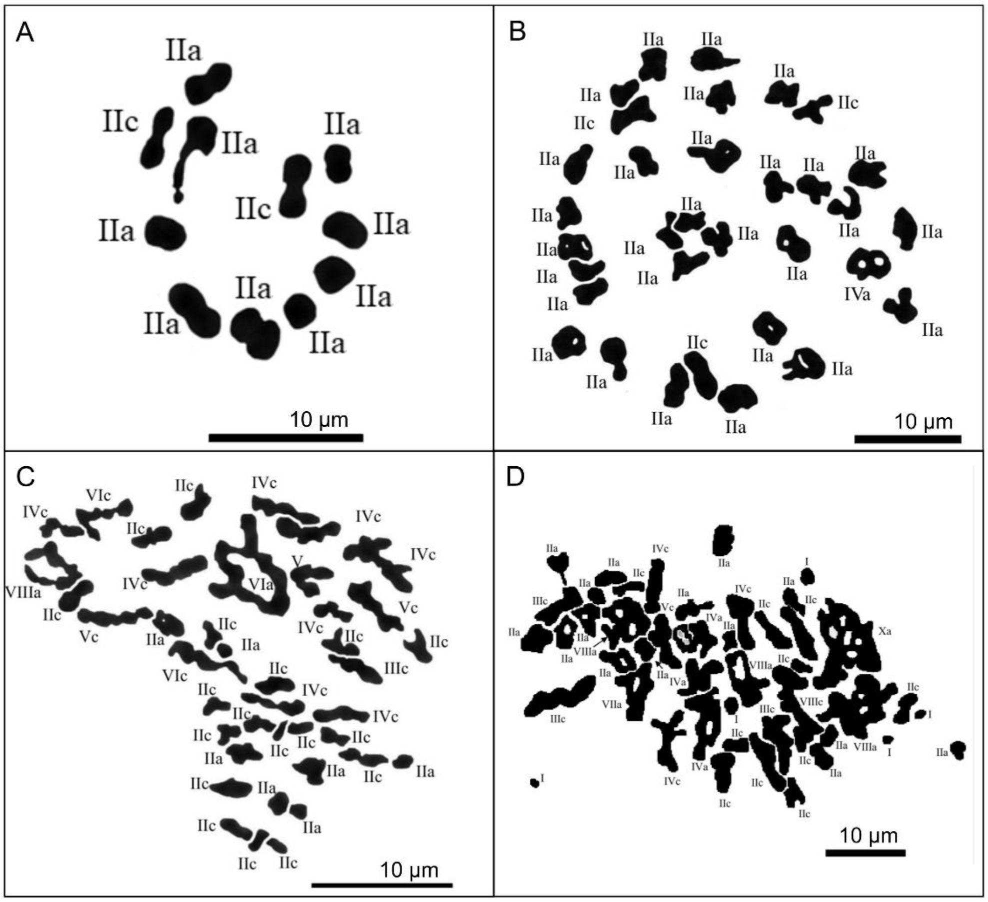
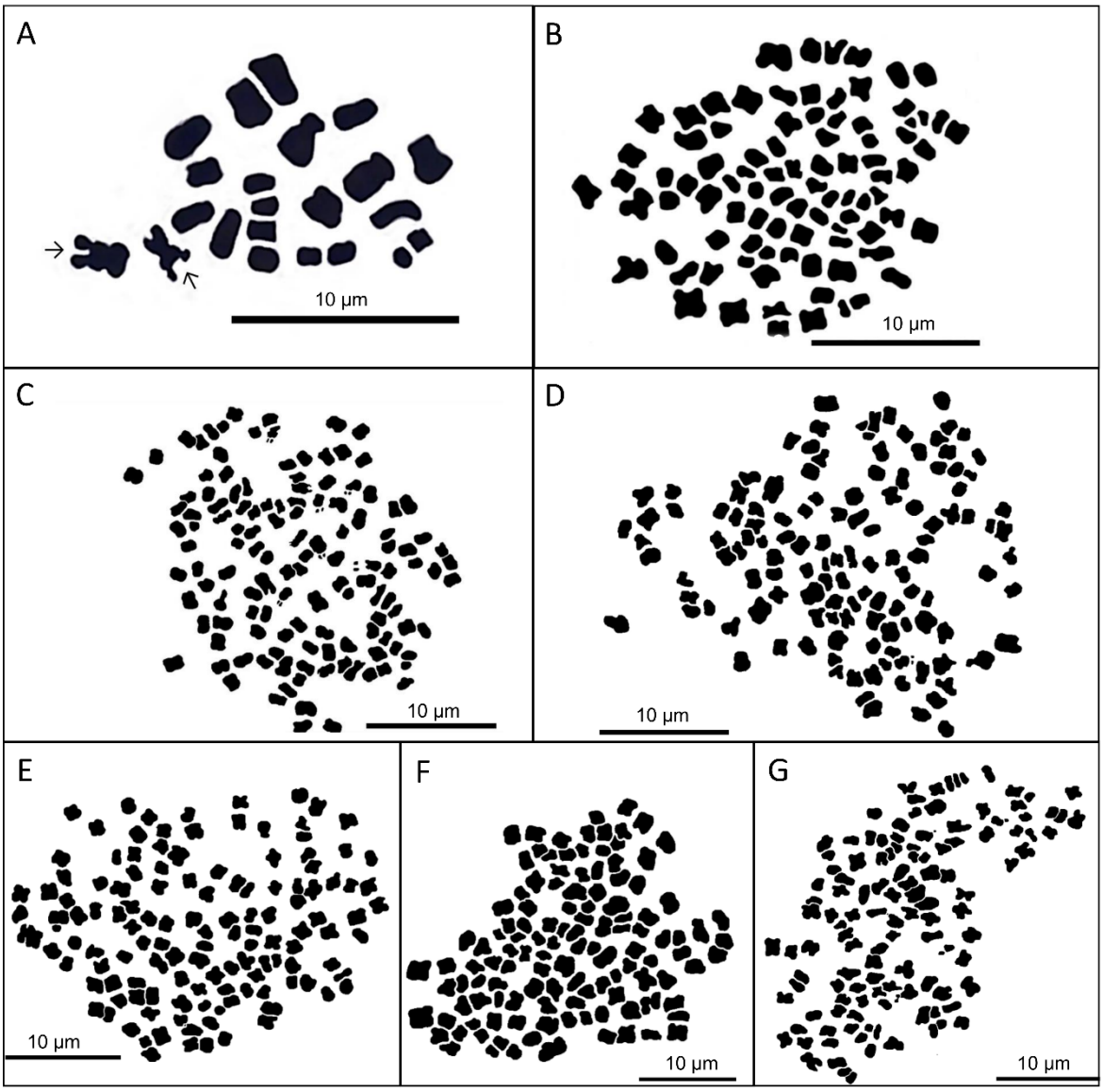
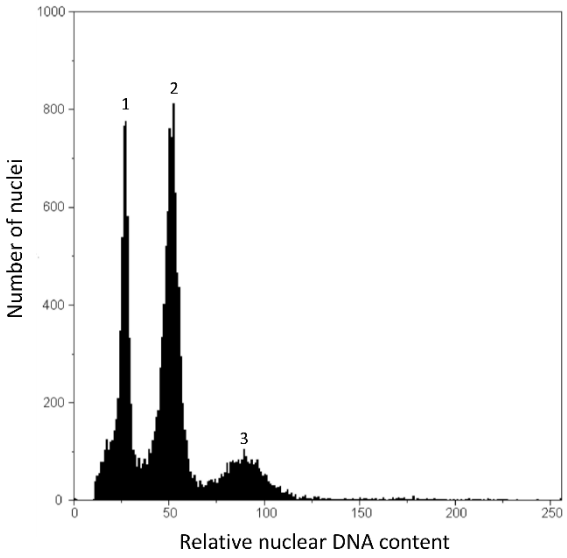


Figure 5



Supplementary material

Supplementary Figure 1



Supplementary Table 1

Specie	Collection number	Chromosomic number *x*= /2n=	Locality/State	Geographical reference
Opuntia tehuacana	GAX 93	x= 11= 11x+5=126	Ajalpan, Puebla	18°21'55.2"N 97°12'55.4"W
Opuntia tehuacana	GAX 100	x= 11= 11x+5=126	Ajalpan, Puebla	18°24'0.9"N 97°12'32"W
Opuntia tehuacana	GAX 101	x= 11= 11x+5=126	Ajalpan, Puebla	18°23'58.6"N 97°12'31.5"W
Opuntia tehuacana	GAX 102	x= 11= 11x+5=126	Ajalpan, Puebla	18° 23' 58.6"N 97° 12' 31.5"W
Opuntia tehuacana	GAX 107	x=11= 12x= 132	Coxcatlán, Puebla	18°16'24.4"N 97°08'11.5"W
Opuntia tehuacana	GAX 111	x=11= 12x= 132	Coxcatlán, Puebla	18°16'45"N 97°07'30.2"W
Opuntia tehuacana	GAX 112	x=11= 12x= 132	Coxcatlán, Puebla	18°17'11.1"N 97°07'48.8"W
Opuntia tehuacana	GAX 113	x=11= 12x= 132	Coxcatlán, Puebla	18°17'20.7"N 97°07'42.9"W
Opuntia tehuacana	GAX 114	x=11= 12x= 132	Coxcatlán, Puebla	18°17'22.1"N 97°07'42.4"W
Opuntia tehuacana	GAX 161	x= 11= 11x+5=126	Cuicatlán, Oaxaca	17°49'29.3"N 96°57'36.5"W
Opuntia tehuacana	GAX 162	x= 11= 11x+5=126	Cuicatlán, Oaxaca	17°49'56.9"N 96°56'04.3"W
Opuntia tehuacana	GAX 164	x= 11= 11x+5=126	Cuicatlán, Oaxaca	17°50'20.8"N 96°55'39.8"W
Opuntia tehuacana	GAX 169	x= 11= 11x+5=126	Cuicatlán, Oaxaca	17°50'32.6"N 96°55'36.8"W
Opuntia tehuacana	GAX 129	x= 11= 11x= 121	Nochixtlán, Oaxaca	17°28'38.8"N 97°03'09.3"W
Opuntia tehuacana	GAX 171	x= 11= 11x= 121	Nochixtlán, Oaxaca	17°28'50.7"N 97°02'35.5"W

Opuntia tehuacana	GAX 173	x= 11= 11x= 121	Nochixtlán, Oaxaca	17°28'50.3"N 97°02'38.6"W
Opuntia tehuacana	GAX 150	x= 11= 11x= 121	Tecomavaca, Oaxaca	17°57'21.7"N 97°00'09.8"W
Opuntia tehuacana	GAX 151	x= 11= 11x= 121	Tecomavaca, Oaxaca	17°57'17.1"N 96° 59'26.8"W
Opuntia tehuacana	GAX 152	x= 11= 11x= 121	Tecomavaca	17°57'14.4"N 96°59'01.6"W
Opuntia tehuacana	GAX 153	x= 11= 11x= 121	Tecomavaca, Oaxaca	17°57'19.1"N 96°58'49.7"W
Opuntia tehuacana	GAX 156	x= 11= 11x= 121	Tecomavaca, Oaxaca	17°57'23.4"N 96°58'47.0"W
Opuntia pilifera	GAX 97	x= 11= 8x= 88	Ajalpan, Puebla	18°21'53.3"N 97°12'58.5"W
Opuntia pilifera	GAX 128	x= 11= 8x= 88	Nochixtlán Oaxaca	17°28'38.8"N 97°03'09.3"W
Opuntia huajuapensis	GAX 118	x= 11= 2x= 22	Cuicatlán, Oaxaca	17°49'26.4"N 97°09'11.3"W

Supplementary Table 2

Specie	Collection number	Locality/State	Geographical reference	GenBank accession numbers *matK, ycf1, psbJ-petA, AT3G48380, AT1G18270*
Opuntia decumbens	GAX 135	Tecomavaca, Oaxaca	17°57'59.1"N 97°00'21.9"W	OK206444, OK206465, OK206484, MT475834, MT475861
Opuntia depressa	GAX 130	Ixcatlán, Oaxaca	17°49'54.70"N 97°9'8.50"W	OK206445, OK206466, OK206485, MT475837, MT475864
Opuntia huajuapensis	GAX 118	Cuicatlán, Oaxaca	17°49'26.4"N 97°09'11.3"W	OK206446, OK206467, OK206486, MT475839, MT475866
Opuntia lasiacantha	GAX 122	Nochixtlán, Oaxaca	17°33'15.9"N 97°12'39.5"W	OK206447, OK206468, OK206487, MT475842, MT475868
Opuntia pilifera	GAX 128	Nochixtlán Oaxaca	17°28'38.8"N 97°03'09.3"W	OK206448, OK206469, OK206488, MT475845, MT475871
Opuntia streptacantha	GAX 127	Nochixtlán, Oaxaca	17°28'19"N 97°03'11.9"W	OK206449, OK206470, OK206489, MT475848, MT475873
Opuntia tehuacana	GAX 93	Ajalpan, Puebla	18°21'55.2"N 97°12'55.4"W	OK206450, OK206471, OK206490, MT475849, MT475874
Opuntia tehuacana	GAX 94	Ajalpan, Puebla	18°21'54.1"N 97°12'57.4"W	OK206451, OK206472, OK206491, OK206510, OK206505
Opuntia tehuacana	GAX 95	Ajalpan, Puebla	18°21'53.3"N 97°12'58.5"W	OK206452, OK206473, OK206492, MT475850, MT475875
Opuntia tehuacana	GAX 107	Coxcatlán, Puebla	18°16'24.4"N 97°08'11.5"W	OK206453, —, OK206493, OK206511, OK206506
Opuntia tehuacana	GAX 126	Nochixtlán, Oaxaca	17°28'19"N 97°03'11.9"W	OK206454, OK206474, OK206494, MT475851, MT475876
Opuntia tehuacana	GAX 137	Tecomavaca, Oaxaca	17° 57' 38.2"N 97° 00' 48.6"W	OK206455, OK206475, OK206495, MT475852, MT475877

Opuntia tehuacana	GAX 141	Ajalpan, Puebla	18°23'18.9"N 97°14'25.2"W	OK206456, OK206476, OK206496, OK206512, OK206507
Opuntia tehuacana	GAX 150	Tecomavaca, Oaxaca	17°57'21.7"N 97°00'09.8"W	OK206457, OK206477, OK206497, MT475853, MT475878
Opuntia tehuacana	GAX 151	Tecomavaca, Oaxaca	17°57'17.1"N 96° 59'26.8"W	OK206458, OK206478, OK206498,
Opuntia tehuacana	GAX 162	Cuicatlán, Oaxaca	17°49'56.9"N 96°56'04.3"W	OK206459, OK206479, OK206499, MT475854, MT475879
Opuntia tehuacana	GAX 163	Cuicatlán, Oaxaca	17°50'20.8"N 96°55'39.8"W	OK206460, OK206480, OK206500, OK206513, OK206508
Opuntia tehuacana	GAX 171	Nochixtlán, Oaxaca	17°28'50.7"N 97°02'35.5"W	OK206461, OK206481, OK206501, OK206514, OK206509
Opuntia velutina	GAX 96	Ajalpan, Puebla	18°21'53.3"N 97°12'58.5"W	OK206462, OK206482, OK206502, MT475856, MT475881
Opuntia tehuantepecana	GAX 170	Tehuantepec, Oaxaca	16°25'38.3"N 95°27'39.2"W	OK206463, OK206483, OK206503, MT475855, MT475880
Grusonia invicta	WLF s/n	UNAM, Botanic Garden, Cactaceae collection		OK206464, —, OK206504, MT475832, MT475859

Capítulo 3. Unraveling Reticulate Evolution in *Opuntia* (Cactaceae) From Southern Mexico

Xochitl Granados-Aguilar, Carolina Granados Mendoza, Cristian Rafael Cervantes,

José Rubén Montes and Salvador Arias

Frontiers in Plant Science 2021. 11: 606809

DOI:10.3389/fpls.2020.606809

(Artículo de Requisito)

frontiers in Plant Science

ORIGINAL RESEARCH
published: 13 January 2021
doi: 10.3389/fpls.2020.606809

Unraveling Reticulate Evolution in *Opuntia* (Cactaceae) From Southern Mexico

Xochitl Granados-Aguilar[1,2], Carolina Granados Mendoza[3], Cristian Rafael Cervantes[1,2], José Rubén Montes[1,3] and Salvador Arias[2]**

[1] Posgrado en Ciencias Biológicas, Instituto de Biología, Universidad Nacional Autónoma de México, Mexico City, Mexico, [2] Jardín Botánico, Instituto de Biología, Universidad Nacional Autónoma de México, Mexico City, Mexico, [3] Departamento de Botánica, Instituto de Biología, Universidad Nacional Autónoma de México, Mexico City, Mexico

OPEN ACCESS

Edited by:
Susann Wicke,
Humboldt University of Berlin, Germany

Reviewed by:
Carolina Moriani Siniscalchi, Mississippi State University, United States
Isabel Larridon, Royal Botanic Gardens, Kew, United Kingdom
Rowan Schley, Royal Botanic Gardens, Kew, United Kingdom
Lucas Charles Majure, University of Florida, United States

***Correspondence:**
Xochitl Granados-Aguilar
xochitl.granados@st.ib.unam.mx
Salvador Arias
sarias@ib.unam.mx

Specialty section:
This article was submitted to Plant Systematics and Evolution, a section of the journal Frontiers in Plant Science

Received: 15 September 2020
Accepted: 10 December 2020
Published: 13 January 2021

Citation:
Granados-Aguilar X, Granados Mendoza C, Cervantes CR, Montes JR and Arias S (2021) Unraveling Reticulate Evolution in Opuntia (Cactaceae) From Southern Mexico. Front. Plant Sci. 11:606809. doi: 10.3389/fpls.2020.606809

The process of hybridization occurs in approximately 40% of vascular plants, and this exchange of genetic material between non-conspecific individuals occurs unequally among plant lineages, being more frequent in certain groups such as *Opuntia* (Cactaceae). This genus is known for multiple taxonomic controversies due to widespread polyploidy and probable hybrid origin of several of its species. Southern Mexico species of this genus have been poorly studied despite their great diversity in regions such as the Tehuacán-Cuicatlán Valley which contains around 12% of recognized Mexico's native *Opuntia* species. In this work, we focus on testing the hybrid status of two putative hybrids from this region, *Opuntia tehuacana* and *Opuntia pilifera*, and estimate if hybridization occurs among sampled southern opuntias using two newly identified nuclear intron markers to construct phylogenetic networks with HyDe and Dsuite and perform invariant analysis under the coalescent model with HyDe and Dsuite. For the test of hybrid origin in *O. tehuacana*, our results could not recover hybridization as proposed in the literature, but we found introgression into *O. tehuacana* individuals involving *O. decumbens* and *O. huajuapensis*. Regarding *O. pilifera*, we identified *O. decumbens* as probable parental species, supported by our analysis, which sustains the previous hybridization hypothesis between *Nopalea* and *Basilares* clades. Finally, we suggest new hybridization and introgression cases among southern Mexican species involving *O. tehuantepecana* and *O. depressa* as parental species of *O. velutina* and *O. decumbens*.

Keywords: Cactaceae, *Opuntia*, reticulate evolution, Tehuacán-Cuicatlán Valley, hybridization, phylogenetic networks, nuclear markers, introgression

INTRODUCTION

Reticulate evolution is a speciation pattern from which new species arise from hybridization and successive reproductive isolation (Soltis, 2013). In plants, hybridization takes place in approximately 40% of vascular species, can lead sometimes to speciation (Whitney et al., 2010), and is considered a major evolutive force (Soltis and Soltis, 2009; Soltis, 2013). Hybridization is the exchange of genetic material between individuals belonging to diagnostically distinct groups based on one or more heritable characters (Riesenberg and Wendel, 1993). This exchange of genetic

material can lead to discordant phylogenetic trees, particularly in groups where hybridization is predominant (Arnold, 1997). Phylogenetic networks improve the representation of hybridization compared to phylogenetic trees (Zhu et al., 2018) because they extend trees with horizontal edges to better model such reticulation events (Elworth et al., 2019) and have been used to better depict the evolutive history of several lineages across vascular plants, including *Pinus* (Gernandt et al., 2018), *Viola* (Marcussen et al., 2011), *Fragaria* (Kamneva et al., 2017), and *Lachemilla* (Morales-Briones et al., 2018).

The genus *Opuntia* is known for its multiple taxonomic controversies resulting from wide morphological variability, potentially due to widespread polyploidy, hybridization between closely related species and, in some cases, homoplasy of selected morphological characters (Majure et al., 2012b). Among *Opuntia* species, natural hybrids are common, and hybridization has been regarded as having an impact on evolution (Pinkava, 2002; Reyes-Agüero et al., 2006; Majure et al., 2012a). Natural hybridization in this group is thought to be facilitated by the absence of reproductive barriers between closely related species, whose offspring are maintained by vegetative propagation and the perennial habit characteristics of this group (Pinkava, 2002; Majure and Puente, 2014). From the 180 recognized *Opuntia* species 93 are distributed in Mexico (Anderson, 2001; Hunt et al., 2006; Majure et al., 2012a). *Opuntia* species from southern Mexico have been poorly studied despite the great diversity found in regions such as the Tehuacán-Cuicatlán Valley, which has 12% (15 species) of the *Opuntia* species diversity of Mexico (Arias et al., 2012). Analyzing evolutive processes such as hybridization in a selected group could help us to better understand the mechanisms that have generated high diversity in this region.

Multiple species of probable hybrid origin have been suggested based on the incongruence of phylogenetic relationships depending on the inheritance patterns (i.e., uniparental vs. biparental; Majure et al., 2012a); however, these hypotheses have not been tested under coalescence reticulation inferences. One such case is *Opuntia pilifera* (**Figure 1E**), a prickly pear with edible fruits and wide trait variation, which was proposed as a hybrid between *Opuntia* species from *Nopalea* and *Basilares* clades (Majure et al., 2012a), without further inference of its potential parental species. Other species have been proposed to have a hybrid origin based only on morphological observation and without being examined in a phylogenetic context. One such case is *Opuntia tehuacana* (**Figure 1A**) which has been proposed as hybrid based on intermediate morphological traits between the sympatric species *O. pilifera* (**Figure 1E**) and *O. huajuapensis* (**Figure 1C**) from the Tehuacán-Cuicatlán Valley (Arias et al., 2012), but this hypothesis has never been formally tested.

Phylogenetic networks are an important approach to distinguish hybridization events in evolutive relationships that cannot be explained in a phylogenetic tree. Most of the phylogenetic analysis developed during the second half of the twentieth century focused on solving dichotomous relationships and were constrained by the low computational power of that time, leaving aside processes such as hybridization or incomplete lineage sorting (ILS) (Arnold, 1997; Elworth et al., 2019). Over the past 10 years improvement on computational power and implementation of statistical methods in phylogenetic software to detect reticulations and ILS led to new approaches based on parsimony, maximum likelihood and Bayesian inference. Under parsimony the inference of phylogenetic networks can be made from heuristic searches within a set of gene-tree topologies (Yu et al., 2013). The maximum likelihood approach is based on the multispecies network coalescent (MSNC) and maximizes the network likelihood (Yu et al., 2012). Bayesian inference, also uses the MSNC but includes a Markov chain Monte Carlo (MCMC) to sample posterior distribution on networks (Zhang et al., 2018). Examples of programs that can infer phylogenetic networks using these three approaches are SplitsTree4 (Huson and Bryant, 2005), BEAST 2 (Zhang et al., 2018), StarBEAST2 (Ogilvie et al., 2017), PhyloNetworks (Solís-Lemus et al., 2017), and PhyloNet (Wen et al., 2018). Among these, PhyloNet is one of the most commonly used (Copetti et al., 2017; Kamneva et al., 2017; Gernandt et al., 2018), since it allows the analysis of data from multiple loci through parsimony, maximum likelihood, pseudolikelihood, and Bayesian inference (Wen et al., 2018). All these software use coalescence theory to model the past of an allele using a stochastic process in order to find its most recent common ancestor. The mathematical approach in this theory also allows the estimation of the nucleotide mutation rate, and this methodology it is not affected by processes such as recombination (Rosenberg and Nordborg, 2002). Other approaches under the coalescent model to explore if hybridization occurs between certain taxa are HyDe, which uses phylogenetic invariants from site pattern probabilities to know the parental species of a putative hybrid (Blischak et al., 2018) and Dsuite, which test the correlations of alleles across populations using the Patterson's *D* statistics (Malinsky et al., 2020).

Hybridization is a common process among *Opuntia* species and it study under phylogenetic networks can help to better understand the relationships of southern Mexico opuntias. This study aims to test the status of two putative hybrids from the Tehuacán-Cuicatlán Valley, *O. tehuacana* (**Figure 1A**) and *O. pilifera* (**Figure 1E**), which were previously proposed by Arias et al. (2012) and Majure et al. (2012a), respectively, and to estimate if hybridization occurs among sympatric *Opuntia* species.

MATERIALS AND METHODS

Taxon Sampling

We sampled wild *Opuntia* species, which occur sympatrically with *O. tehuacana* and *O. pilifera* in the Tehuacán-Cuicatlán Valley (Arias et al., 2012), as well as additional species from the nearby Isthmus of Tehuantepec, Oaxaca, and El Arenal, Hidalgo, Mexico. As outgroup, *Grusonia invicta* was included based on phylogenetic relationships in Opuntioideae according to previous studies (Griffith and Porter, 2009; Guerrero et al., 2019). Plant material was collected through a series of field trips to Tehuacán-Cuicatlán Valley performed from October 2017 to June 2018. Additional plant material was obtained from the Cactaceae collection at Jardín Botánico,

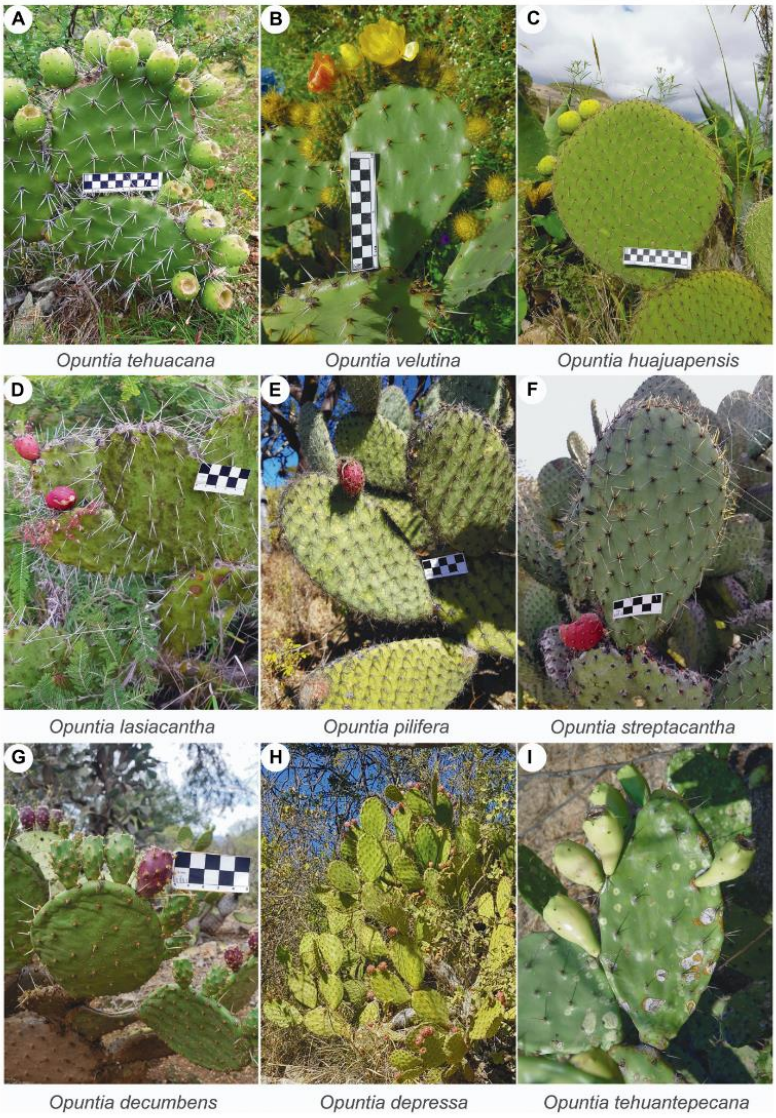


FIGURE 1 | (A–I) Images of the nine *Opuntia* species analyzed on this work. **(C,E)** Species *O. huajuapensis* and *O. pilifera* putative parentals of *O. tehuacana*, as proposed by Arias et al. (2012). Photos by X. Granados and S. Arias.

Instituto de Biología, Universidad Nacional Autónoma de México (UNAM). To ensure the identification and voucher ID of samples at Botanical Garden, sampling of this material was supervised by the Cactaceae specialist Ph.D. SA. In total nine *Opuntia* species were sampled and one to three individuals per species were included, in order to represent the variation of the species throughout its distribution. For the putative hybrids, we included three *O. pilifera* individuals and six *O. tehuacana* individuals. The sampled localities are shown in **Figure 2A**. Voucher specimens were deposited at Jardín Botánico, Instituto de Biología, UNAM and MEXU herbarium. Further collection and locality details are shown in **Supplementary Table 1**.

Nuclear Marker Design

Our objective was to identify single copy nuclear markers potentially useful in Cactaceae species, applying a modification of the mining strategy proposed by Granados Mendoza et al. (2015). Our marker design workflow is summarized in **Figure 3**.

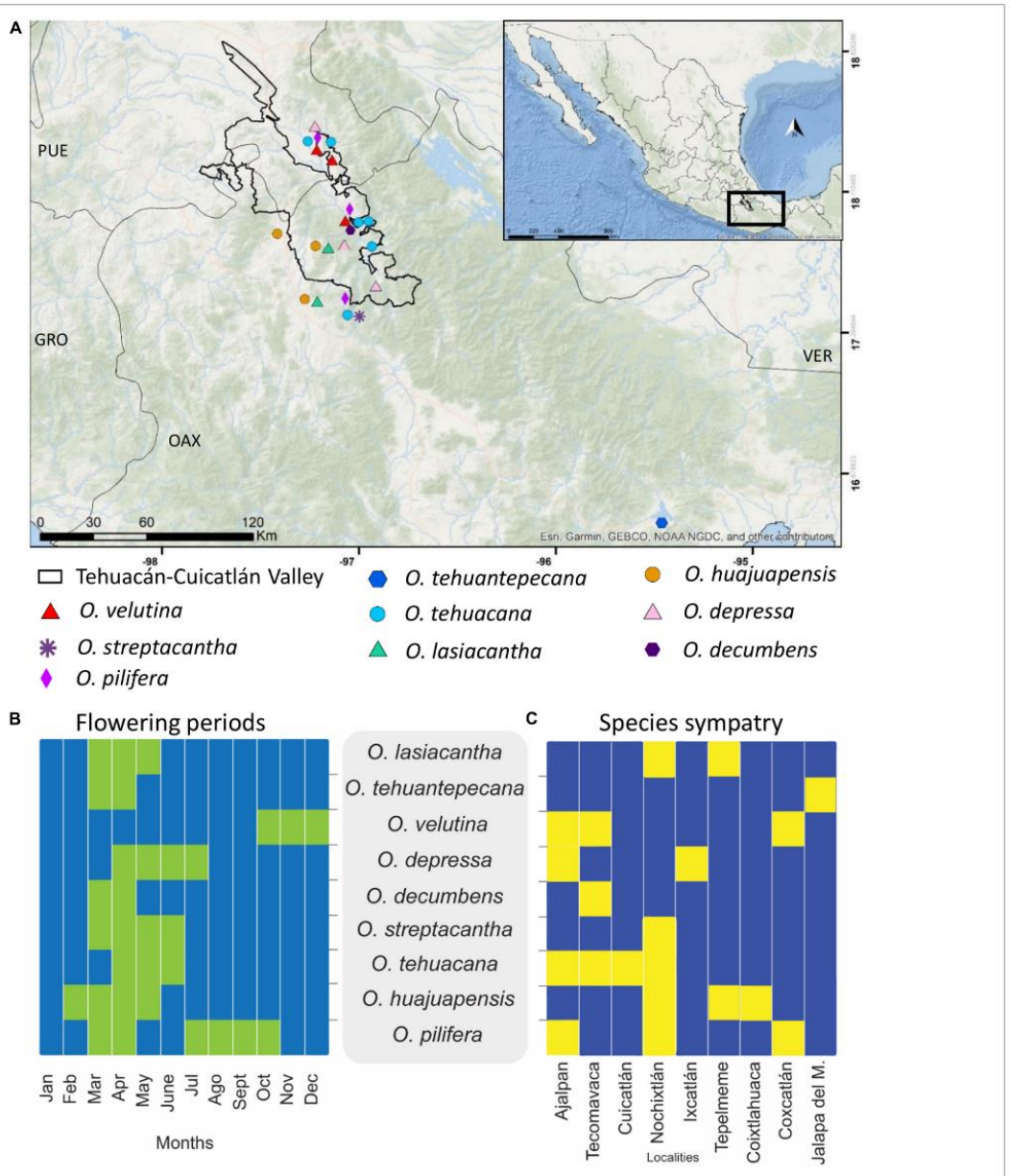


FIGURE 2 | The *Opuntia* species from Tehuacán-Cuicatlán Valley and other species collected in Mexico evaluated on this work. **(A)** Sampled localities in field trips. **(B)** Heatmap showing in green overlap of flowering months between sampled species, data from Arias et al. (2012). **(C)** Heatmap showing in yellow species sympatry per sampled locality.

Using the PLAZA v.4.0 dicots database[1], we first compiled a file with a list of nuclear genes present in low copy in three representatives of the order Caryophyllales (*Amaranthus hypochondriacus*, *Chenopodium quinoa,* and *Beta vulgaris*), as well as two representatives from the Asterids clade (*Daucus carota* and *Actinidia chinensis*) and the model plant *Arabidopsis thaliana* (Order Brassicales, rosid clade). We wanted to obtain a greater number of candidate genes despite the fact that in Asterids and *A. thaliana* could have genes with more copies so, we selected gene families that were present in one copy in Caryophyllales and up to

[1]https://bioinformatics.psb.ugent.be/plaza/versions/plaza_v4_dicots/

three copies on these distant lineages. Resulting in 292 low copy candidate genes, then, we filtered out those genes that were absent in Asterids and *A. thaliana* with an in-house R script (available at https://github.com/cristoichkov/Plaza_filter), retaining 133 candidate genes. To increase sequence variability, markers were designed to span more non-coding than coding regions. Because of this, genes without introns were excluded by visualizing each retained gene family model in the PLAZA v.4.0 dicots database. A total of 28 genes with introns were retained, and the complete and coding sequences of the Caryophyllales representatives were downloaded in fasta format and subsequently aligned in PhyDE v.0.9951 (Müller et al., 2005). Genes with introns between 800 and 1,000 base pairs (bp) and exons greater than 30 bp were further selected. To enrich Cactaceae species representation in our alignments and to improve primer design, we extracted orthologous sequences from the complete genomes of *Carnegiea gigantea*, *Lophocereus schottii*, *Pachycereus pringlei*, *Pereskia humboldtii,* and *Stenocereus thurberi* (NCBI Sequence Read Archive: SRR5036292 to SRR5036296 and SRR5137211 to SRR5137214, respectively; Copetti et al., 2017) with BLAST Command Line Tools v.2.7.1 (Camacho et al., 2009), using as reference the sequences from 28 selected genes. We selected and manually aligned in PhyDE the Caryophyllales genes that matched with at least two Cactaceae genomes. Five candidate genes were recovered, *AT3G05090*, *AT4G24040*, *AT3G48380*, *AT1G18270,* and *AT1G36980* (names based on the *A. thaliana* annotation). Additional Cactaceae species coding sequences from these five genes were mined from oneKP[2] using BLAST in Geneious v.11.1.5 with the transcriptome databases of *Lophophora williamsii*, *Opuntia polyacantha,* and *Pereskia aculeata*, with matches for all genes. Scripts and more detailed information are available at GitHub repository[3].

Finally, primers were designed only on the first four candidate genes (**Table 1**) due to the risk of sequences not overlapping in gene *AT1G36980* because its intron size of more than 1,200 bp for Cactaceae species. We designed up to three pairs of primers per candidate gene to span introns from 800 to 1,200 bp using the online eurofins primer design tool[4].

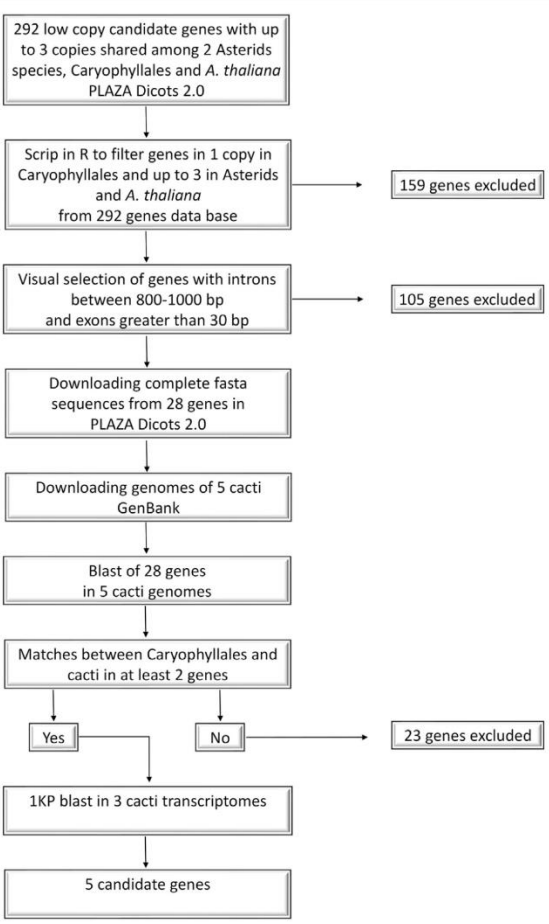


FIGURE 3 | Transcriptome and genome mining workflow.

Nuclear Primer Validation

Total genomic DNA was extracted from silica gel dried tissue following the CTAB protocol (Doyle and Doyle, 1987) with some modifications as reported by Bustamante et al. (2016) to avoid excess mucilage in the samples. DNA quality was tested with NanoDrop 2000. The DNA quality obtained with these modifications was good enough for Sanger sequencing. For each selected nuclear gene, the most promising pair of primers were selected according to previous cacti alignments (**Table 1**; primer ID "a"). To test the feasibility of primer amplification among Cactaceae species, we selected species from distant phylogenetic clades, namely: *Opuntia pilifera*, *O. tehuacana*, *Grusonia invicta*, *Pilosocereus chrysacanthus*, *P. collinsii*, *Melocactus curvispinus*, *Mammillaria albilanata* subsp. *oaxacana*, *M. haageana* subsp. *Meissneri,* and *M. crucigera*. We performed 15 μL PCRs with the commercial mix "Platinum Taq" (Invitrogen), and the reactions included 1.5 μL (1×) of 10× PCR buffer, 0.3 μL of dNTP mix, 0.3 μL of each primer (10 pmol/μL), 0.3 μL of BSA (0.4%), 0.6 μL of $MgCl_2$ (1.5 μM), and 0.075 μL (0.375 units) of Taq DNA polymerase. Amplification tests were made using the touch-up PCR program (**Table 2**) as well as gradient PCR with two $MgCl_2$ concentrations 1.5 and 2.5 μM (**Table 3**). To confirm the presence of PCR amplicons, these were run on 1% agarose gels. PCR cleaning and sequencing was performed at Laboratorio de Biología Molecular de la Biodiversidad y de la Salud, Instituto de Biología, UNAM, for sample sequencing the reactions included 0.4 μl of BigDye Terminator v.3.1 (Applied Biosystems), 2 μl of Buffer 5×, 4 μl of water, 1 μl of primer with a concentration of 10 μM and 3 μl of PCR product. Conditions of reaction sequencing were 30 cycles of 96°C for 10 s, 50°C for 5 s and 60°C for 4 min. After cycling, samples were purified with Centri-Sep (Thermo Fisher Scientific) plates following the manufacturer protocol. To

[2]https://sites.google.com/a/ualberta.ca/onekp/
[3]https://github.com/cristoichkov/Plaza_filter
[4]https://www.eurofinsgenomics.eu/en/ecom/tools/pcr-primer-design/

TABLE 1 | Nuclear primers designed on intron-flanking exons from selected genes.

Gene	Primer ID	Forward sequence 5′–3′	Reverse sequence 5′–3′	Tested
AT3G05090	a	TGACGGGACATTGAAGAGG	GTGGCTCCAGTTTCTAAGTC	Yes
	b	TAGCTACTTGCTCCGCTAC	GTCTAGGTGGGGAGGTTTT	No
	c	GGGCTTCTCTTGAAGGATCTAC	GTGGTGATTCAAGATTATGGCG	No
AT4G24040	a	AGAAATCTCTTCCTGCTCTAC	GACAACATTGGCTACTACATC	Yes
	b	AATGGTGCCAGAGCTTATTAC	AGAAATCTCTTCCTGCTCTAC	No
	c	GACAACATTGGCTACTACATC	GTTTGTGCTGCTGGGATTGC	No
AT3G48380	a	GTCTTACACCCRGTATCARTT	TATTGCATCTCTGGTTCAAGG	Yes
	b	CMGCAATGAAGAATGCAGTCT	GTCTTACACCCRGTATCARTT	No
AT1G18270	a	CAGTCTGATGCARTAGCRAGA	CCACCTTGTTGCTTCAGTTGA	Yes
	b	ACGAGGTGAGCACATGAAGCA	TCTGCCACTTCTTGGAGTTGC	No
	c	AACACTYTCTAGATTCGTGGA	ACCAATGAAGCTCAAGCAGAA	No

each purified sample was added 25 μl of EDTA 0.5 mM and were run in a sequencer Applied Biosystems (Thermo Fisher Scientific) with polymer 7 (Thermo Fisher Scientific).

Primer pairs amplifying a single band in representatives of *Opuntia* and *Grusonia* were further amplified for this work. Sequences were assembled in Sequencher v.5.4.6. Alignments were performed with the program Muscle v.3.8.31 (Edgar, 2004) and subsequently manually adjusted in PhyDE (Müller et al., 2005).

Phylogenetic and Network Analysis

We further amplified the primers with ID "a" for *AT3G48380* and *AT1G18270* genes (**Table 1**) in 26 individuals from the nine *Opuntia* species (**Figure 1**) and the outgroup. PCR amplification followed conditions on **Tables 2**, **3**, respectively.

Phylogenetic networks analyses aimed to identify found hybridization among sampled *Opuntia* species in two levels : all the 27 sampled taxa, and at the individual level in *O. pilifera* and *O. tehuacana*. Therefore, we divided our data in two sets, one that included all 27 taxa and a second group indicated in **Supplementary Table 1** as "hybrid-test," which included only one random individual per species and one putative hybrid. In total we analyzed nine "hybrid-test" matrices with 10 taxa each. To perform the phylogenetic network analysis, we used as a base maximum likelihood (ML) gene trees from RA×ML v.8.2 (Stamatakis, 2014) with the GTR + Γ model, bootstrapping with the autoMRE option and a search for the best-scoring ML tree after the bootstrap searches to obtain the best tree.

The phylogenetic networks analysis was performed in PhyloNet v.3.6.1 (Wen et al., 2018) using the best ML trees for each data block. First, we tested the non-hybridization hypothesis in the complete data block (27 taxa), using the option Infer_ST_MDC to obtain the species tree under the "Minimize Deep Coalescence" (MDC) criterion; next we used all the available reticulation options for the InferNetwork_MP command to test the possible one, two, and three reticulation scenarios. Afterward, we performed nine individual tests for hybridization using each one of the nine "hybrid-test" matrices. For these tests, we first obtained the species trees (Infer_ST_MDC), and thereafter, we used the InferNetwork_MP with the option -h {putative hybrid} to test each individual hybrid hypothesis. For both analyses, each reticulation inference was replicated 10 times. In each one of the 10 replicates, the network with the lowest number of extra lineages was selected and displayed graphically with Dendroscope v.3.0 (Huson and Scornavacca, 2012). Furthermore, we analyze our complete matrix (without outgroup) in SplitsTree v.4.16.1, with a NeighborNet (**Supplementary Figure 1**) to identify potential reticulation between lineages with a non-parametric method.

TABLE 2 | Touch-up PCR program for amplification tests and primer ID "a" in *AT3G48380*.

	Temperature	Time	
Initial denaturation	94°C	2 min	
Denaturation	94°C	35 s	10 cycles
Annealing	46–51°C increasing 0.5°C per cycle	30 s	
Extension	72°C	1 min	
Denaturation	94°C	35 s	30 cycles
Annealing	55°C	30 s	
Extension	72°C	1 min	
Final extension	72°C	5 min	

TABLE 3 | PCR program for gradient test and primer ID "a" in *AT1G18270*, using a concentration of 2.5 μM $MgCl_2$.

	Temperature	Time	
Initial denaturation	94°C	2 min	
Denaturation	94°C	30 s	32 cycles
Annealing gradient	46–57°C	30 s	
Annealing *AT1G36980*	51.5°C		
Extension	72°C	1.5 min	
Final extension	72°C	5 min	

Testing Hybridization With Phylogenetic Invariants

We performed a phylogenetic invariants analysis under the coalescent model on HyDe v.0.4.1a (Hybridization Detection:

Blischak et al., 2018) with the aim to test the following two hypotheses, (1) *O. tehuacana* as a hybrid between *O. pilifera* and *O. huajuapensis*, and (2) *O. pilifera* as a hybrid between species of the *Nopalea* and *Basilares* clades, as well as to confirm the resulting networks from PhyloNet. We used the data set of all 27 taxa first, with the command run_hyde.py to test 11 possible triplet combinations with the hybridization scenarios of *O. tehuacana*, *O. pilifera* and the resultant hybrid scenarios from PhyloNet, then we ran individual hybridization detection analyses (individual_hyde.py) with the file of resultant positive values, to detect hybridization at individual level.

To strengthen our analysis, we performed another hybridization analysis using Dsuite. We used as base the all 27 taxa alignment in Fasta format and we transformed it into VFC in Python v.3.8 with the library cflib-pomo v.1.2.2.1. Then we filtered this file with VCFtools v.0.1.17 to have only biallelic SNPs. After that we obtained 1,000 SNPs which we analyzed with the Dtrios option, using the same 11 possible hybridization scenarios tested in HyDe.

RESULTS

Amplified Nuclear Genes and Data Matrices

From the four pairs of primers tested, only two for intron regions in genes *AT3G48380* and *AT1G18270*, indicated with ID "a" (**Table 1**) presented a single visible PCR product on an agarose gel in *Opuntia, Grusonia, Melocactus,* and *Pilosocereus*.

The aligned complete data block consisted of 27 terminals and 1,976 aligned characters, 1,102 of which correspond to the *AT3G48380* intron and 874 characters corresponding to the intron *AT1G18270*. The reduced data block "hybrid-test" consisted of nine data matrices with 10 terminals in each one and 1,976 aligned characters.

Phylogenetic Networks From Coalescent-Based Methods

Under parsimony the MDC criterion seeks the reconciliation of a set of gene trees in the branches of a species tree with the minimal number of extra lineages, providing an optimal evolutive history for the species tree (Yu et al., 2013). We first inferred the species tree using the MDC criterion for the 27 sampled species, and the species tree had 74 lineages (**Figure 4A**), *O. tehuacana* and *O. depressa* were recovered as successive sister species of a clade containing the remaining sampled *Opuntia* species. Within the latter clade, two main groups were recovered. The first of them was composed of *O. pilifera,* sister to *O. lasiacantha,* and the second was integrated by a clade of *O. velutina,* sister of *O. decumbens,* and a clade where *O. tehuantepecana* is sister to the clade of *O. streptacantha* plus *O. huajuapensis*.

When inferring reticulation events for all sampled species we explored all the possible scenarios with one, two, and three reticulation events, for a total of six possible reticulation events. For the one reticulation event (**Figure 4B**; H1), PhyloNet detected hybridization between *O. tehuantepecana* and *O. depressa* into the clade *O. velutina-O. decumbens* and a reduction of 74 to 45 lineages. In the two reticulation event (**Figure 4C**), we detected hybridization between *O. streptacantha* and *G. invicta* into *O. tehuantepecana* (H1) and introgression from *O. tehuantepecana* into *O. velutina* (H2) with a reduction from 74 to 44 lineages. The three reticulations scenario (**Figure 4D**) resulted only in three hybridization events, of which the first (H1) occurred between *O. streptacantha* and a non-sampled or possible extinct taxon into *O. tehuantepecana*, the second (H2) involved *O. tehuantepecana* and *O. depressa* as a putative parental species for the *O. velutina-O. decumbens* clade and the third (H3) indicated hybridization between *O. lasiacantha* and a non-sampled or possibly extinct taxon into the *O. velutina-O. decumbens* clade. The lineage number remained the same as the two-reticulation event.

For the two hybrids hypothesis test, we sequentially indicate *O. tehuacana* and *O. pilifera* individuals as putative hybrids (**Figures 5A–H**). The first reticulation event (**Figure 5A**) showed 11 lineages for the MDC tree and hybridization (H1) between *O. streptacantha* and *O. huajuapensis* into *O. tehuacana* from Santa Maria Tecomavaca, Oaxaca, and a reduction to six lineages. The other *O. tehuacana* individual from this locality did not present a logical reticulation event. The following reticulation event (**Figure 5B**) depicts the MDC tree (16 lineages) and a hybridization event (H1, eight lineages) between *O. decumbens* and a non-sampled or extinct taxon into *O. tehuacana* from San Juan Bautista Cuicatlán, Oaxaca. The next MDC tree (**Figure 5C**) has 16 lineages, and it shows hybridization between *O. decumbens* and *O. huajuapensis* (H1) into *O. tehuacana* from Asunción Nochixtlán, Oaxaca. The following analysis was performed in *O. tehuacana* individuals from Ajalpan, Puebla, and the MDC tree (**Figure 5D**) has 20 lineages and depicts hybridization between *O. streptacantha* and *O. huajuapensis* and a reduction to 10 lineages. Afterward, the MDC tree (**Figure 5E**) depicts 12 lineages and a hybridization event between *O. streptacantha* and *O. lasiacantha* into *O. tehuacana* from Ajalpan, Puebla, with a reduction to nine lineages.

For *O. pilifera* hybridization tests (**Figures 5F–H**), the first analysis depicts the MDC tree with 11 lineages and a hybridization event that occurred between *O. velutina* and *O. lasiacantha* into *O. pilifera* from Ajalpan, Puebla and a reduction to nine lineages. In the following test (**Figure 5G**), the MDC tree has 21 lineages and a hybridization event between *O. lasiacantha* and the clade *O. streptacantha-O. tehuantepecana* into *O. pilifera* from Asunción Nochixtlán, Oaxaca. The last inferred MDC tree (**Figure 5H**) depicts 21 lineages and hybridization between *O. velutina* and the clade *O. tehuacana-O. huajuapensis* into *O. pilifera* from Ajalpan, Puebla.

Corroboration of Phylogenetic Networks With HyDe and Dsuite

We tested 11 triplets with the command run_hyde.py, and the only significative triplet combination was *O. tehuantepecana-O. depressa* as parental for *O. velutina*. Although for the rest of the triplet combinations we had no significative results, we used the triplets with positive values (**Table 4**) to analyze hybridization at the individual level. We consider the results from individual

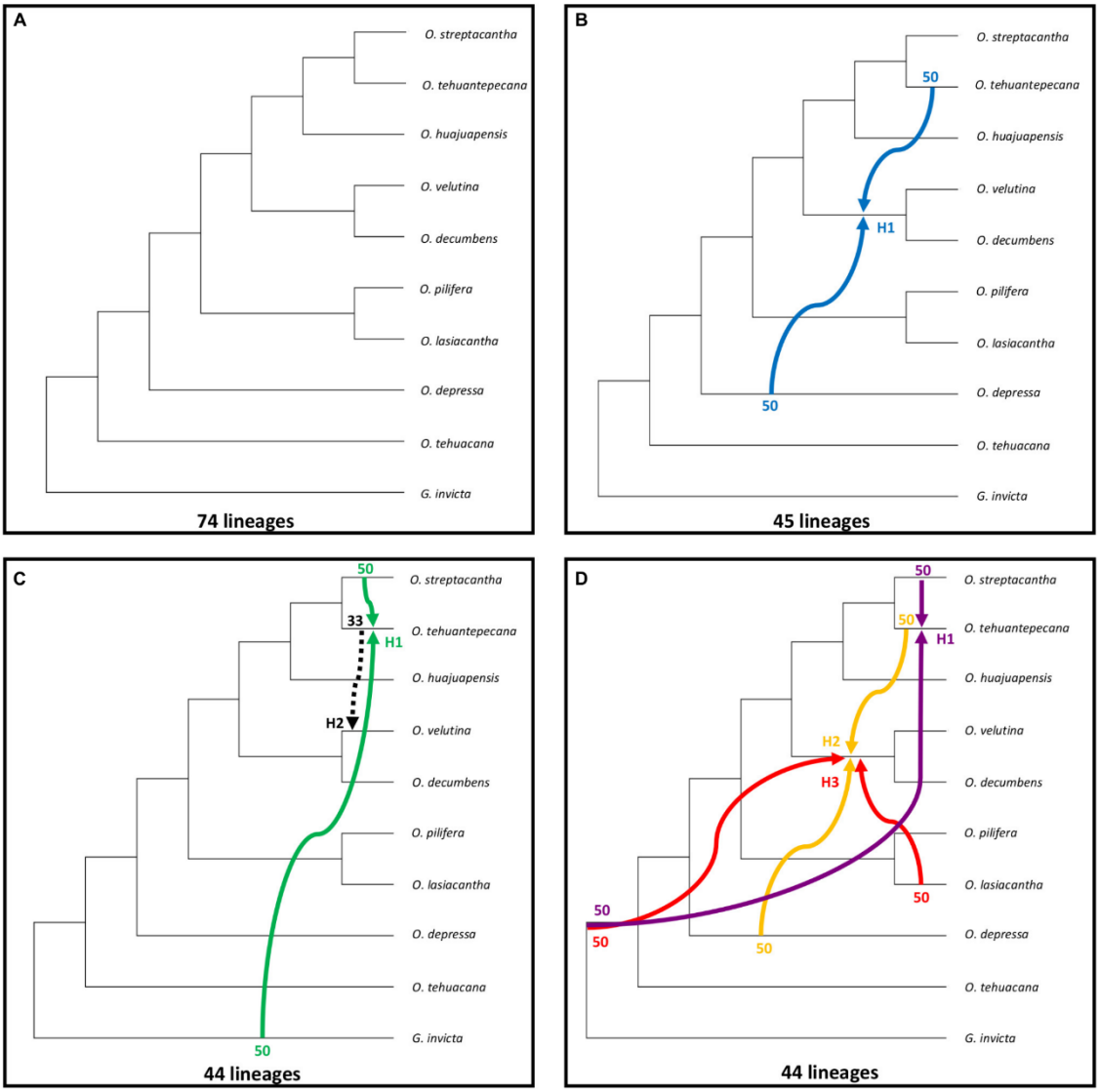


FIGURE 4 | MDC analysis for all sampled *Opuntia* species. **(A)** Species tree without reticulations. **(B–D)** Best reticulation inference from one to three possible reticulations, number of reticulations indicated with H1, H2, H3. Inheritance probabilities are indicated before each arrow. Hybridization events are indicated with solid colored arrows, and the dashed arrow indicates an introgression event.

analysis with a *p*-value lower than 0.05 important and reliable even though they were not significative because we include multiple individuals per species (Blischak et al., 2018). The individual analysis revealed six hybrid individuals with *p*-values lower than 0.05 (**Table 5**).

The same triplets tested on HyDe were used with the Dtrios command on Dsuite, and there were ten combinations with significative *p*-values (**Supplementary Table 2**). The combinations with shared alleles and supported by other analysis were *O. decumbens-O. pilifera*, *O. decumbens-O. tehuacana,* and *O. tehuantepecana-O. velutina*.

DISCUSSION

We tested two hybridization cases, as well as previously unknown hybridization scenarios involving sympatric Opuntia species from Tehuacán-Cuicatlán Valley and southern Mexico. Our

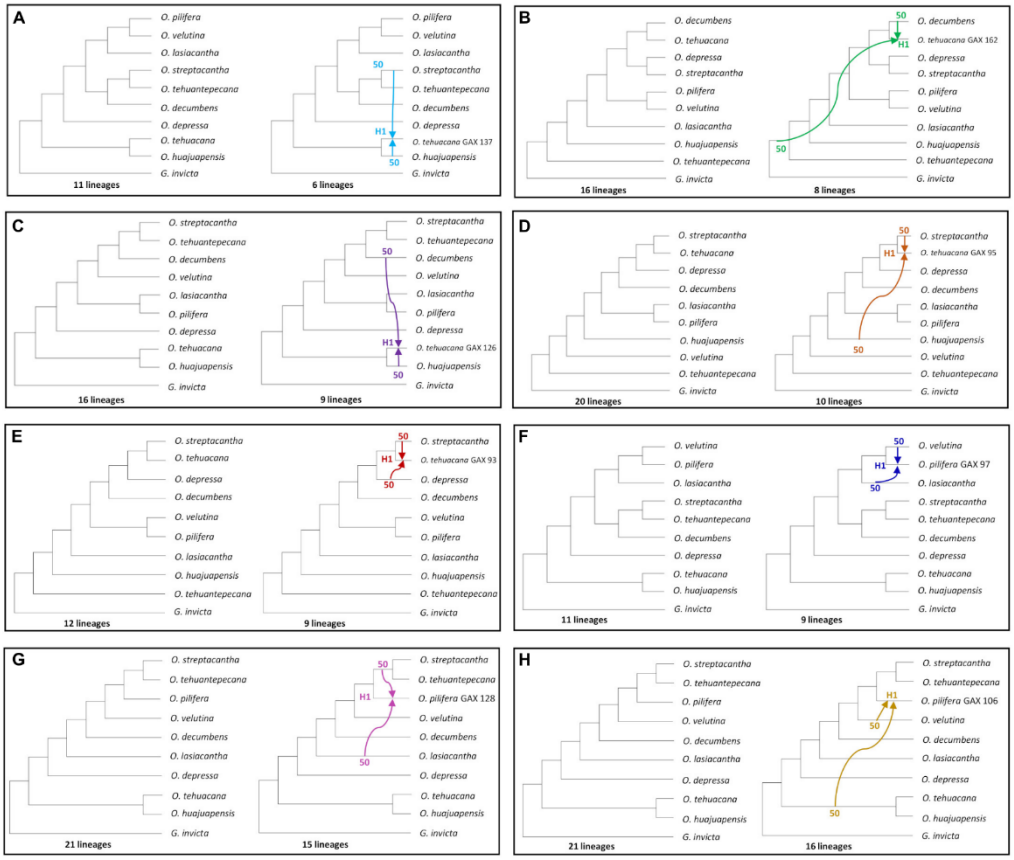


FIGURE 5 | All individual hybridization tests made with MDC; on the left side is the species tree, and on the right side is the inferred reticulation with specific putative hybrids. Inheritance probabilities are indicated before each arrow. **(A–E)** *O. tehuacana* best MDC network. **(F–H)** *O. pilifera* best MDC network.

TABLE 4 | Results from hybridization detection in HyDe with positive values.

P1	Hybrid	P2	Z-scores	*p*-values	Gamma (γ)
O. decumbens	*O. pilifera*	*O. depressa*	0.446	0.327	0.572
O. lasiacantha	*O. pilifera*	*O. velutina*	0.642	0.260	0.351
O. decumbens	*O. tehuacana*	*O. huajuapensis*	1.122	0.130	0.545
O. tehuantepecana	*O. velutina*	*O. depressa*	2.066	0.019	0.484
O. tehuantepecana	*O. decumbens*	*O. depressa*	1.093	0.137	0.310
O. tehuantepecana	*O. velutina*	*O. decumbens*	1.574	0.057	0.374

findings support previous work, in which highlight Opuntia as a genus with multiple hybridization events (Pinkava, 2002; Majure et al., 2012a) but, in this work using new approaches.

We used a multi-individual approach in our analysis to compensate for the low number of nuclear sites (1,976) and obtain reliable results. Phylogenetic networks inferred for all the sampled species showed a lineage decrease compared to the species tree (**Figure 4**); therefore, the scenarios with inferred reticulations can be assumed to be more accurate (Wen et al., 2018). Some of the recovered reticulation scenarios include the outgroup, non-sampled or extinct taxa, we consider these scenarios unlikely, and also probably due to the retention of an ancestral polymorphism, therefore, we only mention them as part of PhyloNet results (Copetti et al., 2017; Wen et al., 2018). Consequently, we emphasize the significance of having a broader sampling for future analysis, to avoid this kind of implausible scenarios. In *Opuntia*, the presence of natural hybrids due to weak reproductive barriers is common (Pinkava, 2002).

TABLE 5 | Detected hybridization on individuals and their putative parental species in HyDe analysis (*p*-values lower than 0.05).

P1	Hybrid	P2	Z-score	*p*-value	Gamma (γ)
O. decumbens	*O. tehuacana* (GAX 162)	*O. huajuapensis*	1.720	0.042	0.686
O. tehuantepecana	*O. velutina* (GAX 108)	*O. depressa*	2.416	0.007	0.507
O. tehuantepecana	*O. velutina* (GAX 139)	*O. depressa*	2.008	0.022	0.460
O. tehuantepecana	*O. velutina* (GAX 96)	*O. depressa*	1.755	0.039	0.482
O. tehuantepecana	*O. decumbens* (GAX 134)	*O. depressa*	1.969	0.024	0.235
O. tehuantepecana	*O. velutina* (GAX 108)	*O. decumbens*	1.972	0.024	0.417

The flowering periods for most of the studied opuntias occur during the spring (**Figure 2B**); hence, pollen exchange could occur between geographically close species. The pollination is mainly carried out by bees known for being generalists favoring cross-pollination between different species (Reyes-Agüero et al., 2006), and there is even evidence of pollination by hummingbirds in the Tehuacán-Cuicatlán Valley, carrying pollen across wider distances than bees (Ortiz-Pulido et al., 2011; Serrano-Serrano et al., 2017).

Implications of Sympatry in Studied Opuntias

It is important to emphasize that most of the sampled species for this study are in sympatry (**Figure 2C**), but we mean not overlapping in potential geographic distributions but rather three to four species inhabiting the same small area. For example, in Ajalpan, Puebla, the species *O. tehuacana*, *O. pilifera*, *O. velutina,* and *O. depressa* coexist in an area of approximately 20 m. This distribution dynamics allows the development of hybrids because of the pollen exchange (Reyes-Agüero et al., 2006) and weak mating barriers (Pinkava, 2002; Majure et al., 2012b). Although we did not formally study ecological dynamics of sampled opuntias we can made some inferences about exchange of genetic material based on our field observations, results, and data from literature about flowering periods (Arias et al., 2012). Hybrid zones in nature are the spatially and temporally place where two distinguishable populations overlap and cross to form viable and sometimes fertile offspring (Arnold, 1997), therefore we can say that most of the sampled localities could be considered as potential hybrid zones. In the cases of the outgroup and additional species like *O. tehuantepecana* and *O. streptacantha,* for which we include sampled individuals geographically distant from Tehuacán-Cuicatlán Valley, we analyzed them to know their relationships with the rest of the species and to have a hybridization context potentially older and wider.

Hybridization and Gene Flow on *O. tehuacana*

The hypothesis of hybrid status in *O. tehuacana* described in previous studies by Arias et al. (2012), was not supported in any individual network. The only scenario that includes one of the proposed parental species occurs in one individual from Ajalpan, Puebla (X. Granados 95; **Figure 5D**). The putative parental species for this individual are *O. streptacantha* and *O. huajuapensis*, which are sister species (**Figure 5D**); however, we did not confirm *O. streptacantha* as a parental with the HyDe or Dsuite analysis, resulting in an implausible scenario. Regarding the reticulation event from *O. huajuapensis* into *O. tehuacana* from Ajalpan, introgression could exist because their floral periods overlap (April–May), their flowers are yellow, sometimes orange in the case of *O. tehuacana*, and there is evidence that the same species of hummingbird visits both opuntias species (Ortiz-Pulido et al., 2011; Arias et al., 2012), making pollen exchange between these species likely. Additional morphological characteristics shared between these species (**Figures 1A,C**) that could support the genetic exchange are the orbicular to suborbicular cladodes and the acid pulp in their fruits (Arias et al., 2012). Despite these shared traits, we did not observe intermediate characters in sampled *O. tehuacana* individuals, as would be expected in a hybrid (Arnold, 1997).

Another proposed parental species for *O. tehuacana* is *O. pilifera* (Arias et al., 2012), but for this scenario, no reticulation event was found either in PhyloNet, HyDe or Dsuite tests. Both species (**Figures 1A,E**) share the presence of hairs in the areolas (sporadic in *O. tehuacana*) and glabrous epidermis. However, *O. tehuacana* is a shrub, the flower is typically orange–yellow and the fruit can remain on the plant for more than a year until it becomes green–yellow, with acidic, light pink pulp. Meanwhile, *O. pilifera* is a tree, the flower is red–pink and the fruit remains on the plant for only one season, turning red to light pink, with sweet and red pulp. Since there are no intermediate traits between these species or another result supporting the parental scenario of *O. pilifera,* this hypothesis is discarded (Arnold, 1997; Reyes-Agüero et al., 2006).

We tested the hypothesis of *O. huajuapensis* and *O. decumbens* as parental species of *O. tehuacana* in order to support the hybridization scenario obtained from PhyloNet (**Figure 5C**). This scenario involves the *O. tehuacana* individual from Nochixtlán. Surprisingly, from the six *O. tehuacana* individuals tested on HyDe, reliable hybridization was detected only in the specimen from Cuicatlán (X. Granados 162), and the reticulation analysis for this individual (**Figure 5B**) involves *O. decumbens* and a non-sampled or extinct taxon. Therefore, we think that the reticulation event between these species was influenced by the fact that these individuals are phylogenetically close, but in fact this relationship is only present on this MDC tree. It is important to emphasize that although HyDe did not detect hybridization in *O. tehuacana* from Nochixtlán, the same gene flow pattern could be shared between individuals from Cuicatlán and Nochixtlán because *O. tehuacana* and *O. huajuapensis* are sympatric in both places (Arias et al., 2012). The pattern of reticulation from *O. decumbens* into *O. tehuacana* appears twice in the inferred individual networks, was recovered in

one individual HyDe analysis, supported by Dsuite analysis in two individuals (**Supplementary Table 2**) and was also found in the NeighborNet (**Supplementary Figure 1**). The floral periods of *O. decumbens* and *O. tehuacana* occur during the same period (**Figure 2B**) and they are sympatric thus, cross pollination can occur between analyzed individuals from both species. Our results confirm that not all *O. tehuacana* individuals have the same reticulation pattern, and we can infer that only introgression among certain individuals is occurring and not hybrid speciation (Blischak et al., 2018).

Hybridization and Gene Flow on *O. pilifera*

Opuntia pilifera is in the *Basilares* clade (Majure et al., 2012a), species of this clade are known for being polyploids and form hybrids. In the analysis performed on PhyloNet with the data set of all individuals, we did not detect hybridization into *O. pilifera* in any inferred network scenario, which could also be related to the low number of loci used and insufficient sampling of closely related species in our study. On the other hand, the test performed on *O. pilifera* individuals revealed hybridization scenarios involving mainly *O. lasiacantha* and *O. velutina*. The pattern of *O. velutina*–*O. pilifera,* and *O. lasiacantha*–*O. pilifera* as sister species is repeated several times on species trees from **Figures 4**, **5**. The changing position of these species could also support the hybridization scenarios obtained in the PhyloNet individual analysis (Wen et al., 2018). Furthermore, in the HyDe analysis to test *O. pilifera* as a hybrid, we obtained positive values (**Table 4**) for the putative *O. pilifera* parental lineages *O. decumbens*–*O. depressa* and *O. lasiacantha*–*O. velutina*, but when we performed the individual analysis, none of the individually tested triplets had a reliable *p*-value; thus, the HyDe analysis cannot confirm the results obtained in PhyloNet. Surprisingly, the analysis performed on Dsuite supports the scenario of *O. decumbens* as parental donor for *O. pilifera*. This sustains the hybridization hypothesis proposed by Majure et al. (2012a), because *O. decumbens* is in clade *Nopalea*. Furthermore, the bloom of these species overlaps and they are sympatric on Tehuacán-Cuicatlán Valley (**Figure 2B**; Arias et al., 2012).

Hybridization in Opuntias From Southern Mexico

In the phylogenetic network analysis with all sampled species, the one reticulation event (**Figure 4B**) depicts hybridization into the *O. velutina*–*O. decumbens* clade with putative parental species *O. tehuantepecana* and *O. depressa*. This scenario is also supported by HyDe analysis with significative hybridization between *O. depressa* and *O. tehuantepecana* intro *O. velutina* (**Table 4**) and at individual level all sampled *O. velutina* and one individual from *O. decumbens* had reliable hybridization results (**Table 5**). The analysis with Dsuite also supports the relationship of *O. tehuantepecana* as parental of *O. velutina* (**Supplementary Table 2**). In the hybridization scenario that includes *O. tehuantepecana* and *O. depressa* as putative parental lineages of *O. velutina,* we infer that the event probably occurred in the past because current distributions of parental species are adjacent but not sympatric (Arias et al., 2012; Barthlott et al., 2015). The *O. depressa* distribution was probably wide enough to contact *O. tehuantepecana,* giving rise to a hybrid zone in which a lineage with similar fitness to its parental species originated after backcrosses and the action of natural selection (Arnold, 1997), which is currently known as *O. velutina*. This assumption is also supported by morphological similarities shared by *O. velutina* and *O. tehuantepecana* (**Figures 1B,I**): both are shrubs, sometimes tree-like with a wide trunk; their glochids are long and yellow, from 5 to 13 mm in *O. velutina* and from 2 to 4 mm in *O. tehuantepecana*; and the traits shared between *O. velutina* and *O. depressa* are the cladodes obovate and pubescent and glochids long and yellow (Anderson, 2001; Arias et al., 2012). The relationship among these species have not been reported elsewhere.

More introgression scenarios between analyzed *Opuntia* species were recovered with the Dsuite analysis, but most of them involve *O. tehuantepecana* and one specie from Tehuacán-Cuicatlán Valley, since these species are not sympatric, the number of SNPs analyzed was too low to assign the true parental donor (Malinsky et al., 2020) and the scenarios were not recovered in other analysis, we consider them unlikely.

Taxonomic Implications of Hybridization in *Opuntia*

The hybridization process in *Opuntia* has evolutive implications on the number of species, the success and survival of his taxa in habitats with extreme weather conditions, but also has significance on the phylogenetic relationships within this group (Pinkava, 2002; Majure et al., 2012a). Not having a bifurcate history complicates the understanding of a group under classical phylogenies where the inheritance pattern is linear (Arnold, 1997). The horizontal exchange of genetic material in opuntias hinders their linear phylogenetic histories and makes the limits among species blurred. *Opuntia* is one of the most complex groups of plants, due to its variable traits, polyploidy, hybridization, and human handling (Pinkava, 2002; Reyes-Agüero et al., 2006). Furthermore, the widespread distribution of species like *O. decumbens*, *O. streptacantha,* and *O. velutina* complicates the collection of multiple individuals throughout its distribution and their inclusion in phylogenetic and network analyzes. New perspectives of reticulate evolution in plants should lead to an integrative vision with sampling multiple individuals, morphometric and biogeographic analysis, and other studies to improve taxonomy of problematic groups.

As future perspectives, we highlight the relevance of this work as a first approach to the hybridization processes in southern Mexico *Opuntia* species, a group that has been little studied despite the large number of species in this region (Arias et al., 2012). Phylogenetic approaches have included some *Opuntia* representatives from the Tehuacán-Cuicatlán Valley (Griffith and Porter, 2009; Majure et al., 2012a), but for most of the representatives of this complex group their evolutive stories remain unknown. This is mainly due to polyploidy and unknown chromosome numbers of species like *O. depressa*, *O. huajuapensis*, *O. tehuacana*,

O. tehuantepecana, and *O. velutina*. Also, the allopolyploid condition in most *Opuntia* species hinders their study due to the presence of homoeologous genes, which are difficult to isolate and to distinguish from paralogous in the subgenomes (Glover et al., 2016).

New hybridization scenarios were found, as expected, in sympatric opuntias such as *O. velutina* as a putative hybrid. Its variable morphological traits and broad distribution (Bravo-Hollis, 1978) make this species an interesting study case for future analysis. Other hybridization scenarios were recovered in our analyses, but we do not have enough information to confirm them.

The case of *O. tehuacana* as a hybrid between *O. pilifera* and *O. huajuapensis* was discarded by our analysis, but due to hybridization detected in some individuals with *O. huajuapensis* and *O. decumbens* as the parental species, this scenario should be tested using more loci and including morphological and morphometric analysis because of the complex relationships among these species. The hybrid status of *O. pilifera* involves two pairs of putative parental species, *O. lasiacantha–O. velutina* and *O. decumben–O. depressa*. The relationship of *O. decumbens* as parental donor of *O. pilifera* was recovered as significative by the analysis of Dsuite, supporting the hypothesis of gene flow between *Nopalea* and *Basilares* clades. Other mentioned putative parental donors should be tested in further analysis.

Although PhyloNet results may not be significative due to the low number of genes used, we performed a multi-individual approach to compensate this disadvantage, and each individual was tested in a hybridization scenario allowing us to detect logical hybridization scenarios, hybrid individuals and introgression. Future studies in *Opuntia* should include more individuals per species and more loci, and, most importantly, carry out integrative analyses that allow elucidation of the reticulate evolution of this complex group of plants with high diversity in Mexico.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

XG-A: investigation, formal analysis, methodology, writing the first draft, text revision, and editing. CGM: methodology conceptualization, text revision, and editing. CC: methodology and complementary analysis. JM: methodology conceptualization, formal analysis, text revision, and editing. SA: researcher leading of this study and obtained the financial support. SA and XG-A: designing the research. All of the authors approved the submitted version of this manuscript.

FUNDING

Financial support to SA for this study was provided by PAPIIT-DGAPA, UNAM (IN208619) and a grant to XG-A provided by Instituto de Biología, UNAM (Open access publication financing).

ACKNOWLEDGMENTS

This study is part of the dissertation of XG-A and is presented as a partial requirement for the Ph.D. in Biological Sciences in the Posgrado en Ciencias Biologicas (PCB), UNAM; she thanks the fellowship of CONACyT (453987). We thank Yolanda Morales, Daniel Franco, Brenda Calderón, Moises Franco, Ulises Rosas and Andrea R. Jiménez (Jardín Botánico and Instituto de Biología, UNAM) for supporting the field and laboratory work, Laura M. Márquez Valdelamar and Nelly María López Ortiz for their support in sequencing (LANABIO, Instituto de Biología, UNAM), Paul D. Blischak (University of Arizona) and Silvia Hinojosa (Jardín Botánico, UNAM) for their support of HyDe analysis, Margarita Santiago (FES Zaragoza, UNAM) and Daniel Franco (Jardín Botánico, UNAM) for their support on map elaboration, the LANABIO laboratories at Jardín Botánico and Instituto de Biología, UNAM and the Posgrado en Ciencias Biologicas, UNAM.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.606809/full#supplementary-material

Supplementary Figure 1 | Neighbor net for 26 *Opuntia* individuals from 9 species.

REFERENCES

Anderson, E. F. (2001). *The Cactus Family*. Oregon: Timber Press.

Arias, S., Gama, S., Vázquez, B., and Guzmán, L. U. (2012). "Cactaceae Juss," in *Flora del Valle de Tehuacán-Cuicatlán*, ed. R. Medina Lemos, (México, D. F: Universidad Nacional Autónoma de México), 1–235.

Arnold, M. L. (1997). *Natural Hybridization and Evolution*. New York, NY: Oxford University Press.

Barthlott, W., Burstedde, K., Geffert, J., Ibisch, P., Korotkova, N., Miebach, A., et al. (2015). "Distribution maps of Cactaceae," in *Biogeography and Biodiversity of Cacti*, ed. D. Metzing, (Alemania: Schumannia), 82–197.

Blischak, P. D., Chifman, J., Wolfe, A. D., and Kubatko, L. S. (2018). HyDe: A Python Package for Genome-Scale Hybridization Detection. *Syst. Biol.* 67, 821–829. doi: 10.1093/sysbio/syy023

Bravo-Hollis, H. (1978). *Las Cactáceas de México*. Ciudad de México: Universidad Nacional Autónoma de México.

Bustamante, E., Búrquez, A., Scheinvar, E., and Eguiarte, L. E. (2016). Population Genetic Structure of a Widespread Bat-Pollinated Columnar Cactus. *PLoS One* 11:152329. doi: 10.1371/journal.pone.0152329

Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., et al. (2009). BLAST+: architecture and applications. *BMC Bioinformatics* 10:421. doi: 10.1186/1471-2105-10-421

Copetti, D., Búrquez, A., Bustamante, E., Charboneau, J. L. M., Childs, K. L., Eguiarte, L. E., et al. (2017). Extensive Gene Tree Discordance and Hemiplasy Shaped the Genomes of North American Columnar Cacti. *Proc. Natl. Acad. Sci. U S A.* 114, 12003–12008. doi: 10.1073/pnas.1706367114

Doyle, J. J., and Doyle, J. L. (1987). A Rapid DNA Isolation Procedure for Small Quantities of Fresh Leaf Tissue. *Phytochem. Bull.* 19, 11–15.

Edgar, R. C. (2004). MUSCLE: Multiple Sequence Alignment with High Accuracy and High Throughput. *Nucleic Acids Res.* 32, 1792–1797. doi: 10.1093/nar/gkh340

Elworth, R. A. L., Ogilvie, H. A., Zhu, J., and Nakhleh, L. (2019). "Advances in Computational Methods for Phylogenetic Networks in the Presence of Hybridization," in *Bioinformatics and Phylogenetics: Seminal Contributions of Bernard Moret*, ed. T. Warnow, (Cham: Springer International Publishing), 317–360.

Gernandt, D. S., Aguirre, Dugua X, Vázquez-Lobo, A., Willyard, A., Moreno, Letelier A, Pérez de la Rosa, J. A., et al. (2018). Multi-locus Phylogenetics, Lineage Sorting, and Reticulation in *Pinus* subsection *Australes*. *Am. J. Bot.* 105, 711–725. doi: 10.1002/ajb2.1052

Glover, N. M., Redestig, H., and Dessimoz, C. (2016). Homoeologs: What Are They and How Do We Infer Them? *Trends Plant Sci.* 21, 609–621. doi: 10.1016/j.tplants.2016.02.005

Granados Mendoza, C., Naumann, J., Samain, M.-S., Goetghebeur, P., De Smet, Y., and Wanke, S. (2015). A genome-scale mining strategy for recovering novel rapidly-evolving nuclear single-copy genes for addressing shallow-scale phylogenetics in Hydrangea. *BMC Evol. Biol.* 15, 1–13. doi: 10.1186/s12862-015-0416-z

Griffith, P., and Porter, M. (2009). Phylogeny of Opuntioideae (Cactaceae). *Int. J. Plant Sci.* 170, 107–116.

Guerrero, P. C., Majure, L. C., Cornejo-Romero, A., and Hernández-Hernández, T. (2019). Phylogenetic Relationships and Evolutionary Trends in the Cactus Family. *J. Hered.* 110, 4–21. doi: 10.1093/jhered/esy064

Hunt, D., Taylor, N., and Charles, G. (2006). *The New Cactus Lexicon*. Milborne Port: DH Books.

Huson, D. H., and Bryant, D. (2005). Application of Phylogenetic Networks in Evolutionary Studies. *Mol. Biol. Evol.* 23, 254–267. doi: 10.1093/molbev/msj030

Huson, D. H., and Scornavacca, C. (2012). Dendroscope 3: An Interactive Tool for Rooted Phylogenetic Trees and Networks. *Syst. Biol.* 61, 1061–1067. doi: 10.1093/sysbio/sys062

Kamneva, O. K., Syring, J., Liston, A., and Rosenberg, N. A. (2017). Evaluating Allopolyploid Origins in Strawberries (*Fragaria*) Using Haplotypes Generated from Target Capture Sequencing. *BMC Evol. Biol.* 17:180. doi: 10.1186/s12862-017-1019-7

Majure, L. C., and Puente, R. (2014). "Phylogenetic Relationships and Morphological Evolution in *Opuntia*s. str. and Closely Related Members of Tribe Opuntieae," in *Further Studies in the Opuntioideae (Cactaceae)*, ed. D. Hunt, (United Kingdam: DH Books), 9–30.

Majure, L. C., Puente, R., and Pinkava, D. J. (2012b). Miscellaneous Chromosome Numbers in Opuntieae Dc. (Cactaceae) with a Compilation of Counts for the Group. *Haseltonia* 67–78. doi: 10.2985/026.018.0109

Majure, L. C., Puente, R., Griffith, M. P., Judd, W. S., Soltis, P. S., and Soltis, D. E. (2012a). Phylogeny of *Opuntia* s.s. (Cactaceae): Clade Delineation, Geographic Origins, and Reticulate Evolution. *Am. J. Bot.* 99, 847–864. doi: 10.3732/ajb.1100375

Malinsky, M., Matschiner, M., and Svardal, H. (2020). Dsuite - fast D-statistics and related admixture evidence from VCF files. *Biorxiv* 634477. Preprint. doi: 10.1101/634477.

Marcussen, T., Jakobsen, K. S., Danihelka, J., Ballard, H. E., Blaxland, K., Brysting, A. K., et al. (2011). Inferring Species Networks from Gene Trees in High-Polyploid North American and Hawaiian Violets (*Viola*. *Violaceae*). *Syst. Biol.* 61, 107–126. doi: 10.1093/sysbio/syr096

Morales-Briones, D. F., Romoleroux, K., Kolář, F., and Tank, D. C. (2018). Phylogeny and Evolution of the Neotropical Radiation of *Lachemilla* (Rosaceae): Uncovering a History of Reticulate Evolution and Implications for Infrageneric Classification. *Syst. Bot.* 43, 17–34. doi: 10.1600/036364418X696897

Müller, J., Müller, K., Neinhuis, C., and Quandt, D. (2005). *PhyDE® - Phylogenetic Data Editor*. Available online at: www.phyde.de (accessed September 28, 2011)

Ogilvie, H. A., Bouckaert, R. R., and Drummond, A. J. (2017). StarBEAST2 Brings Faster Species Tree Inference and Accurate Estimates of Substitution Rates. *Mol. Biol. Evol.* 34, 2101–2114. doi: 10.1093/molbev/msx126

Ortiz-Pulido, R., Díaz, S. A., Valle-Díaz, O. I., and Fisher, A. D. (2011). Hummingbirds and the Plants They Visit in the Tehuacán-Cuicatlán Biosphere Reserve, Mexico. *Rev. Mex. Biodiv.* 83:1139. doi: 10.22201/ib.20078706e.2012.1.1139

Pinkava, D. J. (2002). "On the Evolution of the North American Opuntioideae," in *Studies in the Opuntioideae (Cactaceae)*, ed. D. Hunt, (United Kingdam: DH Books), 59–98.

Reyes-Agüero, J. A., Aguirre, J. R. R., and Valiente-Banuet, A. (2006). Reproductive Biology of *Opuntia*: A Review. *J. Arid. Environ.* 64, 549–585. doi: 10.1016/j.jaridenv.2005.06.018

Riesenberg, L. H., and Wendel, J. F. (1993). "Introgression and its Consequences in Plants," in *Hybrid Zones and the Evolutionary Process*, ed. R. G. Harrison, (New York: Oxford University Press), 70–109.

Rosenberg, N. A., and Nordborg, M. (2002). Genealogical Trees, Coalescent Theory and The Analysis of Genetic Polymorphisms. *Nat. Rev. Genet.* 3, 380–390. doi: 10.1038/nrg795

Serrano-Serrano, M. L., Rolland, J., Clark, J. L., Salamin, N., and Perret, M. (2017). Hummingbird Pollination and the Diversification of Angiosperms: An Old and Successful Association in Gesneriaceae. *P. Roy. Soc. B Biol. Sci.* 284:20162816. doi: 10.1098/rspb.2016.2816

Solís-Lemus, C., Bastide, P., and Ané, C. (2017). PhyloNetworks: A Package for Phylogenetic Networks. *Mol. Biol. Evol.* 34, 3292–3298. doi: 10.1093/molbev/msx235

Soltis, P. S. (2013). "Hybridization in Plants," in *Encyclopedia of Biodiversity* (2nd Edn), ed. S. A. Levin, (Waltham: Academic Press), 166–176.

Soltis, P. S., and Soltis, D. E. (2009). The Role of Hybridization in Plant Speciation. *Annu. Rev. Plant. Biol.* 60, 561–588. doi: 10.1146/annurev.arplant.043008.092039

Stamatakis, A. (2014). RAxML Version 8: A Tool for Phylogenetic Analysis and Post-Analysis of Large Phylogenies. *Bioinformatics* 30, 1312–1313. doi: 10.1093/bioinformatics/btu033

Wen, D., Yu, Y., Zhu, J., and Nakhleh, L. (2018). Inferring Phylogenetic Networks Using PhyloNet. *Syst. Biol.* 67, 735–740. doi: 10.1093/sysbio/syy015

Whitney, K. D., Ahern, J. R., Campbell, L. G., Albert, L. P., and King, M. S. (2010). Patterns of Hybridization in Plants. *Perspect. Plant. Ecol.* 12, 175–182. doi: 10.1016/j.ppees.2010.02.002

Yu, Y., Barnett, R. M., and Nakhleh, L. (2013). Parsimonious Inference of Hybridization in the Presence of Incomplete Lineage Sorting. *Syst. Biol.* 62, 738–751. doi: 10.1093/sysbio/syt037

Yu, Y., Degnan, J. H., and Nakhleh, L. (2012). The Probability of a Gene Tree Topology within a Phylogenetic Network with Applications to Hybridization Detection. *PLoS Genet.* 8:1002660. doi: 10.1371/journal.pgen.1002660

Zhang, C., Ogilvie, H. A., Drummond, A. J., and Stadler, T. (2018). Bayesian Inference of Species Networks from Multilocus Sequence Data. *Mol. Biol. Evol.* 35, 504–517. doi: 10.1093/molbev/msx307

Zhu, J., Wen, D., Yu, Y., Meudt, H. M., and Nakhleh, L. (2018). Bayesian Inference of Phylogenetic Networks from Bi-allelic Genetic Markers. *PLoS Comput. Biol.* 14:1005932. doi: 10.1371/journal.pcbi.1005932

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer LM declared a past co-authorship with one of the authors SA to the handling editor.

Discusión general

La hibridación en Cactaceae documentada para las subfamilias Cactoideae y Opuntioideae presenta diferencias en la forma en la que este proceso ha sido abordado. Para Cactoideae, la hibridación entre distintos géneros es muy común, pero esto se debe probablemente a problemas taxonómicos a nivel de género. Las clasificaciones clásicas para Cactoideae, tienen como base caracteres morfológicos que a consideración del taxónomo pueden ser "iguales", pero realmente no son caracteres homólogos. Por ejemplo, el caso de los tricomas en grandes cactáceas columnares pertenecientes a los géneros *Cephalocereus* y *Pilosocereus* (Zappi, 1994; Anderson, 2001), los cuales fueron incluidos dentro de *Cephalocereus* (Britton and Rose, 1920), pero posteriormente separados por otros caracteres morfológicos y análisis filogenéticos (Zappi, 1994; Hernández-Hernández et al., 2011; Calvente et al., 2016).

Con base en la literatura revisada (capítulo 1), existe poca evidencia del impacto de la hibridación natural en la evolución de Cactoideae, debido a que hay pocos estudios ecológicos (Mandujano et al., 2010) y evolutivos (Tabla 1.1). Los principales factores que impactan en la evolución y diversificación de esta subfamilia son la forma de crecimiento, la capacidad de adaptarse a ambientes áridos, síndromes de polinización variados, así como sus interacciones con factores ambientales (Hernández-Hernández et al., 2014), mientras que el papel de la especiación por hibridación no ha podido ser identificado, probablemente debido a la difícil taxonomía a nivel de género y a la falta de análisis citológicos y ecológicos.

En particular en Opuntioideae el enfoque para estudiar la hibridación es distinto, incluye evidencias citológicas, análisis filogenéticos y cruzas experimentales (Pinkava, 2002; Reyes-Agüero et al., 2006; Majure et al., 2012). Para esta subfamilia la hibridación no ha sido

considerada como un evento aislado que debe reportarse, sino que su papel en la evolución ha sido considerado un importante tema de estudio. En Opuntioideae la hibridación no solo ha sido observada, sino que se han hecho trabajos para probar su existencia y patrones evolutivos. El gran aporte de evidencias provenientes de conteos cromosómicos, análisis filogenéticos y cruzas experimentales han ayudado a tener un mejor entendimiento de la importancia de la hibridación en la evolución de esta subfamilia. La reproducción vegetativa es una característica importante de Opuntioideae, ya que promueve el mantenimiento de los linajes híbridos, ya sea mediante la invasión de nuevos nichos o en el caso de que sean fértiles mediante retrocruzas, a diferencia de la subfamilia Cactoideae en la cual, aunque existe este tipo de reproducción, ocurre en un menor número de taxa, por lo que los linajes híbridos pueden perderse fácilmente (Anderson, 2001; Reyes-Agüero et al., 2006; Mandujano et al., 2010). A pesar de los trabajos realizados en Opuntioideae aún no hay prueba de que alguna especie en particular se haya originado por hibridación, a diferencia de lo que ha ocurrido en familias como Asteraceae (*Helainthus*) e Iridaceae (*Iris*) (Rieseberg, 1991; Arnold, 1994, 1997), pero existen evidencias de introgression y evolución reticulada (Majure et al., 2012a; Granados-Aguilar et al., 2021).

En el género *Opuntia* se han documentado múltiples evidencias de hibridación en sus especies (Pinkava, 2002; Reyes-Agüero et al., 2006; Majure et al., 2012a). Un ejemplo interesante ocurre en la Reserva de la Biósfera del Valle de Tehuacán-Cuicatlán, con la especie *Opuntia tehuacana*, la cual hasta antes del presente trabajo era consideraba de probable origen híbrido (Arias et al., 2012). Esta especie presenta relaciones filogenéticas cercanas con sus especies simpátricas, agrupándose en conjunto dentro del clado *Basilares* (Majure et al., 2012a). Con el uso de tres marcadores de cloroplasto (*matK*, *ycf1* y *psbJ-petA*)

se obtuvo que todas las terminales analizadas de *O. tehuacana* se agruparon en un clado a diferencia de lo que podría obtenerse de un linaje de origen híbrido, los cuales suelen anidarse dentro del clado de la especie donadora de material materno (Rieseberg, 1991; Rieseberg et al., 1993; Arnold, 1997). Además, dos marcadores nucleares (*AT3G48380* y *AT1G18270*) confirmaron el resultado de que *O. tehuacana* se recupera como un clado monofilético, en el que se distinguen dos subclados con una congruencia geográfica. Estos resultados permiten concluir que *O. tehuacana* es una especie con base en el concepto filogenético de especie (Cracraft, 1989), aunque las barreras al intercambio genético sean semi-permeables como se ha reportado en otros grupos de plantas y animales (Harrison and Larson, 2014).

En cuanto a la relación filogenética de *O. tehuacana* con otras especies de *Opuntia* del Valle de Tehuacán-Cuicatlán, utilizando marcadores del cloroplasto (*matK*, *ycf1* y *psbJ-petA*) y núcleo (*AT3G48380* y *AT1G18270*), resultan como especies hermanas *O. huajuapensis*, *O. decumbens* y *O. lasiacantha*. Esto difiere de la propuesta de clasificación de Arias et al. (2012), la cual se estaba basada únicamente en caracteres morfológicos y consideraba a *O. tehuacana* como cercanamente emparentada con las especies *O. huajuapensis* y *O. pilifera* dentro de la serie Criniferae. Por su parte, los análisis filogenéticos con marcadores del cloroplasto (*matK*, *ycf1* y *psbJ-petA*) comparados con los de cloroplasto y núcleo concatenados (*matK*, *ycf1*, *psbJ-petA*, *AT3G48380* y *AT1G18270*), presentaron incongruencias en cuanto a la posición de las especies *O. huajuapensis*, *O. decumbens* y *O. lasiacantha*, las cuales se ubicaron en el clado *Nopalea* en la filogenia de cloroplasto, mientras que en el análisis concatenado se agruparon en un mismo clado hermano del clado de *O. tehuacana*, esto podría ser evidencia de procesos como hibridación o introgresión (Rieseberg et al., 1993; Arnold, 1997; Majure et al., 2012a).

El número cromosómico de *O. tehuacana* fue de 11x en cuatro de cinco localidades analizadas y sólo en la localidad de Coxcatlán fue de 12x. También se registraron aneuploidías, las cuales pueden estar relacionadas con la poliploidía y el flujo génico interespecífico que se encontró en ciertos individuos. El análisis de cromosomas en células madre del polen mostró univalentes en un individuo de la localidad de Ajalpan, Puebla, interpretándose como un híbrido de primera generación (F1) (Mestiri et al., 2010). Es importante resaltar que aunque hay localidades aneuploides cuyos gametos podrían ser menos aptos (Henry et al., 2009; Barke et al., 2020), la supervivencia de estos individuos está respaldada por la reproducción vegetativa común en este género (Reyes-Agüero et al., 2006). Además, el número cromosómico tan alto en *O. tehuacana* indica que esta especie puede ser un complejo poliploide maduro o en declive (Stebbins, 1971). Este tipo de complejos probablemente se originaron en el Plioceno-Pleistoceno, lo cual corresponde con la edad aproximada de origen del clado Basilares (Majure et al., 2012a).

El tamaño del genoma en *O. tehuacana* varía significativamente entre las cinco localidades muestreadas. Esto es relevante porque esta variación se relaciona con su poliploidía, la probable mezcla de nuevos citotipos, y por lo tanto con su evolución (Soltis and Soltis, 1999; Chen et al., 2007; Palomino et al., 2016). La variación del tamaño de genoma al interior de una especie es poco frecuente y puede estar relacionado con ADN satélite, elementos transponibles o a la presencia de diferentes genes ribosomales (Biémont, 2008; Leitch et al., 2008).

Los principales argumentos del estatus híbrido de *O. tehuacana* son los análisis de redes filogenéticas que evidencian la introgresión en algunos individuos de las localidades de

Ajalpan y Cuicatlán, proveniente probablemente de las especies *O. decumbens* and *O. huajuapensis*. Es importante resaltar que los conteos cromosómicos somáticos y meióticos para estas mismas localidades presentaron aneuploidías y univalentes, por lo cual se tienen dos fuentes de evidencia que indica flujo génico interespecífico en las mismas zonas de estudio.

Conclusiones generales

La hibridación es un proceso complicado, el cual puede originar nuevos linajes, pero aún para la ciencia moderna, es muy difícil de probar. Para las plantas es un proceso que ocurre frecuentemente, por lo que lejos de la percepción humana es un proceso natural y el cual aún tiene más preguntas que respuestas. Con el desarrollo de nuevas metodologías tanto citogenéticas como moleculares y sobre todo algorítmicas podemos tener un mejor entendimiento de este proceso. En Cactaceae probablemente la hibridación documentada juega un papel muy relevante en el número de especies, así como en la supervivencia de los linajes que podemos observar en la actualidad, ya que en algún momento este intercambio genético entre distintas especies les pudo haber permitido colonizar nuevos ambientes. En este contexto también es de suma relevancia la poliploidía en esta familia, la cual en algunos casos se relaciona con una acelerada evolución.

Es importante resaltar la congruencia entre distintas metodologías para la presencia de intercambio génico, tanto en los análisis de redes filogenéticas, así como citogenéticos, por lo que el uso tanto de técnicas modernas como tradicionales nos lleva a hipótesis más robustas, para poder probar la presencia de hibridación.

En Opuntioideae la reproducción vegetativa permite el establecimiento y dispersión de individuos híbridos, por lo que esta característica es una de las principales razones de que se presenten muchos más casos de hibridación (o que estos hayan sido mejor documentados), que en Cactoideae.

Finalmente, en cuanto a *Opuntia tehuacana*, se concluye que es una especie con ploidía de 11x y 12x, que presenta aneuploidías, las cuales pueden deberse a flujo génico interespecífico, se encuentra en el clado Basilares al igual que muchas de sus especies simpátricas en el Valle de Tehuacán-Cuicatlán y a pesar de ser un complejo poliploide maduro aún tiene poblaciones grandes. Para comprender la evolución de una especie tan compleja es necesario realizar estudios sobre barreras al intercambio genético, viabilidad de polen, así como cruzas controladas, para poder entender mejor el papel de la hibridación en una especie poliploide.

Referencias bibliográficas

Anderson, E. F. (2001). *The Cactus Family*. Portland, Oregon: Timber Press.

Arias, S., Gama, S., Vázquez, B., and Guzmán, L. U. (2012). Flora del Valle de Tehuacán-Cuicatlán. Fascículo 95, Cactaceae. *Instituto de Biología, Universidad Nacional Autónoma de México* 95.

Arnold, M. L. (1994). Natural Hybridization and Louisiana Irises. *BioScience* 44, 141–147. doi:10.2307/1312250.

Arnold, M. L. (1997). *Natural Hybridization and Evolution*. Nueva York: Oxford University Press.

Arnold, M. L. (2006). *Evolution Through Genetic Exchange*. Nueva York: Oxford University Press Available at: https://books.google.com.mx/books?id=R5QUDAAAQBAJ.

Baker, M. A., and Johnson, R. A. (2000). Morphometric Analysis *of Escobaria sneedii* var. *sneedii*, *E. sneedii* var. *leei*, and *E. guadalupensis* (Cactaceae). *Systematic Botany* 25, 577–587. doi:10.2307/2666722.

Baker, M. A., and Pinkava, D. J. (1987). A Cytological and Morphometric Analysis of a Triploid Apomict, *Opuntia* x *kelvinensis* (Subgenus Cylindropuntia, Cactaceae). *Brittonia* 39, 387–401. doi:10.2307/2807141.

Baker, M., and Pinkava, D. (2018). Chromosome Numbers in Some Cacti of Western North America — IX. *Haseltonia* 25, 2–29. doi:10.2985/026.025.0103.

Barke, B. H., Karbstein, K., Daubert, M., and Hörandl, E. (2020). The relation of meiotic behaviour to hybridity, polyploidy and apomixis in the *Ranunculus auricomus* complex (Ranunculaceae). *BMC Plant Biology* 20, 523. doi:10.1186/s12870-020-02654-3.

Barthlott, W., Burstedde, K., Geffert, J. L., Ibisch, P. L., Korotkova, N., and Miebach, A. (2015). *Biogeography and Biodiversity of Cacti*. Alemania: Schumannia.

Biémont, C. (2008). Within-species variation in genome size. *Heredity* 101, 297–8.

Bravo-Hollis, H. (1978). *Las cactáceas de México*. vol. 1. Universidad Nacional Autónoma de México Available at: https://books.google.com.mx/books?id=ii0mAQAAMAAJ.

Bravo-Hollis, H. (1991). vol. 3. *Las cactáceas de México*. Universidad Nacional Autónoma de México Available at: https://books.google.com.mx/books?id=c81gAAAAMAAJ.

Britton, N. L., and Rose, J. N. (1920). *The Cactaceae: descriptions and illustrations of plants of the cactus family*. Washington: Carnegie Institution of Washington, Available at: https://www.biodiversitylibrary.org/item/100134.

Bustamante, E., Búrquez, A., Scheinvar, E., and Eguiarte, L. E. (2016). Population Genetic Structure of a Widespread Bat-Pollinated Columnar Cactus. *PLOS ONE* 11, e0152329. doi:10.1371/journal.pone.0152329.

Calvente, A., Moraes, E., Lavor, P., Bonatelli, I., Nacaguma, P., Versieux, L., et al. (2016). Phylogenetic analyses of *Pilosocereus* (Cactaceae) inferred from plastid and nuclear sequences. *Botanical Journal of the Linnean Society*. doi:10.1111/boj.12491.

Chen, Z. J., Ha, M., and Soltis, D. (2007). Polyploidy: genome obesity and its consequences. *New Phytologist* 174, 717–720. doi:10.1111/j.1469-8137.2007.02084.x.

Copetti, D., Búrquez, A., Bustamante, E., Charboneau, J. L. M., Childs, K. L., Eguiarte, L. E., et al. (2017). Extensive gene tree discordance and hemiplasy shaped the genomes of North American columnar cacti. *PNAS* 114, 12003–12008. doi:10.1073/pnas.1706367114.

Cracraft, J. (1989). "Speciation and its ontology: the empirical consequences of alternative species concepts for understanding patterns and processes of differentiation," in *Speciation and its Consequences*, eds. D. Otte and J. A. Endler (Sinauer Associates), 29–59.

Elworth, R. A. L., Ogilvie, H. A., Zhu, J., and Nakhleh, L. (2018). Advances in computational methods for phylogenetic networks in the presence of hybridization. arXiv:1808.08662 [q-bio]. Available at: http://arxiv.org/abs/1808.08662.

Gontier, N. (2015). "Reticulate Evolution Everywhere," in *Reticulate Evolution*: *Symbiogenesis, Lateral Gene Transfer, Hybridization and Infectious Heredity* Interdisciplinary Evolution Research., ed. N. Gontier (Cham: Springer International Publishing), 1–40. doi:10.1007/978-3-319-16345-1_1.

Granados-Aguilar, X., Granados Mendoza, C., Cervantes, C. R., Montes, J. R., and Arias, S. (2021). Unraveling Reticulate Evolution in *Opuntia* (Cactaceae) From Southern Mexico. *Front. Plant Sci.* 11. doi:10.3389/fpls.2020.606809.

Griffith, M. P. (2001). Experimental hybridization of northern Chihuahuan desert region *Opuntia* (Cactaceae). *Aliso* 20, 37–42.

Griffith, M. P. (2003). Using molecular evidence to elucidate reticulate evolution in *Opuntia* (Cactaceae). *Madroño* 50, 162–169.

Griffith, P., y Porter, M. (2009). Phylogeny of Opuntioideae (Cactaceae). *International Journal of Plant Sciences* 170, 107–116.

Harrison, R. G. (1993). "Hybrid Zones: historical perspective," in *Hybrid Zones and the Evolutionary Process*, ed. R. G. Harrison (Oxford, New York: Oxford University Press), 378.

Harrison, R. G., and Larson, E. L. (2014). Hybridization, introgression, and the nature of species boundaries. *Journal of Heredity* 105, 795–809. doi:10.1093/jhered/esu033.

Henry, I. M., Dilkes, B. P., Tyagi, A. P., Lin, H.-Y., and Comai, L. (2009). Dosage and parent-of-origin effects shaping aneuploid swarms in *A. thaliana*. *Heredity* 103, 458–468. doi:10.1038/hdy.2009.81.

Hernández-Hernández, T., Hernández, H. M., De-Nova, J. A., Puente, R., Eguiarte, L. E., and Magallón, S. (2011). Phylogenetic relationships and evolution of growth form in Cactaceae (Caryophyllales, Eudicotyledoneae). *American Journal of Botany* 98, 44–61. doi:10.3732/ajb.1000129.

Hernández-Hernández, T., Brown, J. W., Schlumpberger, B. O., Eguiarte, L. E., and Magallón, S. (2014). Beyond aridification: multiple explanations for the elevated diversification of cacti in the New World succulent biome. *New Phytologist*. 202, 1382–1397. doi:10.1111/nph.12752.

Hunt, D. R., Taylor, N. P., and Charles, G. (2006). *The New Cactus Lexicon: Text*. Inglaterra: DH books Available at: https://books.google.com.mx/books?id=OHyZAQAACAAJ.

Leitch, I. J., Hanson, L., Lim, K. Y., Kovarik, A., Chase, M. W., Clarkson, J. J., et al. (2008). The ups and downs of genome size evolution in polyploid species of *Nicotiana* (Solanaceae). *Annals of Botany* 101, 805–814. doi:10.1093/aob/mcm326.

Linder, C., Moret, B., Nakhleh, L., and Warnow, T. (2004). Network (reticulate) evolution: biology, models, and algorithms. *The Pacific Symposium on Biocomputing*.

Machado, M. C. (2008). What is the role of hybridization in the evolution of the Cactaceae? *Bradleya*, 1–18. doi:10.25223/brad.n26.2008.a1.

Majure, L. C., Puente, R., Griffith, M. P., Judd, W. S., Soltis, P. S., and Soltis, D. E. (2012a). Phylogeny of *Opuntia s.s.* (Cactaceae): clade delineation, geographic origins, and reticulate evolution. *American Journal of Botany* 99, 847–864. doi:10.3732/ajb.1100375.

Majure, L. C., Puente, R., and Pinkava, D. J. (2012b). Miscellaneous chromosome numbers in Opuntieae Dc. (Cactaceae) with a Compilation of Counts for the Group. *Haseltonia*, 67–78. doi:10.2985/026.018.0109.

Mallet, J. (2007). Hybrid speciation. *Nature* 446, 279–283. doi:10.1038/nature05706.

Mandujano, M. del C., Carrillo-Angeles, I., Martínez-Peralta, C., and Golubov, J. (2010). "Reproductive Biology of Cactaceae," in *Desert Plants*: Biology and Biotechnology, ed. K. G. Ramawat (Berlin, Heidelberg: Springer), 197–230. doi:10.1007/978-3-642-02550-1_10.

Mayr, E. (1942). *Systematics and the Origin of Species, from the Viewpoint of a Zoologist*. Harvard University Press Available at: https://books.google.com.mx/books?id=mAIjnLp6r_MC.

Mestiri, I., Chagué, V., Tanguy, A.-M., Huneau, C., Huteau, V., Belcram, H., et al. (2010). Newly synthesized wheat allohexaploids display progenitor-dependent meiotic stability and aneuploidy but structural genomic additivity. *New Phytologist* 186, 86–101. doi:10.1111/j.1469-8137.2010.03186.x.

Montanucci, R. R. (2015). Experimental evidence for reproductive isolation between *Astrophytum coahuilense* (H. Möller) Kayser and *A. capricorne* var. *senile* (Fric) Okumura (Cactaceae). *Haseltonia*, 13–21. doi:10.2985/026.020.0104.

Palomino, G., Martínez, J., Méndez, I., Muñoz-Urías, A., Cepeda-Cornejo, V., and Pimienta-Barrios, E. (2016). Nuclear genome size, ploidy level and endopolyploidy pattern in six species of *Opuntia* (Cactaceae). *Caryologia* 69, 82–89. doi:10.1080/00087114.2015.1109956.

Pinkava, D. J. (2002). “On the evolution of the North American Opuntioideae,” in *Studies in the Opuntioideae (Cactaceae)* Succulent Plant Research. (DH Books), 59–98. Available at: https://books.google.com.mx/books?id=lTAmAQAAMAAJ.

Reyes-Agüero, J. A., Aguirre R., J. R., and Valiente-Banuet, A. (2006). Reproductive biology of *Opuntia*: A review. *Journal of Arid Environments* 64, 549–585. doi:10.1016/j.jaridenv.2005.06.018.

Rieseberg, L. H. (1991). Homoploid reticulate evolution in *Helianthus* (Asteraceae): Evidence from ribosomal genes. *American Journal of Botany* 78, 1218–1237. doi:10.2307/2444926.

Rieseberg, L. H., Ellstrand, N. C., and Arnold, D. M. (1993). What can molecular and morphological markers tell us about plant hybridization? *Critical Reviews in Plant Sciences* 12, 213–241. doi:10.1080/07352689309701902.

Scheinvar, L., Olalde-Parra, G., and Gallegos-Vázquez, C. (2015). Una nueva especie del género *Opuntia* (Cactaceae) para el estado de Veracruz, México. *Botanical Sciences* 93, 33–39.

Segura, S., Scheinvar, L., Olalde, G., Leblanc, O., Filardo, S., Muratalla, A., et al. (2007). Genome sizes and ploidy levels in Mexican cactus pear species *Opuntia* (Tourn.) Mill. series Streptacanthae Britton et Rose, Leucotrichae DC., Heliabravoanae Scheinvar and Robustae Britton et Rose. *Genetic Resources and Crop Evolution* 54, 1033–1041.

Soltis, D. E., y Soltis, P. S. (1999). Polyploidy: recurrent formation and genome evolution. *Trends in Ecology & Evolution* 14, 348–352. doi:10.1016/S0169-5347(99)01638-9.

Soltis, P. S. (2013). “Hybridization in Plants,” in *Encyclopedia of Biodiversity* (Second Edition), ed. S. A. Levin (Waltham: Academic Press), 166–176. doi:10.1016/B978-0-12-384719-5.00202-1.

Stebbins, G. L. (1971). *Chromosomal evolution in higher plants*. 1st ed. Londres, UK: Edward Arnold Publishers Available at: http://wellcomelibrary.org/item/b18036594 [Accessed July 13, 2019].

Tel-Zur, N., Abbo, S., Bar-Ziv, D., and Mizrahi, Y. (2004). Genetic relationships among *Hylocereus* and *Selenicereus* vine cacti (Cactaceae): Evidence from hybridization and cytological studies. *Annals of Botany* 94, 527–534. doi:10.1093/aob/mch183.

Whitney, K. D., Ahern, J. R., Campbell, L. G., Albert, L. P., and King, M. S. (2010). Patterns of hybridization in plants. *Perspectives in Plant Ecology, Evolution and Systematics* 12, 175–182. doi:10.1016/j.ppees.2010.02.002.

Zappi, D. C. (1994). *Pilosocereus (Cactaceae)*: The genus in Brazil. D. Hunt.