Evolução e Sistemática de Cactos-Palma nas Américas:

do Campo à Genômica

Matias Köhler

EVOLUÇÃO E SISTEMÁTICA DE CACTOS-PALMA NAS AMÉRICAS:

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PORTO ALEGRE, RS

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TESE APRESENTADA AO PROGRAMA DE PÓS-GRADUAÇÃO EM BOTÂNICA DA UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL COMO REQUISITO PARCIAL À OBTENÇÃO DO GRAU DE DOUTOR EM CIÊNCIAS: BOTÂNICA.

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MATIAS KÖHLER

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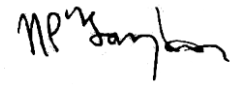
O presente trabalho, em nível de doutoramento, foi avaliado e aprovado por banca examinadora composta pelos seguintes membros:



Prof. Dr. Nigel Taylor, Gibraltar Botanic Gardens, University of Gibraltar.

Profa. Dra. Alice Calvente, Universidade Federal do Rio Grande do Norte.

Prof. Dr. Evandro Marsola de Moraes, Universidade Federal de São Carlos.

Certificamos que esta é a **versão original e final** do trabalho de conclusão que foi julgado aprovado, com méritos de louvor, para obtenção do título de doutor em Ciências: Botânica.

Autor da tese, Matias Köhler

Supervisora, Prof. Dra. Tatiana T. Souza-Chies

Supervisor, Prof. Dr. Lucas C. Majure

Dedico:

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"Nem todos preferem as rosas, nem todos desistem dos cactos."

Fabíola Simões

RESUMO

Plantas são fundamentais na natureza – e para a vida humana, fornecendo alimentos, remédios, fibras e vários outros recursos –, de modo que é imprescindível expandir nossos conhecimentos sobre a biodiversidade vegetal para seu uso sustentável. Cactos formam um dos grupos de plantas com flores mais fascinantes do mundo. Praticamente endêmicos das Américas, são conspícuos elementos das principais paisagens áridas, semiáridas, subúmidas e até tropicais do continente, exibindo uma alta diversidade de espécies, acompanhada por peculiaridades morfológicas, ecológicas e fisiológicas que despertam atenção. Dentre essa diversidade, os cactos-palma (*Opuntia* spp.) se destacam não só pela importância econômica e cultural, mas também por serem um dos mais ricos em número de espécies, e amplamente distribuídos ao longo das Américas. Visando preencher lacunas de conhecimento sobre os cactos-palma, especialmente no sul da América do Sul, integramos expedições de campo, coleções e pesquisas de herbário, observações morfológicas, dados moleculares e citogenéticos para investigar aspectos da sistemática e evolução do grupo. Nossos resultados revelaram-se extremamente importantes para contribuir com o conhecimento do grupo. Reportamos duas novas ocorrências para a flora do Brasil (*Opuntia rioplatensis* e *O. bonaerensis*) e uma para a do Uruguai (*O. bonaerensis*), além de reavaliar e tipificar uma espécie endêmica do Uruguai (*O. canterae*). Combinando dados morfológicos, moleculares e de modelagem de nicho ecológico, sugerimos que eventos de mudanças climática do Pleistoceno impactaram a distribuição de *O. bonaerensis* no sul da América do Sul; e revelamos que *O. penicilligera*, considerada endêmica da Argentina, é, provavelmente, derivada de espécies da América do Norte; e discutimos as afinidades morfológicas de *O. ventanensis* com *O. fragilis*, que também pode estar associada a uma origem Norte Americana. Nossos dados moleculares revelaram de maneira inédita a estrutura e o conteúdo do genoma plastidial (plastoma) de *Opuntia quimilo*, com importantes novidades acerca da evolução do plastoma em Cactaceae e na subfamília Opuntioideae. Baseado em dados do plastoma, circunscrevemos de maneira robusta Opuntioideae em três tribos (Opuntieae, Tephrocacteae e Cylindropuntieae) e exploramos marcadores úteis para futuros estudos filogenéticos no grupo. Nossos dados moleculares também contribuíram para identificar uma nova linhagem de vírus de DNA de fita simples infectando cactos, especialmente *Opuntia.* Análises filogenéticas moleculares e macroevolutivas demonstraram que *Opuntia* representa uma rápida e recente radiação evolutiva, que vem diversificando-se nos últimos 3 milhões de anos, com eventos de dispersão por longa-distância influenciando a distribuição atual do grupo, e com a homoplasia de vários caracteres morfológicos. A integração dos nossos dados de campo com moleculares também sustentou novidades nomenclaturais, combinando *O. schickendantzii* em *Salmonopuntia*, e revelando *O. leoglossa* como uma nova espécie para acomodar um táxon historicamente mal identificado. Combinando observações morfológicas, análises citogenéticas e inferências filogenéticas, sugerimos que hibridação é um processo que continua gerando diversidade em *Opuntia*, ao descrever um putativo novo híbrido que tem como possíveis parentais uma espécie do sul da América do Sul e uma espécie Norte Americana introduzida na América do Sul. Nossos estudos impulsionam um novo paradigma para o conhecimento dos cactos-palma nas Américas, ao integrar as mais diversas ferramentas para o estudo da biodiversidade, que deve contribuir para uma futura e necessária monografia do grupo.

Palavras-chave: Arumbeva. Botânica. Cactaceae. *Opuntia.* Taxonomia. Tuna.

ABSTRACT

Plants are a keystone for nature – including for human life by providing food, medicine, fibers as well as many other resources –, so that it is essential to expand our knowledge about plant biodiversity for sustainable use. Cacti are one of the most fascinating groups of flowering plants. Mostly endemic of the Americas, they are conspicuous elements of major arid, semiarid, subhumid, and even tropical landscapes of the continent, exhibiting a high level of species diversity accompanied by morphological, physiological, and ecological peculiarities that have called attention. Among that diversity, the prickly-pears cacti (*Opuntia* spp.) stands out not only for their economic and cultural importance but also for being one of the most species-rich groups and widely distributed across the Americas. Aiming to contribute to our knowledge about the prickly-pears cacti, especially in southern South America, we have integrated fieldwork, collection-based research, morphological, molecular, and cytogenetics data to investigate aspects of the systematics and evolution of the group. Our study revealed to be extremely important to contribute with the knowledge regarding the prickly-pears diversity. We reported two new records for the Brazilian flora (*Opuntia rioplatensis* and *O. bonaerensis*) and for the Uruguayan (*O. bonaerensis*), in addition to the reassessment and typification of an endemic species of Uruguay (*O. canterae*). By combining morphological, molecular data and ecological niche modelling, we suggested that Pleistocene climatic events have impacted the distribution of *O. bonaerensis* in southern South America; and we showed that *O. penicilligera*, considered endemic of Argentina, is putatively derived from North American species; and we discussed morphological affinities between *O. ventanensis* and *O. fragilis*, which can also be associated with a North American origin. Our molecular data revealed for the first time the content and structure of the chloroplast genome (plastome) of an *Opuntia*, *O. quimilo,* with important insights into plastome evolution across Cactaceae and Opuntioideae. Based on plastome data, we robustly circumscribe Opuntioideae in three tribes (Opuntieae, Tephrocacteae, and Cylindropuntieae), and explored useful molecular markers for future phylogenetic studies in the group. Our molecular data also contributed to identify novel divergent lineages of single-stranded DNA virus infecting cacti, especially *Opuntia*. Molecular phylogenetics inferences and macroevolutionary analyses showed that *Opuntia* represents a rapid and recent evolutionary radiation, which are diversifying in the last 3 million years, with long-distance dispersal shaping the current distribution of the group, and the evolution of homoplasious morphological characters underlying their diversification. The integration of our field and molecular data also supported nomenclatural novelties, combining *O. schickendantzii* in *Salmonopuntia,* and unraveling *O. leoglossa* as a new species to accommodate a historically misidentified taxon. By combining morphological observations with cytogenetic analyses and molecular phylogenetic inferences, we suggested that hybridization is a process that still produces diversity within *Opuntia*, describing a putative new hybrid that has as likely parentals a southern South American species and a North American species introduced in South America. Our studies promote a new paradigm for the knowledge of prickly pear cacti in the Americas, by integrating diverse approaches for the biodiversity study, which should contribute to a future and needed botanical monograph of the group.

Keywords: Botany. Cactaceae. Opuntia. Prickly-pear cactus. Taxonomy. Cacti.

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APRESENTAÇÃO

Essa tese reúne contribuições científicas desenvolvidas durante o meu doutoramento, tangendo a temática "Evolução e sistemática de cactos-palma nas Américas". Longe de ser uma síntese da temática, se aproxima mais de um *pot-pourri,* combinando fragmentos de um estudo maior que segue em desenvolvimento.

A motivação desse trabalho nasce durante minha graduação em Ciências Biológicas (2010-2014), e amadurece durante meu mestrado em Botânica (2015-2016). O contato com a natureza e o interesse pela flora vem de antes – talvez com meu pai, acompanhando jardinagens, pescarias, trilhas e passeios.

Desde antes da minha graduação, tive contato e experiências com o Viveirismo, a Agroecologia, o Movimento Ambientalista e a Educação Ambiental. Permeado nisso, fui orientado pelo ilustre Prof. Dr. Paulo Brack, e tive contato com as Plantas Alimentícias Não- Convencionais (PANC) e a Agrobiodiversidade. Com Brack e suas referências, aprofundei-me no conhecimento da flora local e nativa, e me vi deslumbrado com sua diversidade, riqueza e potencial.

Naquela época, ao colaborar na identificação da flora nativa com frutos alimentícios – projeto em parte resultante nas frutas nativas do Rio Grande do Sul[1] –, uma carência de conhecimento acerca das espécies de cactos-palma (*Opuntia* spp.) do estado foi percebida. A partir de então, aos poucos, explorando regiões, deparando-me com a diversidade, com questionamentos que meus mestres e tutores não confiavam respostas, ou aportavam informações controversas, fui percebendo a importância e a necessidade de produzir conhecimento acerca desses cactos.

Esse é o vir a ser do meu trabalho, e foi a motivação para a realização desse doutorado. Ainda que com metas e objetivos bem estabelecidos, o percurso foi se construindo ao longo do caminhar. Ao me deparar com espinhos e gloquídios – isto é, as mais diversas complexidades taxonômicas e práticas em diferentes esferas do trabalho –, várias metas foram se adaptando ou readequando. Findo parte dessas metas com essa tese, tendo cumprido parte do que me propus. Mas com a certeza de que isso não é um fim, e de que ainda há muito mais a ser feito.

[1] Brack, P., Köhler, M., Corrêa, C.A., Ardissone, R.E., Sobral, M.E.G. & Kinupp, V.F. (2020). Frutas nativas do Rio Grande do Sul, Brasil: riqueza e potencial alimentício. *Rodriguésia*, 71, e03102018. https://doi.org/10.1590/2175-7860202071091

"[...] Por eso debes tener siempre presente que un camino es sólo un camino; si sientes que no deberías seguirlo, no debes seguir en él bajo ninguna condición. [...] Sólo entonces sabrás que un camino es nada más un camino, y no hay afrenta, ni para ti ni para otros, en dejarlo, si eso es lo que tu corazón te dice. Pero tu decisión de seguir en el camino o de dejarlo debe estar libre de miedo y de ambición.

[...] Antes de embarcarte en cualquier camino tienes que hacer la pregunta: ¿tiene corazón este camino? Si la respuesta es no, tú mismo lo sabrás, y deberás entonces escoger otro camino. [...] ¿Tiene corazón este camino? Si tiene, el camino es bueno; si no, de nada sirve. [...] Uno hace gozoso el viaje; mientras lo sigas, eres uno con él. El otro te hará maldecir tu vida. Uno te hace fuerte; el otro te debilita.

[...] El problema es que nadie hace la pregunta, y cuando uno por fin se da cuenta de que ha tomado un camino sin corazón, el camino está ya a punto de matarlo. [...] Un camino sin corazón nunca es disfrutable. Hay que trabajar duro tan sólo para tomarlo. En cambio, un camino con corazón es fácil: no te hace trabajar por tomarle gusto.

[…] Para mí sólo recorrer los caminos que tienen corazón, cualquier camino que tenga corazón. Esos recorro, y la única prueba que vale es atravesar todo su largo. Y esos recorro mirando, mirando, sin aliento."

– Don Juan (1968), em "*The Teachings of Don Juan: A Yaqui way of knowledge*", "*Las enseñanzas de Don Juan*", ou "A erva do Diabo", de Carlos Castañeda.

INTRODUÇÃO

As plantas são parte fundamental da natureza, e essenciais para a vida na Terra. Produtoras primárias, transformam – com a energia solar – moléculas simples em complexas, tornando-se a base das cadeias tróficas (Corner, 2002). Do ponto de vista humano, fornecem alimento, abrigo, fibras, corantes, remédios, madeira, dentre tantas outras utilidades (Lewis, 1996). Contudo, para esses fins, é necessário conhecer e estudar a biodiversidade.

Estima-se a existência de ~500,000 espécies vegetais no planeta (Viridiplantae, *Viridophytes* – Soltis et al., 2018a), habitando os mais distintos ambientes e exibindo as mais diversas morfologias. De longe, as plantas com flores (Angiospermas, *Angiospermae* – Cantino et al., 2007) são as mais diversas atualmente, representando cerca de 80% dessa diversidade, com 350,000 (The Plant List, 2010) a 400,000 espécies (Govaerts, 2001).

Cactos compreendem um dos grupos de plantas com flores mais fascinantes do mundo. Possuindo variadas formas e tamanhos de crescimento, flores vistosas, suculência, partes comestíveis, substâncias farmacológicas, peculiares associações ecológicas, e uma ampla capacidade de tolerar ambientes adversos pelo calor e pelo estresse hídrico, despertam atenção e curiosidade de cientistas, colecionadores, horticultores e de um variado público não-especializado (Anderson, 2001; Nobel, 2002).

Atualmente, os cactos têm recebido destaque por práticas de *merchandising*, sendo associados aos mais diversos objetos de consumo – canecas, camisetas, almofadas, postais, utensílios domésticos etc. Essa prática, por um lado carismática e de valoração das plantas no cotidiano da vida humana, também deve ser analisada com atenção, pois pode provocar pressão sobre o extrativismo ilegal de cactos para comercialização – prática que, associada à perda e destruição de habitats, tem colocado o grupo como um dos mais ameaçados de extinção no mundo (Goettsch et al., 2015).

A família dos cactos (Cactaceae) compreende cerca de 1,700 espécies reconhecidas em 144 gêneros (Korotkova et al., 2021), com estimativas variando entre 1,400–1,800 espécies (Hunt et al., 2006; Nyffeler & Eggli, 2010). A família faz parte da ordem Caryophyllales, uma importante linhagem de Angiospermas (~13,000 espécies), peculiar por conter plantas com diversas características morfológicas, anatômicas e bioquímicas especializadas à hábitats extremos

(Hernández-Ledesma et al., 2015). Porém, cactos são únicos pelo desenvolvimento de aréolas, i.e., pequenas estruturas meristemáticas reduzidas, concentradas e altamente modificadas, das quais diversas estruturas secundárias podem se originar, como botões florais, ramos, folhas, espinhos e gloquídios (Mauseth, 2006).

Cactos são naturalmente endêmicos das Américas (Anderson, 2001), com exceção de uma espécie epífita – *Rhipsalis baccifera,* registrada na África Tropical (provavelmente introdução recente, Cota-Sánchez & Bomfim-Patrício, 2010). São elementos conspícuos das paisagens áridas e semiáridas do continente – desde o sul da Argentina até o Canadá, passando pelos arquipélagos caribenhos e pela América Central – mas também habitam florestas tropicais úmidas, especialmente como epífitas (Anderson, 2001; Taylor & Zappi, 2004). Associado aos diferentes ambientes em que ocorrem, a família também apresenta altos graus de endemismo ao longo de seus principais centros de diversidade (Desertos da América do Norte, Região Centro-Sul dos Andes, e o Nordeste/Leste do Brasil, incluindo os biomas Caatinga e Mata Atlântica), mas fora deles também (Ortega-Baes & Godínez-Alvarez, 2006; Mutke et al., 2015).

A ausência de fósseis dificulta o conhecimento acerca do passado e da origem de Cactaceae. Estimativas baseadas em divergências moleculares e fósseis de grupos externos sugerem que a linhagem dos cactos divergiu de suas linhagens-irmãs (Anacampserotaceae + Portulacaceae, Walker et al., 2018; Wang et al., 2019) cerca de 35–32 milhões de anos atrás (Mya) (Arakaki et al., 2011; Hernández-Hernández et al., 2014). Porém, a diversificação do grupo parece ter sido mais recente, intensificando-se no fim do Mioceno (~10–5 Mya), provavelmente associada à aridificação e à redução de CO_2 atmosférico, quando os principais e mais diversos grupos de cactos surgiram (Arakaki et al., 2011).

Cactaceae é representada por cinco principais linhagens evolutivas: *Leuenbergeria*, *Pereskia*, Opuntioideae, Maihuenioideae e Cactoideae (Guerrero et al., 2019), sendo as três últimas circunscritas como subfamílias. *Leunbergeria* e *Pereskia* (~18 espécies), previamente circunscritas como Pereskioideae, formam um grado na base da filogenia dos cactos (Edwards et al., 2005; Butterworth & Edwards, 2008; Walker et al., 2018) e são reconhecidas por exibir caracteres morfológicos plesiomórficos, como folhas perenes, fotossintetizantes, e caules usualmente não-fotossintetizantes. Parte desses caracteres, como folhas perenes fotossintetizantes, também são observados em Maihuenioideae (~2 espécies), uma peculiar linhagem de cactos endêmicos da região da Patagônia Argentina e Chilena. Contrariamente, Cactoideae é identificada pela completa ausência de folhas (com raras exceções) e uma diversidade morfológica de caules suculentos

fotossintetizantes – desde colunares até globulares –, sendo o grupo mais diverso dentro da família (Nyffeler & Eggli, 2010; Hernández-Hernández et al., 2011).

Opuntioideae compreende os cactos que produzem gloquídios (i.e., pequenos tufos de espinhos retrorsos – caducos ou facilmente destacáveis – presentes nas aréolas), e possuem as sementes cobertas por um arilo denso, ósseo, proveniente de um funículo originado de um óvulo campilótropo (Stuppy, 2002). Os membros desse grupo estão distribuídos ao longo de quase todas áreas das Américas (desertos, campos/savanas, florestas e regiões temperadas), do nível do mar aos mais de 4,500 m de altitude nos Andes Peruanos (Anderson, 2001).

Dentro de Opuntioideae, a tribo Opuntieae é a mais diversa (~230 espécies), englobando os cactos com caule fotossintetizante achatado, i.e., cladódios – salvo raras exceções (Griffith & Porter, 2009). Atualmente, sete gêneros são reconhecidos na tribo Opuntieae, mas isso não foi sempre assim – sendo *Opuntia* s.l. um gênero "guarda-chuva", ao qual a maioria dos táxons da subfamília Opuntioideae já foram associados (Schumann, 1899; Britton & Rose, 1919). Porém, avanços na sistemática molecular demonstraram que *Opuntia* s.l. é polifilético (Wallace & Dickie, 2002; Griffith & Porter, 2009), incluindo membros de outras tribos, o que fez com que diferentes gêneros fossem segregados com base em caracteres morfológicos e clados bem sustentados estatisticamente (Stuppy, 2002; Taylor et al., 2002; Majure et al., 2012b; Majure et al., 2013; Majure & Puente, 2014).

Notoriamente, cactos da tribo Opuntieae apresentam uma desafiadora taxonomia associada à (1) variabilidade morfológica das espécies, onde muitas vezes os fenótipos podem estar associados à variáveis ambientais; (2) ausência ou escassa representação de materiais informativos conservados em herbários e coleções científicas; (3) limitados caracteres morfológicos tradicionalmente utilizados para delimitação de táxons; (4) frequente hibridação e introgressão entre espécies simpátricas e alopátricas; e (5) ausência de dados biológicos provenientes da completa e ampla distribuição dos táxons (Britton & Rose, 1919; Anderson, 2001; Pinkava, 2002; Majure & Puente, 2014).

Opuntia é o gênero de Cactaceae mais importante economicamente (Nobel, 2002; Ranjan et al., 2016; Inglese et al., 2017), e talvez um dos mais icônicos e emblemáticos dos cactos. Possui distinta relevância cultural e ecológica – especialmente associado aos povos originários e nativos das Américas, como no México, onde está diariamente no prato de comida da população, e é ilustrado na bandeira do país (Bravo-Hollis, 1937; Anderson, 2001; Griffith, 2004; Chávez-Moreno et al., 2009; Inglese et al., 2017). Vários estudos reportam as características nutracêuticas e medicinais especiais dos frutos e cladódios de diferentes espécies de *Opuntia*, além de finalidades

cosméticas e biotecnológicas (Feugang, 2006; Shedbalkar et al., 2010; Ranjan et al., 2016). Devido à característica quase intrínseca das espécies de *Opuntia* crescerem em ambientes com baixa disponibilidade hídrica e fertilidade do solo, seu cultivo tem sido uma importante produção agrícola em regiões desérticas, áridas, semiáridas ou com baixa fertilidade (Russel & Felker, 1987; Pimienta-Barrios et al., 1995; Inglese et al., 2017), e tem recebido cada vez mais importância em cenários atuais de mudanças climáticas e aridificação do clima (Ben-Gal et al., 2006; Nefzaoui, 2009).

Popularmente, membros do gênero *Opuntia* são conhecidos como tuna, arumbeva, urumbeva, palma, cacto-palma, *nopal* (em Espanhol, originalmente da língua asteca *Nahuatl,* fazendo referência à palma, raquete, cladódio), *prickly-pear cactus* (em Inglês, "cacto de pera espinhosa", tradução livre). Além desses, existem muitos outros nomes mais singulares associados a determinadas espécies, e de conhecimento geograficamente limitado a determinadas populações e/ou comunidades tradicionais (e.g., *utkilio, cintos, themas, sainimbé, ninpuomowasu* etc.).

Cactos-palma ocorrem do sul da América do Sul até o norte da América do Norte, especialmente ao longo das regiões áridas, semiáridas e até subúmidas do continente. Aparte de obras clássicas que tratam da diversidade geral de cactos (Schumann, 1989; Britton & Rose, 1919; Backeberg, 1958, 1966; Anderson, 2001; Hunt et al., 2006) – estimando de 85 a 180 espécies –, o conhecimento acerca da diversidade e da biologia das espécies de *Opuntia* é díspar ao longo de sua distribuição.

Marcadamente, as espécies de cacto-palma da América do Norte são historicamente mais estudadas e catalogadas (Engelmann, 1856; Britton & Rose, 1908; Bravo-Hollis, 1937; Pinkava & McLeod, 1971; Benson, 1982; Hernández et al., 2001; Rebman & Pinkava, 2001; Pinkava, 2002; Pinkava, 2003). Enquanto na América do Sul os estudos focados na diversidade de cactos-palma são poucos (Arechavaleta, 1905; Spegazzini, 1905; Ritter, 1979, 1980) – sendo a diversidade aparentemente negligenciada, e tratamentos taxonômicos, em geral, pontuais, com foco na Argentina (Kiesling, 1999, 2005; Leuenberger, 2002; Font, 2014).

Recentemente, contribuições têm sido feitas para expandir o conhecimento acerca dos cactos-palma, combinando dados de morfologia e taxonomia clássica, com citogenética e sistemática molecular, com especial foco nas espécies da América do Norte (Majure et al., 2012a; Majure et al., 2012b; Majure et al., 2014; Majure et al., 2017).

A partir da lacuna de conhecimento acessível observada sobre os cactos-palma no sul do Brasil, objetivou-se, nessa tese, direcionar esforços para obtenção de dados acerca do grupo nessa

região. Porém, tendo em vista que as plantas nem sempre respeitam limites cartográficos ou fronteiras políticas, o objeto de estudo foi expandido para incluir a área de distribuição dos cactos-palma que ocorrem no sul da América do Sul, circunscritas por Majure et al. (2012b) como pertencentes ao clado Elatae.

Para esse fim, duas principais estratégias foram exploradas: o campo e a genômica. Para ambas estratégias, colaborações primordiais com diferentes pesquisadores de cactos-palma foram estabelecidas, a saber: Fabián Font (*Herbario Museo de Farmacobotánica "Juan A. Domínguez" (BAF), Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires*, Buenos Aires, Argentina), Luis J. Oakley (*Cátedra de Botánica, Facultad de Ciencias Agrarias, Universidad Nacional de Rosario,* Zavalla, Argentina), Raúl Puente (*Desert Botanical Garden,* Phoenix, AZ, USA) e Lucas C. Majure (*University of Florida Herbarium (FLAS), Florida Museum of Natural History,* Gainesville, FL, USA).

Por meio de expedições de campo, uma ampla área do centro-sul e leste da América do Sul (incluindo áreas do Brasil, Uruguai, Argentina, Paraguai e Bolívia) foi amostrada e explorada para obtenção de dados acerca de populações de cactos-palma e relativos (*Opuntia* s.l.). Dados de distribuição, morfologia e ecologia dos táxons foram obtidos por meio de observações e anotações de campo, fotografias e estudo de materiais armazenados em herbários visitados ao longo da região. Coletas, quando possível, também foram realizadas para depósito de testemunho em herbários e coleções científicas. Além disso, amostras de interesse, quando possível, foram mantidas em cultivo para facilitar posteriores estudos morfológicos, citogenéticos, biológicos e moleculares.

Uma parte desse material amostrado e estudado foi selecionado para obtenção de dados moleculares genéticos. Para isso, amostras de raízes ou epiderme dos cladódios foram armazenadas em sílica-gel, e posteriormente tiveram DNA total extraído. As amostras de DNA foram sequenciadas utilizando a plataforma Illumina HiSeq X aplicando bibliotecas de sequenciamento de genoma com baixa cobertura, *genome skimming* (Straub et al., 2012), na empresa *Rapid Genomics LLC* (Gainesville, FL, USA - http://rapid-genomics.com/home/).

A seguir, são apresentados resultados parciais obtidos a partir da execução das estratégias acima mencionadas, durante o período dos anos 2017-2020. As contribuições envolvem o conhecimento sobre a evolução e sistemática dos cactos-palma nas Américas – desde aspectos taxonômicos e florísticos locais, até hipóteses e novidades sistemáticas, macroevolutivas e genômicas –, e estão disponíveis em formato de artigos científicos. Outras contribuições estão em processo de construção, e serão publicadas à parte dessa tese.

ESTRUTURA DA TESE

Para fins de apresentação, a tese está dividida em três partes, com cada parte contendo capítulos em formato de artigos científicos. A divisão em partes é representativa – tentando demonstrar as diferentes esferas em que o trabalho foi desenvolvido: do campo à genômica.

Por campo, refere-se à obtenção de dados baseadas em expedições de estudo e/ou coleta para observação das plantas em seu habitat – buscando-se uma compreensão da diversidade morfológica, ecológica, florística e taxonômica do grupo ao longo de sua distribuição. Por genômica, refere-se à obtenção de dados resultantes da extração e sequenciamento de DNA de amostras de plantas, fazendo o uso dessa informação para revelar aspectos evolutivos e moleculares do grupo estudado.

É claro que essa distinção entre campo e genômica muitas vezes não existe – e esse trabalho buscou a integração dessas duas esferas. Desse modo, eventualmente o leitor ou a leitora poderá se questionar em algum momento porque tal artigo está em determinada parte. Por isso, ressalto que essa divisão é para fins de apresentação e valorização da integração das diferentes esferas em que o trabalho foi realizado.

Na Parte I (Campo – Florística e Taxonomia) são apresentados três capítulos. Na Parte II (Genômica – Plastomas e Vírus) são apresentados dois capítulos. Na Parte III (Do Campo à Genômica – Sistemática, Evolução e Diversificação) são apresentados cinco capítulos, totalizando 10 capítulos na tese.

Por fim, são apresentadas conclusões e perspectivas a partir do estudo apresentado.

"Hay cosas que no se compran en la botica de la esquina. Hay que hacer la enorme y costosa diligencia de adquirirlas con el espíritu, y eso cuesta"

Atahualpa Yupanqui,
que embalou *coplas* em viagens de campo, estudos, herbários,
revisões, escritas, mates, e tanto mais.

PARTE I

CAMPO – FLORÍSTICA E TAXONOMIA

Expedições de campo – juntamente com a revisão de herbários e de literatura – são fundamentais para o conhecimento da biodiversidade. Permitem a observação das espécies na natureza, revelando dados e características morfológicas e ecológicas, além de sua ocorrência. Nos próximos capítulos: (1) reportamos o primeiro registro de *Opuntia rioplatense*, para a flora do Brasil; (2) reavaliamos o conhecimento acerca de *Opuntia canterae*; (3) e discutimos acerca da identidade de dois táxons considerados endêmicos da Argentina – *Opuntia penicilligera* e *O. ventanensis*.

FIRST RECORD OF *Opuntia rioplatense* (CACTACEAE) FOR THE BRAZILIAN FLORA

Esse artigo está publicado de forma editorada e diagramada no periódico *Phytotaxa* 379 (4): 293-296 (2018), e pode ser acessado no endereço: https://doi.org/10.11646/phytotaxa.379.4.3, o qual deve-se referir para citação. É aqui reproduzido com permissão e autorização dos autores, e da editora *Magnolia Press*.

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First record of *Opuntia rioplatense* (Cactaceae) for the Brazilian Flora

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The genus *Opuntia* Miller (1754) (Cactaceae Juss., Opuntioideae K.Schum., Opuntieae DC.) is native to the Americas, where is distributed from southern Argentina to Canada (Anderson 2001), and represents one of the largest genus of Cactaceae with ca. 150 species (see e.g., Stuppy 2002). Members of *Opuntia* shares a combination of peculiar morphological traits, including shrubs or trees with flattened photosynthetic stem shoots (cladodes), areoles with large smooth or retrorsely barbed spines and glochids, reduced and small caducous leaves , rotaceous diurnal flowers with inferior ovaries inner the pericarpels and stigma plurilobed, stamens with thigmonasty, reticulate semitectate pollen, and seeds covered by a sclerified funicular aril (Buxbaum 1953, Anderson 2001, Stuppy 2002, Hunt 2006, Majure & Puente 2014, Majure et al. 2017).

Species delimitation within *Opuntia* is not a simple issue especially concerning the high morphological plasticity (often related to environmental factors), a common hybridization, the poor conservation of collected specimens, the few data about biological information (morphology, chromosome counts, etc.) and phylogeny (see e.g., Majure & Puente 2014). These issues are especially relevant for the species occurring in southern parts of South America (sSA), that have been historically less studied. However, recently studies for the sSA species have brought advances in the understanding of their distribution and circumscription (Leuenberger 2002, Font 2014, Las Peñas et al. 2017).

As part of the taxonomic and floristic studies of *Opuntia* in sSA, we found a new record for the Brazilian flora [*Opuntia rioplatense* Font (2014: 85)] based on fieldworks carried out in the west of the state of Rio Grande do Sul. The collected material was deposited at the Federal University of Rio Grande do Sul herbarium, "Instituto de Ciências Naturais" (ICN) (acronym according to Thiers 2018).

Opuntia rioplatense Font (2014: 85) (Fig. 1) nom. nov. pro *Opuntia elata* Salm-Dyck (1834: 361) var. *obovata* Walther (1930: 204) non *O. obovata* Griffiths (1919: 202).

Lectotype (designated by Crook & Mottram 1996: 140):—[Icon] Figure 1 in Walther (1930: 203).

Epitype (designated by Las Peñas et al. 2017: 113):—ARGENTINA. Santa Fe, Rosario, Zavalla, 33º01'27.89"S, 60º54'07.69"W, 10 November 2012, Galetti s.n. (UNR).

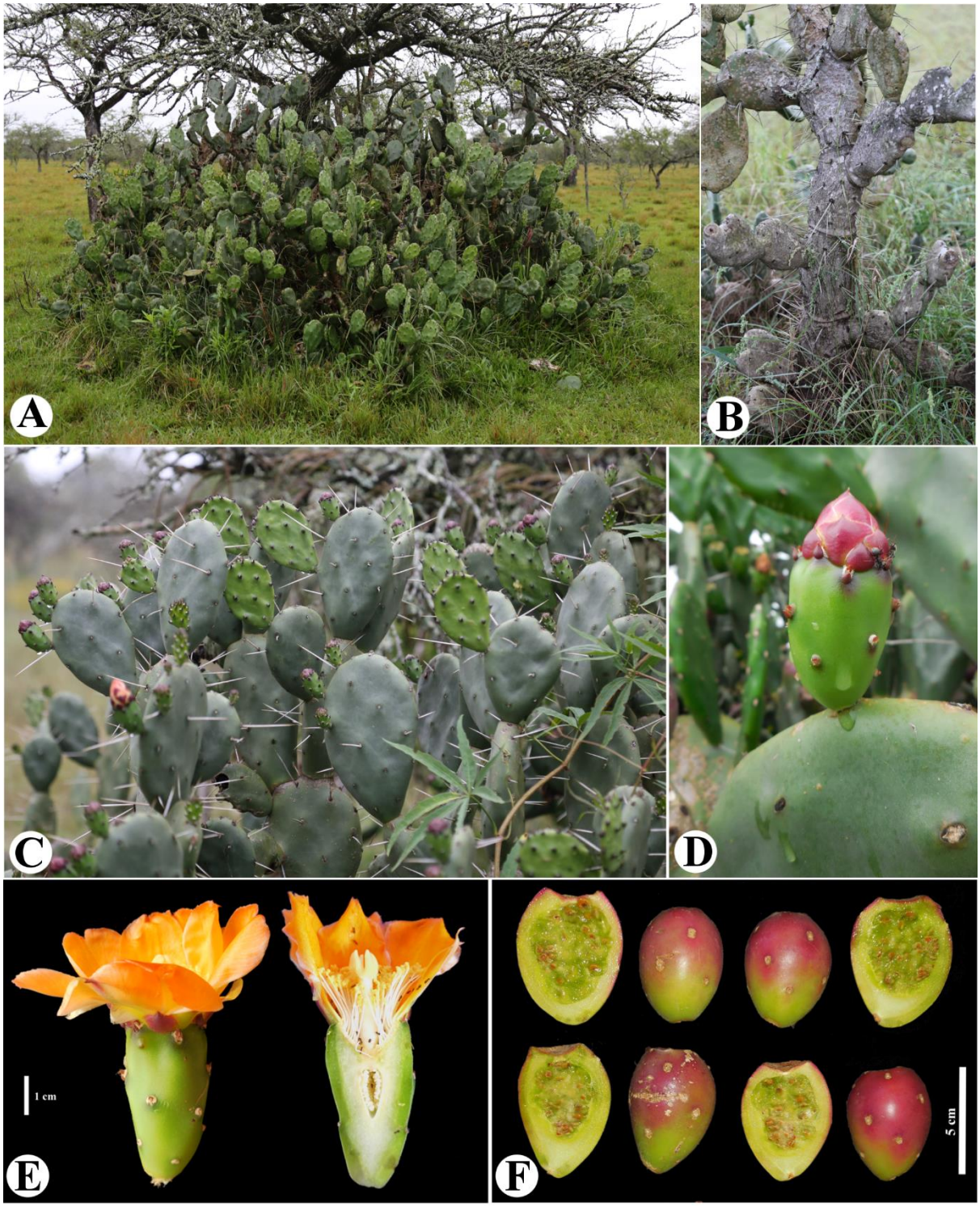

Figura 1 (Figure 1). *Opuntia rioplatense* **A.** Habit (*M. Köhler et al. 218*) **B.** Trunk with spines (*M. Köhler et al. 217*) **C.** Details of cladodes (*M. Köhler et al. 221*) **D.** Floral bud with acute apex (*M. Köhler et al. 221*) **E**. Flowers in longitudinal section (*M. Köhler et al. 220*) **F.** Fruits with longitudinal section (*M. Köhler et al. 221*).

Distribution and habitat:—*Opuntia rioplatense* occurs in central and eastern Argentina (Buenos Aires, Córdoba, Corrientes, Entre Ríos, La Pampa and Santa Fe Provinces), central-western Uruguay (Paysandú, Soriano, Montevideo, Maldonado) and extreme southwestern Brazil (west of Rio Grande do Sul State, our new record). In Brazil, the species is apparently restricted to the Pampa biome, specifically in a steppic-savanna environment known as Espinilho Park. The Espinilho Park is characterized by the occurrence of *Prosopis affinis* Sprengel (1825: 326), *P. nigra* (Grisebach 1879: 118) Hieronymus (1881: 283) and *Vachellia caven* (Molina 1782: 174) Seigler & Ebinger (2005: 148), exhibiting similarities to the Chaco vegetation in Argentina, the largest South American seasonally dry forest, which is a mosaic of xerophytic forests, woodlands, shrubs, savannas and grasslands (Roesch *et al.* 2009).

Notes on taxonomy, karyology and phylogeny:—*Opuntia rioplatense* is a taxon recently resurrected within Elata group species based on *Opuntia elata* var. *obovata* Walther (Font 2014), non *O. obovata* Griffiths (1919: 202). *O. rioplatense* differs from *O. elata* because of its acute flower bud apex (Fig. 1D), obovate-elliptic cladodes (Fig. 1C) and the obovate fruits (Fig. 1F), in contrast to the obtuse-rounded bud apex, elongated-oblong cladodes and the shortly obpyriform fruits. *O. rioplatense* was already known by the end of the 19th century by the first Argentine cactologists, but it was always erroneously cited as *O. vulgaris* Miller (1768) or *O. paraguayensis* Schuman (1899: 149). *Opuntia rioplatense* is reported as a tetraploid species ($2n = 4x = 44$) (Realini *et al.* 2014a; Las Peñas *et al.* 2017), and our counts have reinforced that ploidy level (Köhler unpublished data). The phylogenetic position of *O. rioplatense* is still uncertain, as the only phylogeny of the sSA species cannot be considered as conclusive (see Realini *et al.* 2014b), although there is a clear distinct haplotype between *O. elata*, and *O. rioplatense*. The species is probably nested in the *Elatae* clade (see Majure *et al.* 2012), and further molecular data should provide for more robust phylogenetic hypothesis regarding the species of this clade (Köhler unpubl. data).

Notes on conservation status:—Although the species is widely distributed in Argentina and Uruguay, we emphasize its restricted occurrence in Brazil in a small area of the State of Rio Grande do Sul. Considering that to date, the species has not been cited or considered for the Brazilian flora (Flora do Brasil 2018), we highlight the importance of investigating the state of conservation of the species in Brazilian territory to ensure its maintenance.

Diagnostic key to the southern Brazilian *Opuntia* species with orange flowers:
1. Stigma-lobes green to greenish; ripe fruits with purple to reddish inner pericarpel tissue..... *O. megapotamica*
- Stigma-lobes creamy white or yellowish; ripe fruits with green inner pericarpel tissue.....2
2. Flower buds with rounded or depressed apex; cladodes elongated to oblong..... *O. elata*
- Flower buds with acute or conical apex; cladodes obovate to elliptic..... *O. rioplatense*

Specimens examined:—BRAZIL. Rio Grande do Sul: **Barra do Quaraí**, Parque Estadual do Espinilho, 56 m, 30º11'16.10"S 57º 29'51.54"W, 12 October 2017, *M. Köhler et al. 217* (ICN); 57 m, 30º11'7.60"S7º29'44.78"W, 12 October 2017, *M. Köhler et al. 218* (ICN); 42 m, 30º11'30.00"S 57º31'37.47"W, 12 October 2017, *M. Köhler et al. 220* (ICN); 44 m, 30º11'26.78"S 57º31'33.91"W, 12 October 2017, *M. Köhler 221* (ICN). URUGUAY. Paysandú: **Lorenzo Geyres**, Camping Queguay, 36 m, 32º7'26.45"S 57º55'51.33"W, 3 December 2017, *M. Köhler et al. 288* (ICN). Soriano: **Mercedes**, Ruta 14, 29 m, 33º14'22.98"S 57º58'17.52"W, 3 December 2017, *M. Köhler et al. 291* (ICN). Montevideo: **Montevideo**, Camino a Punta Espinillo, 37 m, 34º49'55.95"S 56º21'44.10"W, 4 December 2017, *M. Köhler et al. 312* (ICN).

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REASSESSMENT AND TYPIFICATION OF *Opuntia canterae* (OPUNTIOIDEAE, CACTACEAE), AN ENDEMIC PRICKLY PEAR CACTUS OF URUGUAY

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SHORT COMMUNICATION

Reassessment and typification of *Opuntia canterae* (Opuntioideae, Cactaceae), an endemic prickly pear cactus of Uruguay

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Background and aims – *Opuntia* is the most widespread genus of Cactaceae, naturally occurring throughout arid and semi-arid areas of the Americas. Many of the species have taxonomic problems resulting from incomplete original descriptions, lack of type designations, a paucity of taxonomic revisions and, in general, difficult species delimitation resulting from hybridization, morphological plasticity, and poor specimen preparation. However, efforts are being undertaken to fill the gaps in our distributional, morphological and phylogenetic knowledge of the group. Here, we reassess the name *Opuntia canterae*, providing an updated description, typification, photographs, distribution map, conservation assessment and additional notes.

Material and methods – Extensive fieldwork was conducted, along with comprehensive herbarium and literature review. Morphological characters were assessed based on the commonly used characters used for prickly pears. Species delimitation is proposed based on our morphological studies, taxonomic and literature revision, as well as preliminary phylogenetic studies. The IUCN guidelines were followed to provide a conservation assessment of the species.

Key results – *Opuntia canterae* is reassessed as a distinct species separated from its previous synonym (*O. elata*) by the elliptic to long-oblanceolate stem segments, acute to conical flower bud apex and long-obconic fruits. An epitype is here designated to further clarify the morphological features of the species, which, heretofore, were only represented by a photo. The species is considered endemic to Uruguay and is provisionally assessed as Endangered (EN) using IUCN criteria, but more fieldwork will be necessary to provide a further precise conservation status.

Keywords – Biodiversity; Caryophyllales; cacti; endemic; Pampa; Pampean; threatened species.

INTRODUCTION

Opuntia Mill. is the most widespread genus of Cactaceae, naturally occurring from southern South America (Argentina) to northern North America (Canada) (Britton & Rose 1919; Anderson 2001; Majure et al. 2012). The group has a putative origin during the Late Miocene (11.6–5.3 Mya) in southwestern South America with subsequent dispersal events of lineages to northern South America, the Caribbean region, Central America and to the North American deserts (Arakaki et al. 2011; Majure et al. 2012). The group exhibits a variety of morphological characters such as a shrubby or tree growth form, dry/fleshy fruit, epidermis and seeds either pubescent or glabrous, dioecy/monoecy, ornithophilic/melittophilic pollination, as well as other discrete and phenotypically plastic characters (Schumann 1899; Britton & Rose 1919; Backeberg 1958; Anderson 2001; Hunt et al. 2006; Majure & Puente 2014; Majure et al. 2017).

Eight major clades have been recovered within *Opuntia* s. str. (Majure et al. 2012; Köhler et al. unpubl. res.), and the South American species are mainly nested in two of these clades: *Macbridei* and *Elatae* (*sensu* Majure et al. 2012; Köhler et al. unpubl. res.). The *Macbridei* clade includes species occurring in the northern part of South America, from central Peru to central Colombia (Britton & Rose 1919; Anderson 2001; Madsen 1989; Vega 2013; Majure & Puente 2014), while the *Elatae* clade includes the southern South American lineages occupying mainly the Pampa and the Chaco regions, as well as the Galapagos Islands (Britton & Rose 1919; Leuenberger 2002; Majure et al. 2012; Font 2014; Las Peñas et al. 2017; Köhler et al. 2018; Köhler et al. unpubl. res.).

Some of the southern South American (sSA) species of *Opuntia* have a confused taxonomic history. Many of these taxa were described based on materials collected by Old World naturalists that were travelling to the New World and sending biological materials to European gardens (Pontes et al. 2017). That routine led to many names, which were poorly described, based on morphotypes grown under greenhouses conditions, with insufficient diagnoses or use of characters and usually without the designation of nomenclatural types (Haworth 1812; Pfeiffer 1837; Salm-Dyck 1850). Beyond that, many European naturalists that migrated to the New World and started to contribute to the knowledge of local floras also often failed to cite original materials or provide precise descriptions of the novel species proposed (Spegazzini 1899, 1901, 1902, 1905, 1925; Arechavaleta 1905). Recently, major efforts have been made to better assess the identity and the interpretation of many of these names with typifications and a handful of taxonomic revisions

(Crook & Mottram 1995, 1996, 1997, 1998, 1999, 2000, 2001, 2002, 2003, 2004, 2005; Leuenberger 1993, 2001a, 2001b, 2002; Font 2014; Las Peñas et al. 2017).

Opuntia canterae was described by Arechavaleta (1905) as a distinct species based on his knowledge of the Uruguayan flora and neighbouring areas. The description included a comprehensive diagnosis with a complementary description accompanied by personal observations about the ecology and distribution of the species (Arechavaleta 1905). This taxon was later treated as a doubtful species by Britton & Rose (1919), whom merely transcribed the original description of Arechavaleta without mentioning the detailed knowledge about the ecology, etc., of the species. Bertram (1929, 1931) reported his success in growing *Opuntia* species in Germany, illustrating a flowering prickly pear identified as *O. canterae* by Hern W. Weingart. Herter (1957) included the species in his study of the Uruguayan flora and illustrated the species with narrow and spineless stem segments, with acute flower buds. Backeberg (1958) reproduced Arechavaleta's description providing a photograph of ambiguous assignment, and without providing any additional information. Anderson (2001) listed the species in his treatment based on these prior, depauperate descriptions. Leuenberger (2002), in the first attempted taxonomic revision of a series from the sSA species of *Opuntia* (series *Armatae* K.Schum. = *Elatae* Britton & Rose), was unable to critically access the identity of the taxon, and suggested that it may belong in *O. elata* Salm- Dyck or *O. cardiosperma* K.Schum.

Later, Font (2014), in an attentive revision of the series *Armatae*, proposed a novel set of morphological characters for a comprehensive circumscription of the species within the group. Besides the commonly used morphological characters of the stem segments (cladodes), spination and habit of the species, Font (2014) introduced the morphology of the flower bud apices, stigma colour and the inner pericarpel tissue colour as useful characters to diagnose taxonomic entities that have been problematic historically. Even so, Font (2014) broadly circumscribed *O. elata* to include *O. canterae*, and later Las Peñas et al. (2017) retained it in the synonymy of *O. elata* s. lat.

During a broad taxonomic, systematic and floristic revision of the southern South American species of *Opuntia,* a distinct morphotype was observed in the Pampean region of Uruguay, and further analyses suggested that it conformed to *Opuntia canterae*. Here, we propose a reassessment of *O. canterae*, providing a typification, an updated description, photographs, conservation assessment and additional notes about the species.

MATERIAL AND METHODS

Extensive field trips were carried out in southern South America encompassing the principle ecoregions to obtain data about natural populations of *Opuntia* in the area. The region is represented by subtropical grasslands permeated by rocky outcrops that compose the Pampa or Río de La Plata grassland (Andrade et al. 2018) and the Chaco region (Pennington et al. 2000). Major herbaria from the region have been revised to check distribution records and specimen identification of all *Opuntia*: BA, BAF, CORD, CTES, HAS, ICN, LIL, LP, MCN, MVFA, MVJB, MVM, SI (herbarium abbreviations following Thiers continuously updated). The digital database of Brazilian collections was also consulted through the SpeciesLink platform (2019) to check herbaria from disparate geographical regions.

A literature review was carried out comprising the main magna opera that contain descriptions of southern South American *Opuntia* species (Arechavaleta 1905; Spegazzini 1901, 1905, 1925; Schumann 1890, 1899a, 1899b; Britton & Rose 1919; Backeberg 1958, 1966; Ritter 1979, 1980), as well as recent revisions, floras and taxonomic treatments (Kiesling 1999, 2005; Kiesling & Ferrari 2005; Kiesling et al. 2008; Machado et al. 2008; Leuenberger 2002; Font 2014; Las Peñas et al. 2017). The morphological characters used for identification of the southern South American species of *Opuntia* followed those proposed by Font (2014) and Las Peñas et al. (2017), which have been reported as useful for species delimitation in other sSA *Opuntia* species (Köhler et al. 2020). For the conservation status assessment of the species, the GeoCAT tool (Bachman et al. 2011) was used to evaluate the area of occupancy (AOO) and the extent of occurrence (EOO), using a cell width of 5 km based on our observations. The criteria followed those proposed by the IUCN Red List (IUCN 2019). A distribution map was generated using the free and Open Source Geographic Information System QGIS v. 3.10.2 (QGIS Development Team 2020) with the public domain map dataset available at Natural Earth (https://www.naturalearthdata.com/).

RESULTS AND DISCUSSION

Opuntia canterae has been treated as a doubtful taxon (Britton & Rose 1919; Leuenberger 2002; Kiesling et al. 2008), valid species (Anderson 2001), or more recently as a synonym of *O. elata* (Font 2014; Las Peñas et al. 2017). During our recent field expeditions, a distinct morphotype was observed in the Pampean region of Uruguay, and none of the previous taxonomic treatments included the morphological features that are found in our circumscription of the species. The combined features in *O. canterae* of the elliptic to long-oblanceolate stem segments, acute flower bud apices and long-obconic ripe fruits (fig. 1), separate the species from *Opuntia elata*, which includes specimens with obovate-oblong stem segments, rounded/globose flower bud apices and pyriform fruits. Our preliminary phylogenetic studies of the sSA species of *Opuntia* (Köhler et al. unpubl. res.) reinforces *O. canterae* as a distinct evolutionary lineage of the *Elatae* clade (*sensu* Majure et al. 2012), which led us to propose a reassessment of the species.

Knowledge about the biology of the species is still lacking. As pointed out by Arechavaleta (1905) and confirmed by our field work, *O. canterae* frequently has sterile stamens and fruits (fig. 1E–F). All populations we were able to study had sterile stamens and fruits, but Arechavaleta (1905), although mentioning these features, also reported developed seeds. Thus, it will be necessary to further investigate putative dioecy in this species, as reported for other *Opuntia* species (Reyes-Agüero et al. 2006; Díaz & Cocucci 2008; Majure & Puente 2014). However, a hybrid origin for *O. canterae* must also be considered. Natural hybridization is widely reported and well known in *Opuntia* (Grant & Grant 1979; Griffith 2001; Rebman & Pinkava 2001; Majure et al. 2012; Majure & Puente 2014). During our fieldwork, we observed *O. canterae* occurring both isolated, as well as in sympatry with other *Opuntia* species, such as *O. rioplatense* Font, *O. elata* Link & Otto, *O. aurantiaca* Lindl. and *O. anacantha* Speg., but no obvious putative parents can be inferred. So, additional studies must be carried out to assess the chromosome number and reproductive biology of *O. canterae*. If evidence is generated suggesting that *O. canterae* may be of hybrid origin and always is a sterile, with only vegetative reproduction, wherein cladodes disarticulate and are later deposited on the ground rooting and forming clones of the parent plant, it would be an efficient way for the species to maintain its dispersion over time. However, regardless of the origin of *O. canterae*, considering the currently known distribution of the species over several sites, phylogenetic position (Köhler et al. unpubl. res.) and the ease of recognition based on several morphological characters, satisfying the morphological phenetic (Judd 2007) and diagnostic (Wheeler & Platnick 2000) species concepts, its specific status is warranted and justified.

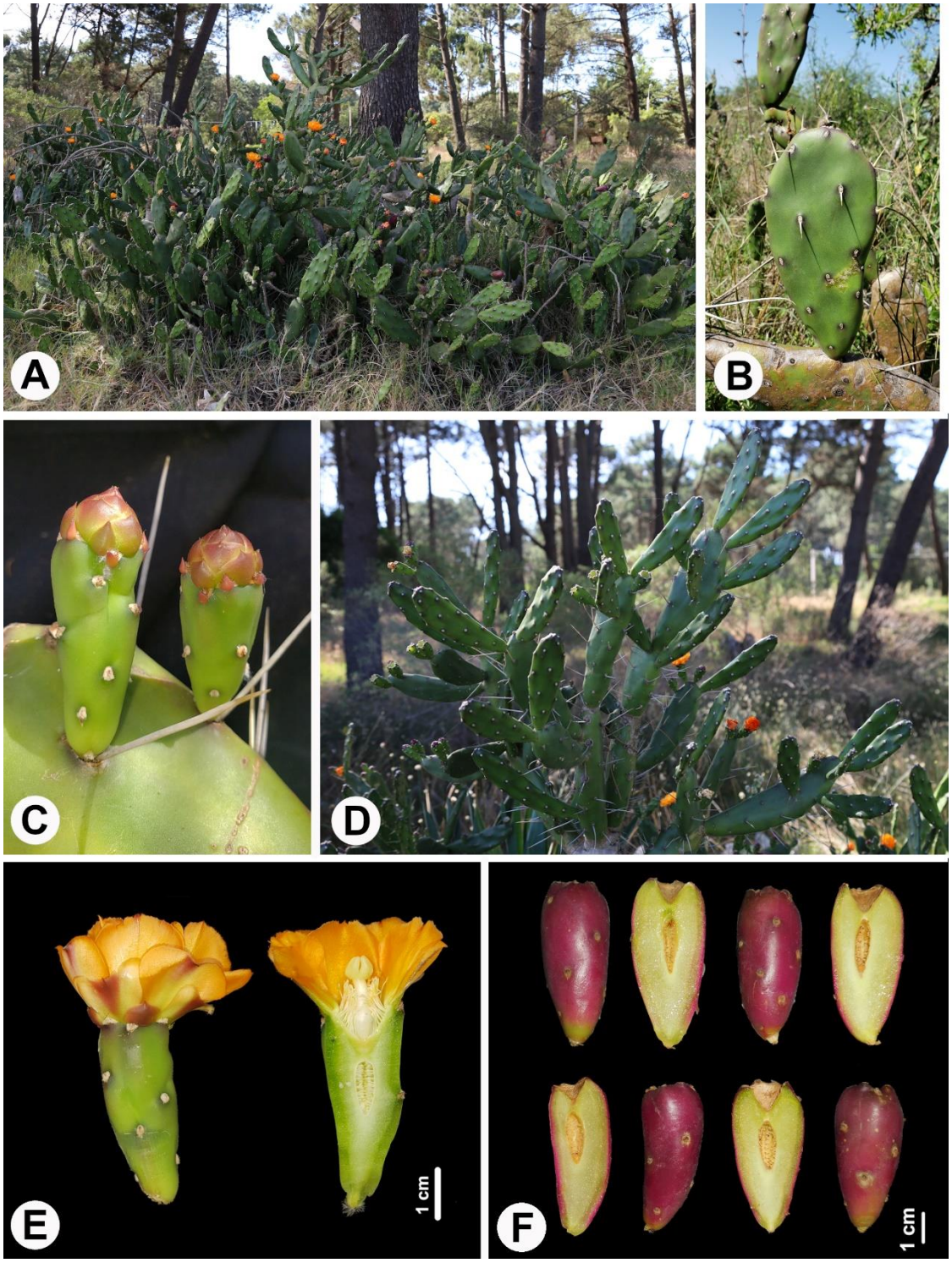

Figura 2 (Figure 1). Morphological features of *Opuntia canterae*. **A.** Plant in habitat (*M. Köhler 316*). **B.** Detailed stem segment resembling morphotype designated as neotype (*M. Köhler 550*). **C.** Detail of the acute flower bud apex (*M. Köhler 550*). **D.** Elliptic to long-oblanceolate stem segments, showing growing cladodes with protuberant areoles encircled with dark-violet coloration from betalain pigmentation (*M. Köhler 316*). **E.** Flower in longitudinal section showing orange tepals, obconic pericarpel, sterile stamens and obovate to elliptical ovary (*M. Köhler 550*). **F.** Longitudinal section of the long-obconic dark-purple ripe fruits showing the sterile ovaries and light green inner pericarpel tissue (*M. Köhler 316*). All photographs by M. Köhler.

Taxonomic treatment

Opuntia canterae Arechav. (Arechavaleta 1905: 278–280, as O. canterai). Figs 1–4
Type – Not designated.
Neotype – Designated by Las Peñas et al. 2017, Lám. LX in Osten (1941). See photograph on fig. 3.
Epitype (designated here) – Uruguay: Canelones, Neptunia, 34°47'2.73"S, 55°53'11.75"W, 6 Dec. 2017, M. Köhler et al. 316 (holoepitype: ICN, ICN 201773, barcode 00043878; isoepitype: MVM).

Description – Shrub, erect, 1.5–2(> 2) m tall. Stem segments (cladodes) 13–30 × 4–6 cm, 2–2.5(–3) cm thick, elliptic to long-oblanceolate, dark green, apex rounded to obtuse, base attenuate, occasionally forming subterete proximal stems. Areoles 6–9 per cladode face, 0.4–0.6 cm in diameter, circular to elliptic, frequently protuberant on growing cladodes, encircled with dark-violet betalain pigments. Leaves conic, dark-violet, 3–4 mm long, usually only on the apex of new cladodes or pericarpel, quickly ephemeral. Glochids present, but not well-developed (hardly exserted above the areoles), ferruginous. Spines 0–1(–2) per areole, acicular, white to light grey, reflexed (when < 3 cm) to straight (when > 4–10 cm). Pericarpel 3.5–4 × 1.5(–2) cm, obconic. Flower bud apex acute to conical, external tepals reddish, obcordate with mucronulate apex; inner tepals orange, largely obovate with mucronulate apex; flower at anthesis 3–5 cm in diameter. Stamens numerous with pale yellow filaments and anthers when present; frequently sterile. Stigma 6–7 lobed, connivent, cream-colored. Style cylindric to obclaviform, 1.7–2 × 0.3–0.5 cm. Ovary 1–1.3 × 0.4–0.7 cm, obovoid, in the upper third of the pericarpel. Fruit 5.5–7 × 2.5–3 cm, long-obconic, red to dark-purple when ripe, inner pericarpel light greenish. Seeds flattened (not seen in recent specimens).

Specimens examined – **Uruguay**: **Montevideo**: Pocitos, Dec. 1921, *C. Osten 16016* (MVM). **San José**: Rincón del Pino, 34°30'8.61"S, 56°50'7.37"W, 4 Dec. 2017, *M. Köhler et al. 299* (ICN), *M. Köhler et al. 302* (ICN); Libertad, 34°39'48.17"S, 56°35'3.69"W, 4 Dec. 2017, *M. Köhler et al. 303* (ICN). **Canelones**: Neptunia, 34°47'2.73"S, 55°53'11.75"W, 6 Dec. 2017, *M. Köhler et al. 316* (ICN). **Río Negro**: Nuevo Berlin, 32°53'10.9"S, 58°02'42.4"W, 23 Jan. 2020, *M. Köhler et al. 550* (ICN).

Distribution – Only recorded in Uruguay near Río de la Plata and Río Uruguay (Esteros and Algarrobales del Río Uruguay) in the departments of Canelones, Río Negro, San José and Montevideo (fig. 2).

Habitat – The species is originally described as occurring along the Uruguayan coastal plain of the Río de La Plata, on sandy or rocky (granite) soils, where it has been sparsely observed. New records have been observed in the extreme northwest part of the Río de La Plata, on the margins

of the Río Uruguay, in the Esteros y Algarrobales del Río Uruguay, suggesting a broader distribution that must be further investigated.

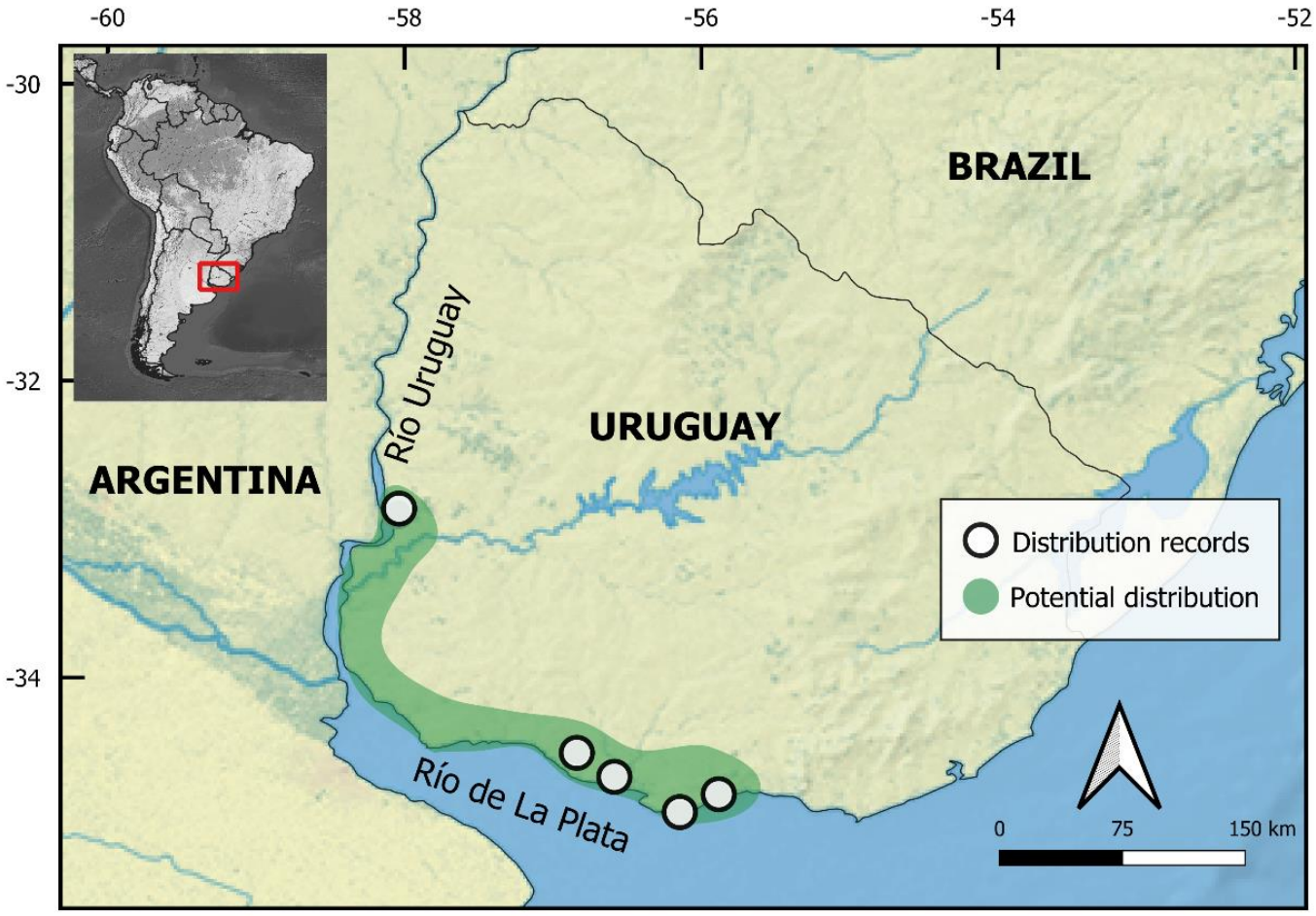

Figura 3 (Figure 2). Distribution map of *Opuntia canterae*. The white dots indicate the known records of distribution, while the green area indicates a potential distribution of the taxon that must be further investigated. Map created using QGIS v.3.10.2 (QGIS Development Team 2020) with dataset available at Natural Earth (https://www.naturalearthdata.com/).

Conservation assessment – Currently, 6 herbarium specimens of *Opuntia canterae* are known, collected between 1921 and 2020. These represent 5 unique occurrences, but the species has not been found again at the oldest locality (*C. Osten 16016* - Pocitos, Montevideo), where the capital city of Uruguay has developed. So, based on the presently known distribution, the extent of occurrence (EOO) of the species is estimated to be ~6,400 km$_2$, which places it under the Vulnerable (VU) category under criterion B1, whereas its area of occupancy (AOO) is estimated to be 100 km$_2$, which places it under the Endangered (EN) category under criterion B2 (IUCN 2019). The 6 herbarium specimens represent 2 locations, which places the species in the Endangered category under subcriterion 'a' of criterion B2. Many of the natural areas of Uruguay have been converted to agroindustry plantations of *Eucalyptus* spp., *Glycine max* (L.) Merr. (soybean), threatening one of the locations, whereas residential and commercial development threatens the other location. We therefore infer a reduction in the extent and quality of the habitat of *O. canterae*. Because of the low AOO (< 500 km$_2$), the low number of locations (2) and the inferred reduction in the extent and quality of the habitat, we give a provisional IUCN assessment of Endangered EN B2ab(ii,iii). We suggest that more fieldwork is necessary to increase our knowledge of *O. canterae*, its distribution and the threats it faces after which the conservation status of this species should be re-evaluated.

Phylogenetic relationships – This species was not sampled in previous phylogenetic analyses (Majure et al. 2012; Majure & Puente 2014; Realini et al. 2015; Majure et al. 2020). However, newly generated data has revealed the species as a distinct lineage in the *Elatae* clade (Köhler et al. unpubl. res.; *sensu* Majure et al. 2012), being closely related to some species treated in series *Armatae* K.Schum., such as *O. elata* and *O. megapotamica* (*sensu* Font 2014).

Notes – Las Peñas et al. (2017) designated a neotype based on a photographic plate provided by Osten (1941: Lám. LX). The same photograph was found in the MVM herbarium on a duplicate herbarium sheet, which was also accompanied by personal notes of C. Osten (fig. 3) that were almost entirely transcribed in Osten (1941). Our field studies allowed us to observe the same features provided by the photograph, as well as the original descriptions of Arechavaleta (1905), in those populations sampled (fig. 1A–B, D). However, considering that the neotype is a photograph of a putatively juvenile plant, which lacks important characters to be critically identified, we here designate an epitype showing the morphological features necessary for the precise identification and designation of the name of the species (fig. 4).

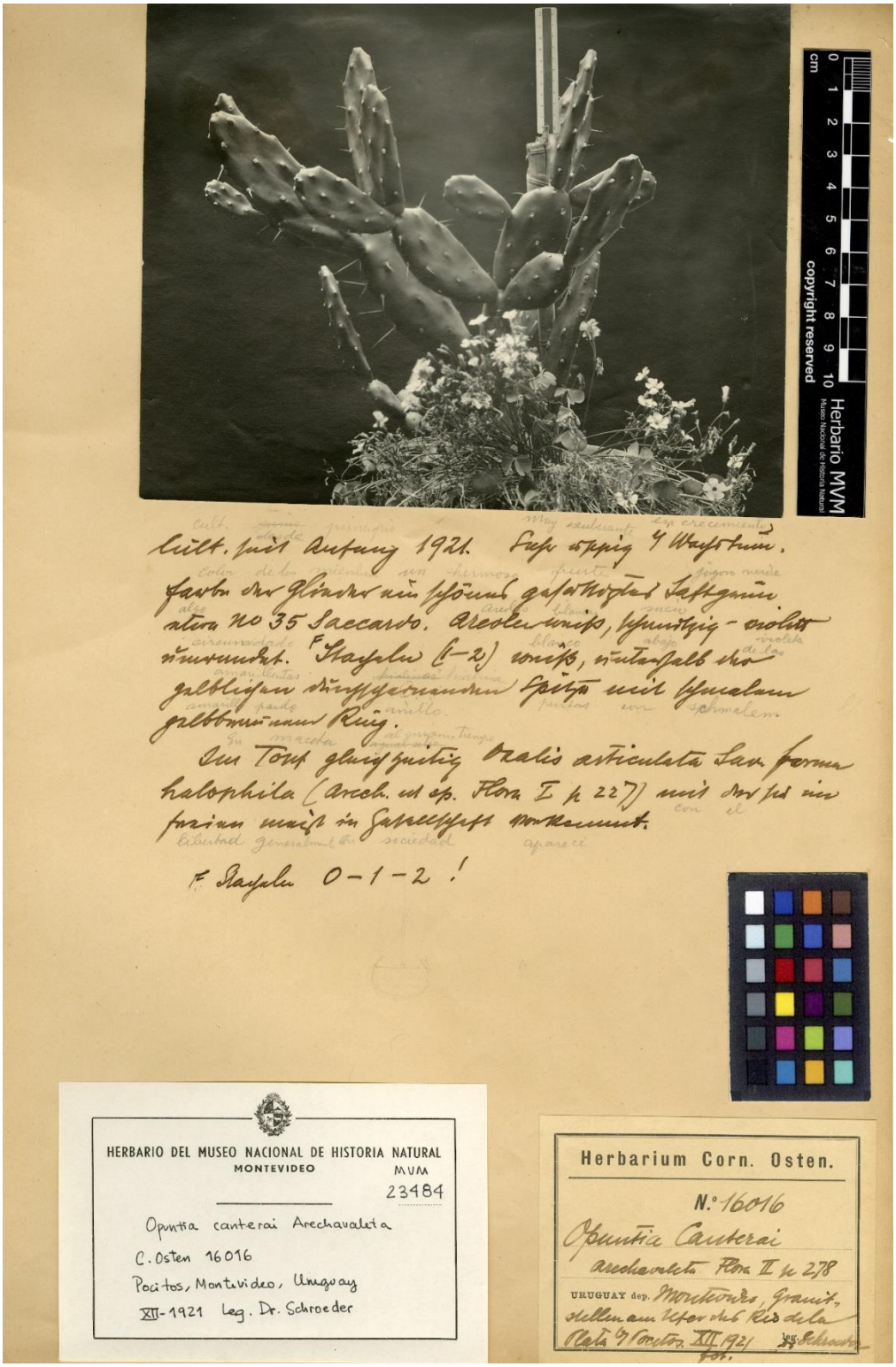

Figura 4 (Figure 3). Herbarium specimen from the Cornelius Osten Herbarium (MVM 23484, *C. Osten 16016*), which includes the photograph designated as the neotype by Las Peñas et al. (2017), accompanied by personal notes from C. Osten. © Museo Nacional de Historia Natural (Uruguay), all rights reserved; reproduced with permission. This image is not distributed under the terms of the Creative Commons licence of this publication. For permission to reuse, please contact the rights holder.

Figura 5 (Figure 4). Epitype of *Opuntia canterae* (ICN 201773, barcode 00043878, *M. Köhler et al. 316*), which includes important characters to critically identify and apply the name to the taxon, such as the elliptic to long-oblanceolate stem segments, acute flower bud apices and long-obconic fruits.

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North American Opuntias (Cactaceae) in Argentina? Remarks on the phylogenetic position of *Opuntia penicilligera* and a closer look at *O. ventanensis*

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ABSTRACT

Opuntia, the most widespread genus of cacti, occurs throughout the Americas from Patagonia to Canada. Various species have very wide distributions in the Americas, and thus may be considered as both native and aliens. We reexamined data based on recent work on the phylogenetics and taxonomy of *Opuntia* from southern S-America and showed that two presumed endemic species to Argentina—*O. penicilligera* and *O. ventanensis*—are likely derived from, or may be conspecific, with North American species. In particular, *O. penicilligera* is most closely related to members of the *O. macrorhiza* species complex and is morphologically similar to both *O. macrorhiza* and *O. cymochila*. *O. ventanensis* shares nearly all vegetative and reproductive morphological characters with *O. fragilis* and likely is conspecific with that taxon. Owed to the wide distribution of *Opuntia* species and the movement of many taxa by people, extra care must be exercised when describing new species or for carrying out taxonomic treatments. A phylogenetic perspective, as well as a careful study of species across the distribution of the genus, is recommended.

Keywords – nopales, Opuntia, Opuntioideae, phylogenetics, prickly pears, South America

INTRODUCTION

Members of the prickly pear cacti, tuna or nopales, *Opuntia* Miller (1754: without page) (Cactaceae Juss.) are naturally widely distributed throughout and are endemic to the Americas (see e.g., Anderson 2001). The group most likely originated in southern South America with subsequent dispersals and further diversification in North America (Majure *et al.* 2012a). The genus *Opuntia* (including *Nopalea* Salm-Dyck (1850: 233) is composed of two South American clades and one large and very diverse North American clade composed of six subclades (Majure *et al.* 2012a, Majure & Puente 2014). Many taxa endemic to northern Colombia and Venezuela [e.g., *O. boldinghii* Britton & Rose (1919: 155), *O. schumannii* F.A.C.Weber ex A.Berger (1904: 34)] were actually derived from the North American clade, and were shown to have originated via the dispersal of North American clade members back to South America (Majure *et al.* 2012a).

Species of *Opuntia* have been introduced throughout the world for use as ornamentals, forage for livestock, as well as for the production of agricultural products for human consumption, such as nopales and tunas, i.e., stem segments and prickly pear fruit, respectively (Casas & Barbera 2002, Nefzaoui & Ben Salem 2002). The range of many species within the Americas also has been substantially modified, as a result of the long-term use by humans for the above-stated reasons. Likewise, some taxa that do not appear to be widely used by humans also have been broadly dispersed, presumably by natural forces, such as large herbivores (Janzen 1986).

Opuntia ficus-indica (Linnaeus 1753: 468) Miller (1768: without pagination), a domesticated polyploid derivative of Mexican prickly pear species, is found throughout most of tropical America where it was presumably dispersed by humans (Griffith 2004), and its arrival in South America is assumed to have been as early as 8,000 years ago (Kiesling 1998, Ervin 2012). *O. pubescens* Wendland ex Pfeiffer (1837: 149), another native species of Mexico, also occurs in South America (Bolivia, Colombia, Ecuador, Peru, Venezuela), although there are no known human uses for this species, at least outside of cultivation as an ornamental. Moreover, *O. fragilis* (Nuttall 1818: 296) Haworth (1819: 82), one of the most widespread species in North America, has long been presumed to have been dispersed via migrating buffalo, where the easily disarticulating stem segments (cladodes) easily stick into the fur of passing animals by way of their strongly retrorsely-barbed spines (see e.g., Ribbens 2008). Thus, these cladodes act as vegetative propagules for this hexaploid species, which very seldomly reproduces sexually (Ribbens *et al.* 2011). Perhaps the same method of dispersal (via migrating animals) could have been involved in the production of the wider

distribution of *O. pubescens* outside of its original range in Mexico, and could potentially explain other known disjunctions of some species of *Opuntia*.

Opuntia penicilligera Spegazzini (1902: 291) was described from the southern part of Argentina, and it is treated as an endemic taxon by various authors (e.g., Cabrera & Fabris 1965, Kiesling 1988, Kiesling 1999, Zuloaga *et al.* 1999), although its morphological similarity with the other southern South American (sSA) species has never been clear (Spegazzini 1905, Spegazzini 1925, Britton & Rose 1919). However, a recent taxonomic revision of the *Opuntia* ser. *Elatae* Britton & Rose (1919: 156) based on morphological data, tentatively included the species in the sSA species group (Font 2014). Likewise, *O. ventanensis* Long (2012: 79) is a recently described taxon also from the southern Argentina region, and it has been treated as an endemic species. Although the morphological affinities of *O. ventanensis* with other sSA species have been discussed (Long 2012), the affinities with non-sympatric species in a broad context of the genus have not yet been considered.

Recent phylogenetic and cytogenetic analyses of sSA taxa of *Opuntia* aimed at determining the relationships among species occurring in Argentina and neighboring areas (Realini *et al.* 2014a, 2014b). Unfortunately, their work did not obtain the level of topological resolution using only the plastid *trnL-trnF* and *psbJ-petA* intergenic spacers, as well as ISSR markers, to fully understand relationships of the sSA taxa or test hypotheses regarding the putative relationships of some taxa with North American species due to their lack of taxon sampling.

Here, we propose a re-evaluation of the phylogenetic relationship of *Opuntia penicilligera* using previous molecular data generated for a broad scale phylogeny of the prickly pears (Majure *et al.* 2012a), as well as for southern South American species (Realini *et al.* 2014a). We also used data from the literature and herbarium materials to compare morphological characters of *O. ventanensis* and *O. fragilis*, a potentially closely related and phenetically very similar species from North America. Our primary goal was to test the hypothesis that those two presumably endemic species from Argentina could have putative origins from North American *Opuntia* species.

MATERIAL AND METHODS

DNA sampling, sequence alignment and phylogenetic analyses

Newly generated plastid sequences (*pshJ-petA, trnL-trnF*) from Realini *et al.* (2014a) were downloaded from GenBank and incorporated into a plastid dataset composed of the six plastid loci (*atpB-rbcL, matK, ndhF-rpl32, pshJ-petA, trnL-trnF, ycf1*) used in Majure *et al.* (2012a). These included both diploids and some polyploids for all major clades (two South American and six North American clades). Some species names used in Majure *et al.* (2012a) have been updated or corrected (e.g., Majure *et al.* 2013, Majure *et al.* 2014, Majure *et al.* 2017), and those corrections are given in Appendix 1. Sequences were aligned using the MAFFT (Katoh & Standley 2016) plugin in Geneious (v. 11.1.5, Biomatters Ltd.) and then corrected manually. Maximum likelihood (ML) in RAxML (Stamatakis 2014) was employed for phylogeny reconstruction using the GTR+G model of molecular evolution and undertaking 1000 bootstrap pseudoreplicates.

Morphological data

Morphological comparisons between *Opuntia fragilis* and *O. ventanensis* were based on observations in the field (*Majure 6762, 6781, 6791*; DES), examination of herbarium specimens (BAF, DES, FLAS, acronym according to Thiers 2019+), and analysis of relevant literature (Parfitt 1991, Pinkava 2003, Ribbens 2008, Long 2012). The following characters were analyzed: growth form, morphology of the stem, number and presence of the spines, and floral and fruit characters (see Table 1).

RESULTS

The phylogeny of tribe Opuntieae is not completely resolved with the few plastid loci used here, although major clades are often supported. Thus, the backbone of the phylogeny and relationships among major clades were not resolved (i.e., there are no bootstrap values over 50 for the backbone of the phylogeny). The *Opuntia* clade and one large subclade, including the *Scheerii*, *Macrocentra* and *Humifusa* clades, were all well supported. Likewise, the *Nopalea, Macrocentra, Humifusa*, and *Basilares* subclades are supported. Although the southern South American *Elatae* clade is not supported, several subclades are supported within the *Elatae* clade, and the *Elatae* clade was resolved as well supported in Majure *et al.* (2012a) based on a larger dataset.

Opuntia penicilligera was resolved in a well-supported Northern American clade (bs=93, Fig 1), i.e. the *Humifusa* clade, in a subclade with *O. macrorhiza* Engelmann (1850: 206) and relatives (i.e., the *O. macrorhiza* species complex), with which the taxon is morphologically similar (Fig. 2). Only a partial *trnL-trnF* sequence was available for *O. ventanensis*, which did not provide any useful phylogenetic information to place that species in a phylogenetic context. So, we only explored the morphological characters of that taxon, as compared to the proposed closely related species *O. fragilis*.

Opuntia ventanensis shares nearly all morphological characters, including vegetative and reproductive features, with the North American species *O. fragilis* (Tab. 1). Both *O. ventanensis* and *O. fragilis* are low, sprawling shrubs (Fig. 3) and have small, easily disarticulating cladodes and strongly, retrorsely-barbed spines. Both have yellow inner tepals and pinkish or reddish staminal filaments, as well as green stigma lobes, and both taxa exhibit spiny pericarpels. Chromosome number is apparently 2*n*=55 in *O. ventanensis*, and 2*n*=66 in *O. fragilis*.

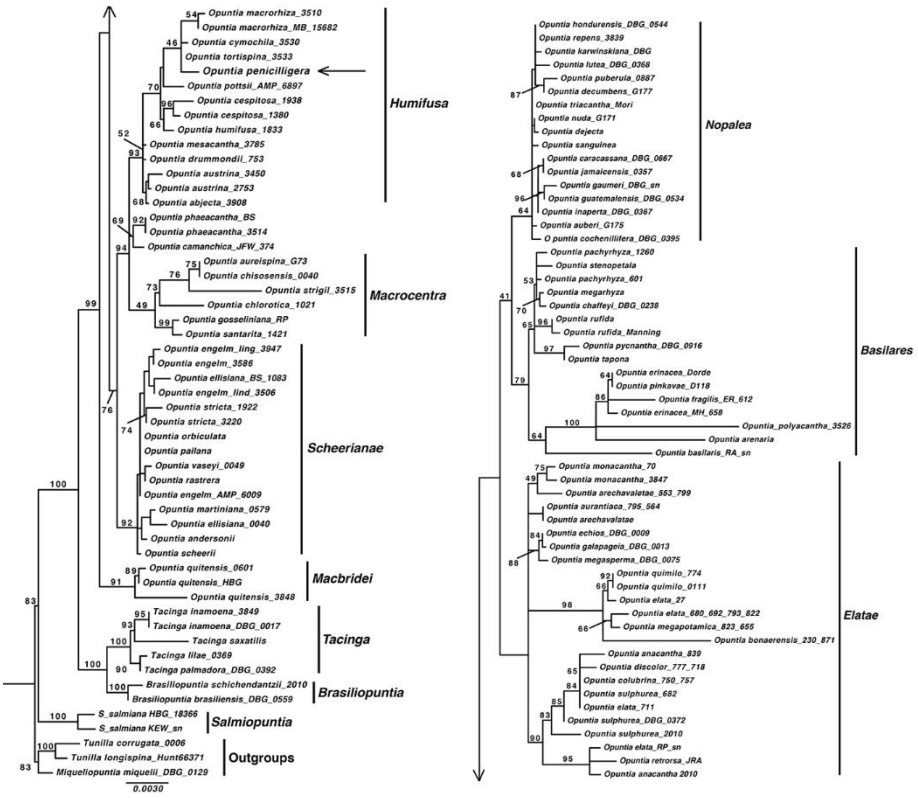

Figura 6 (Figure 1). Phylogeny of tribe Opuntieae (excluding the genus *Consolea*) including *Opuntia penicilligera* (arrow; *Humifusa* clade) based on the *trnL-F* and *petA-psbJ* intergenic spacer data generated by Realini *et al.* (2014) and the 6-locus dataset of Majure *et al.* (2012a). See comments about topology in the Results.

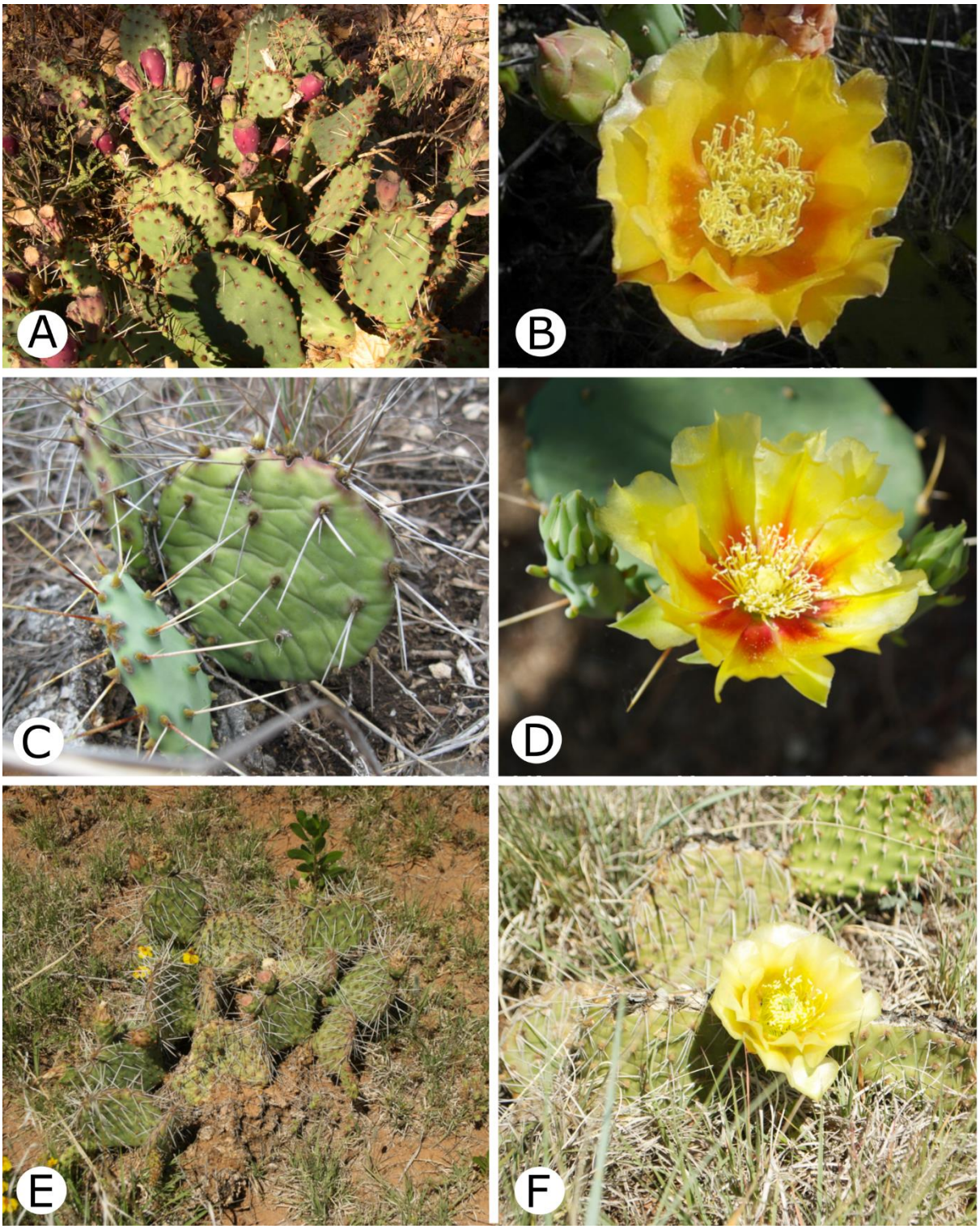

Figura 7 (Figure 2). Members of the *Opuntia macrorhiza* complex compared with *O. penicilligera*. **A.** *O. penicilligera* from Mendoza, Tunuyán (Argentina) (*Logarzo 547 - BAF*), **B.** flowers of *O. penicilligera* from Mendoza, Tunuyán (Argentina) (*photo only*), **C.** *O. macrorhiza* from Kerr Co., Texas, USA, in habitat, **D.** *O. macrorhiza* in flower (*Majure 3510* - FLAS), **E.** *O. cymochila* (spiny form) from Quay Co., New Mexico, USA, in habitat and **F.** in flower (*Majure 6854* - DES, FLAS). (photo A by L. Varone, B by C. Inchauspe, and C-D by L.C. Majure).

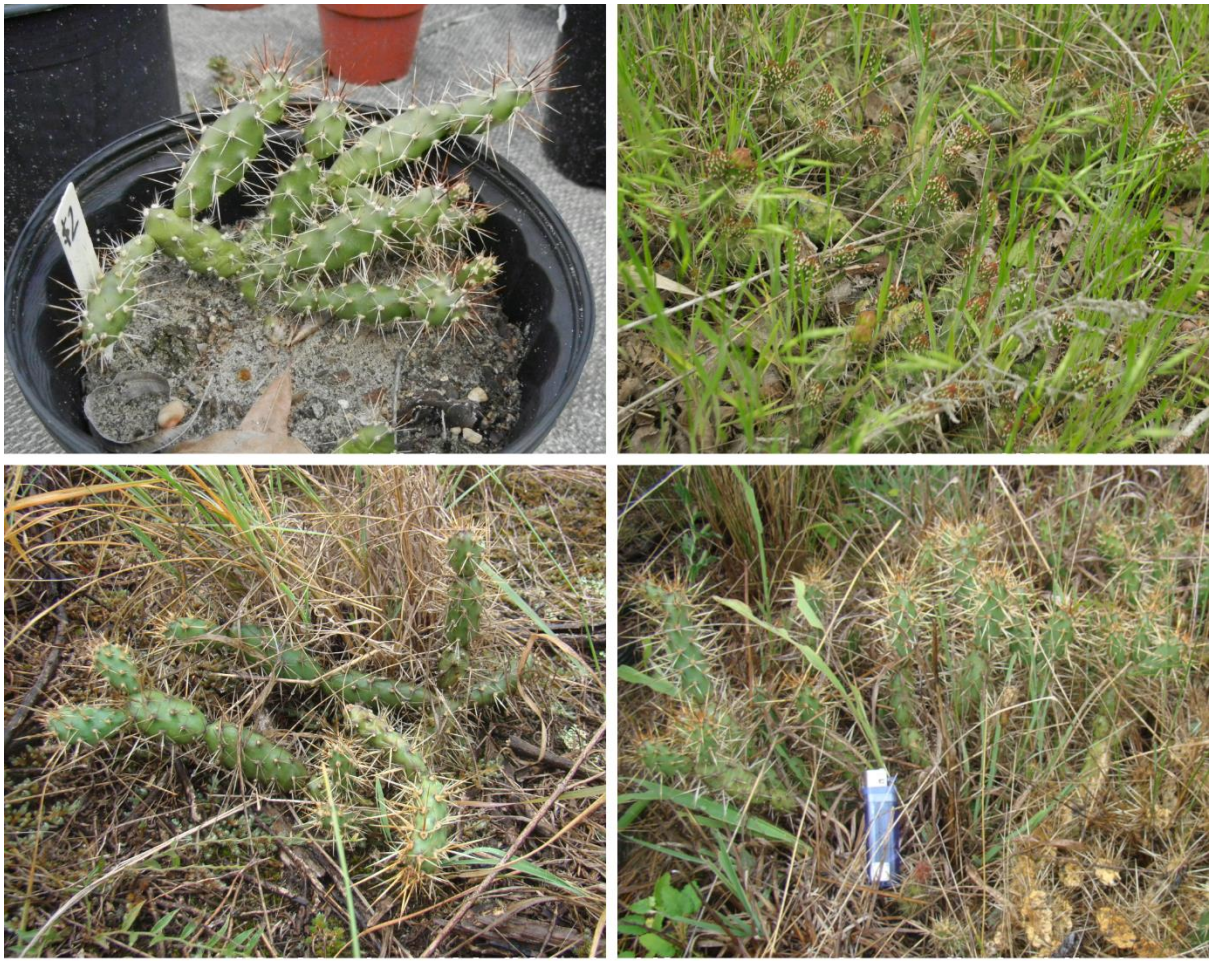

Figura 8 (Figure 3). Morphological characters of *Opuntia fragilis* compared with *O. ventanensis*. **A.** in habitat showing **A.** clump-forming habit of *O. fragilis* (*Ribbens s.n.*-FLAS), from Marquette Co., Michigan, USA, **B.** *O. fragilis* in habitat from Buffalo Co., Nebraska, USA (*Majure 6762*-DES, FLAS), **C-D**. *O. ventanensis* in habitat from Córdoba (*Font 634*-BAF) (photos A–B by L.C. Majure and C–D by F. Font).

DISCUSSION

Opuntia penicilligera has long been a puzzling name for the Argentinian flora. Although it was described in 1902 by C. Spegazzini, its identity remained unresolved for a long time, since no original material had been designated and the type never located (see Spegazzini 1902, Kiesling 1984, Katinas 2004, Leuenberger & Arroyo-Leuenberger 2014). During this time, the taxon has been reported as a native and endemic to the north Patagonian Argentinian region, occurring in the hills and dry plains south of Buenos Aires, north of Río Negro, south of La Pampa, northwest of Neuquén and west of Mendoza provinces (Cabrera & Fabris 1965, Kiesling 1988, Kiesling 1999, Zuloaga *et al.* 1999, Kiesling *et al.* 2008, Font 2014).

Tabela 1 (Table 1). Morphological comparisons between *Opuntia fragilis* and *O. ventanensis*.

	Opuntia fragilis	***Opuntia ventanensis***
Growth form	Sprawling shrubs	Sprawling shrubs
Cladodes	Easily detached	Easily detached
Spine number	3–8	3–6
Spine type	Radials + centrals	Radials + centrals
Stigma lobes	Green	Green
Fruit size (cm)	1–3 × 0.8–1.5	2 × 1.2
Filaments	Red to red-brown	pink
Pericarpel	Spiny	Spiny

Recently, a first step was made to elucidate the correct application of the name *Opuntia penicilligera*, with the designation of a neotype (see postscript of Leuenberger & Arroyo-Leuenberger 2014). Although the selected specimen is incomplete lacking information regarding flowers, it is assumed to correspond with the original description (Spegazzini 1902) exhibiting the conspicuous penicillate rusty brown glochids on orbicular to obovate stems with one principal spine up to 5 cm long, and none or 3–4 much shorter spines, with the receptacles cylindric to conical and the fruits reddish. However, as previously reported by Leuenberger & Arroyo-Leuenberger (2014), plants identified by collectors and botanists hitherto as *O. penicilligera* may belong to one, two or up to three morphotypes potentially related to North American species.

Phylogenetic analysis of those data generated by Realini *et al.* (2014a) and Majure *et al.* (2012a) placed *Opuntia penicilligera* in the North American *Humifusa* clade composed of *O. macrorhiza* and relatives, in accordance with both morphology and ploidy. *O. penicilligera* is morphologically very similar to *O. macrorhiza* and close relatives based on observation of photos of live plants, as published in Font (2014) and Leuenberger & Arroyo-Leuenberger (2014), and on examination of herbarium specimens (B, BAF, LP, SI, acronym according to Thiers 2019+). So, the placement with members of a clade containing *O. macrorhiza* is not surprising. However, it also appears that what is being called *O. penicilligera* may be a range of taxa in the *Macrorhiza* complex and possibly even the *Macrocentra* clade (*sensu* Majure *et al.* 2012a). Likewise, the material sequenced by Realini *et al.* (2014a, *Font 531* at BAF), is one of numerous morphotypes (Font, pers. obsv.) that may or may

not represent typical material of *O. penicilligera* as described by Spegazzini (1902). As revealed in our phylogenetic analysis here, based on the specimens sequenced by Realini *et al.* (2014), it is very clear from the phylogenetic placement that *O. penicilligera* is not at all closely related to other members of the *Elatae* clade, as tentatively proposed by Font (2014).

Although *Opuntia macrorhiza* has been treated by several authors (e.g., Britton & Rose 1919, Benson 1982, Pinkava 2003), it has not been taxonomically revised and, on the basis of personal observations (Majure, unpubl. data), this taxon forms a species complex consisting of numerous morphotypes (both diploid and tetraploid) that are currently under study. However, the majority of the taxa belonging to the *O. macrorhiza* complex is composed of tetraploids (2*n*=44; see Majure & Ribbens 2012, Majure *et al.* 2012b), the same ploidy as that reported for *O. penicilligera* by Realini *et al.* (2014b). Likewise, another close relative in the *Macrorhiza* complex, *O. cymochila* Engelmann & Bigelow (1856: 295), which shows some morphological affinities to *O. penicilligera*, has been reported as both tetraploid and hexaploid (Majure *et al.* 2012b). Thus, we cannot rule out the possibility that what is referred to as *O. penicilligera* could be represented in Argentina by both *O. cymochila* and *O. macrorhiza*.

Opuntia cymochila is a morphologically variable species, likely partly a result of its putative hybrid origin between *O. macrorhiza* and *O. polyacantha* Haworth (1819: 82) (see Majure 2012), and it is probable that what we refer to as *O. cymochila* has been derived numerous times through repeated hybridization events (Majure 2012), as is common for most hybrid derived species (Soltis and Soltis 2009). *O. cymochila* displays characters that are mosaics of both putative parents, with fruit that may be fleshy or mostly dry and spiny or mostly spineless. Cladodes of *O. cymochila* may be extremely spiny, as in *O. polyacantha*, or with much fewer spines, as in *O. macrorhiza*. The number of areoles per diagonal row in *O. cymochila* (6–8) normally exceeds that of *O. macrorhiza* (4–6) and is fewer than that of *O. polyacantha* (8–10). The inner tepals of *O. cymochila* may be entirely yellow as in *O. polyacantha* or with a reddish to reddish-brown base, as in *O. macrorhiza*.

Opuntia ventanensis is morphologically very similar to *O. fragilis* based on several characters, i.e. growth habit (low, sprawling shrubs forming mats or cushions; Fig. 3), disarticulating cladodes (vegetative propagules) with strongly retrorsely-barbed spines (these vegetative propagules are easily dispersed by animals in both taxa; Ribbens 2008, Long 2012, Majure & Ribbens 2012 & refs. therein), numbers of spines per areole which overlap [3–8 in *O. fragilis* vs. 1–3(–5) in *O. ventanesis*]. Although Long (2012) stated that *O. ventanensis* only has up to 3 spines per areole, the photos of cladodes in her paper show up to six spines per areole including both central and radial spines, and it should be noted that both central and radial spines are present, as in *O. fragilis* (a common

character in the *O. polyacantha* complex; Parfitt 1991). Both species rarely flower (see Bennett *et al.* 2003, Pinkava 2003, Ribbens 2008, Long 2012). Both taxa have yellow inner tepals, these may have a pink midrib in *O. ventanensis* (Long 2012) or be reddish or greenish at the base in *O. fragilis* (Ribbens 2008). Flowers of both exhibit green stigma lobes, as well as small fruit (2 × 1.2 cm in *O. ventanensis* vs. 1–3 × 0.8–1.5 cm in *O. fragilis*; Pinkava 2003, Long 2012). The staminal filaments in *O. fragilis* are red to reddish-brown, and those in *O. ventanensis* were described as pink (Long 2012). Figure 2B and 3A-B in Long (2012) show a spiny pericarpel in *O. ventanensis*, yet another character shared with *O. fragilis*.

Opuntia fragilis has only been reported as a hexaploid (2*n*=66) from throughout parts of its range (Parfitt 1991, Majure & Ribbens 2012). Interestingly, Realini *et al.* (2014b) reported a pentaploid chromosome number for *O. ventanensis* (2*n*=55). So, if indeed *O. ventanensis* proves to be closely related or even synonymous with *O. fragilis*, then either the chromosome number is variable in the species, or perhaps chromosome numbers should be re-analyzed for the Argentinian populations, since just one count has been reported. On the other hand, that *O. fragilis* has not been analyzed for ploidy across its native range leaves open the possibility of pentaploid individuals in North American populations as well. Likewise, although it is clear that *O. fragilis* is part of the *Polyacantha* clade (Parfitt 1991, Majure *et al.* 2012a), it is still unclear as to the origin of the hexaploid species.

Numerous non-native species of *Opuntia* and other cacti have been introduced into Argentina for the purpose of producing agricultural products, as well as for use as ornamentals (Castellanos & Lelong 1934). When and where *O. macrorhiza* (and potentially *O. cymochila* and *O. fragilis*) could have been introduced into the country is unknown, but they likely could have been introduced as ornamentals as well. The colonization of Argentina, especially the Buenos Aires region, goes back to the 16th century, and the alien flora has been relatively well documented and studied (Hauman 1925, 1927, Molfino 1926, Rapoport & Brion 1991, Söyrinki 1991, Zuloaga & Morrone 1996, 1999, Zuloaga *et al.* 1999). Thus, it is known that the northern Patagonia floras include a high number of exotic plants (Speziale & Ezcurra 2011). Along those lines, the description of presumably new taxa from that region should include comparisons, not only with South American species, but also with potential alien relatives. This should also be the standard for taxa being described from other parts of the distribution of *Opuntia*, since so many species have been so widely cultivated and introduced outside of their natural range.

Although phylogenetic data are available regarding the relationships of major clades within *Opuntia* s.str. (Majure *et al.* 2012a, Majure & Puente 2014), those results are not always taken into

consideration for revisions of closely related species or for nomenclatural changes (Guiggi 2015). On the contrary, the outdated system of using traditional groupings based on phenetic similarity and oftentimes geography (Britton &Rose 1919, Backeberg & Knuth 1935) is still being utilized. Likewise, certain characters, for example, associated with hummingbird pollinated flowers (reddish tepals, nectar chambers, lack of staminal thigmonasty), when not placed in a phylogenetic context are overemphasized as being unique (e.g., Oakley & Kiesling 2016; *O. quimilo*), when on the contrary they are common throughout tribe Opuntieae (e.g., *Tacinga*, *Macbridei* clade, *O. stenopetala*, *Nopalea* clades), and likewise *O. quimilo* is deeply nested within the *Elatae* clade (Fig. 1). It is clear that traditional taxonomic species groups in specific instances, such as *Opuntia* series *Aurantiacae* and *Curassavicae sensu* Britton & Rose (1919), each composed of members from two different clades, are not natural groupings in the broad sense. Although the phylogenetic relationships of the sSA species are still not fully understood, and are currently under study (Köhler *et al.* 2018; Köhler *et al.*, unpubl. data), *Opuntia* series *Elatae* Britton and Rose (1919: 156) or *Opuntia* series *Armatae* Schumann (1899: 743) *sensu* Leuenberger (2002: 413), for example, clearly excludes many of the close relatives of that clade, such as *O. quimilo* Schumann (1898: 746), *O. retrorsa* Spegazzini (1905: 571) and potentially the Galapagos' Island species (Griffith & Porter 2009, Majure *et al.* 2012a). Thus, it is most appropriate where relationships are known that taxa traditionally circumscribed under such names be re-evaluated when carrying out taxonomic revisions. The combination of morphological and cytological work, coupled with phylogenetic work have the potential to yield more robust hypotheses of species relationships, as well as species limits.

Considering the morphological characters outlined above, it seems likely that *Opuntia ventanensis* is very closely related or may even belong within the species concept of *O. fragilis*. However, further morphological and phylogenetic study, including samples from the type locality, are necessary to clarify the relationship of *O. ventanensis* with other species. The possible origin of these North American taxa in Argentina is of much interest, and it would be fascinating to understand when and where they were originally introduced, as well as the intention of the introduction. It seems likely that cattle introduced from the United States could have been one source of prickly pears in the country, as well as introduction for ornamental purposes or even long-distance dispersal via migrating birds.

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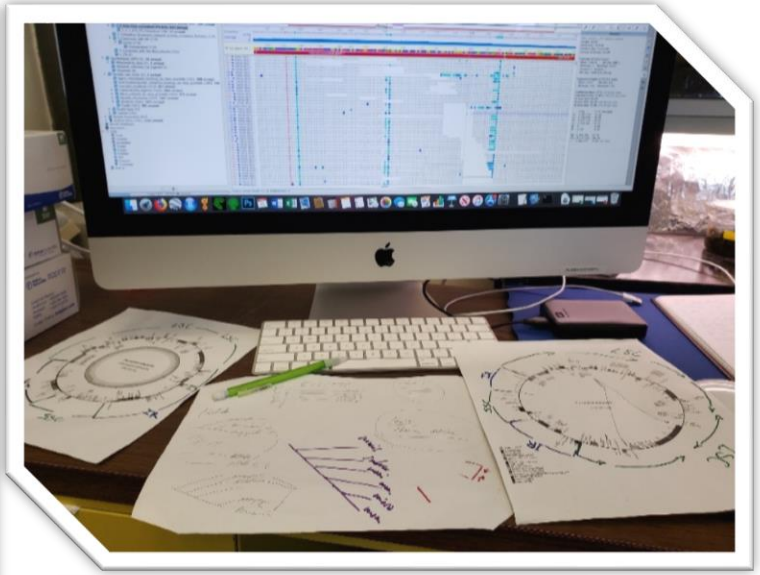
“Somewhere, something incredible is waiting to be known.”

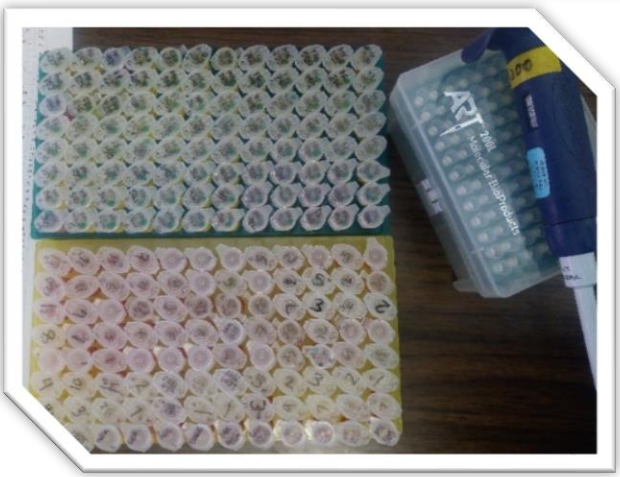
— Carl Sagan

PARTE II

GENÔMICA – PLASTOMAS E VÍRUS

Informações moleculares, reveladas a partir da extração e do sequenciamento do DNA presente nas células e nos tecidos vegetais, reúnem uma infinidade de dados hereditários que codificam e estruturam as macromoléculas dos organismos. Através de técnicas específicas, e de uma miríade de processos associados à bioinformática e estudos evolutivos, diversas análises permitem investigar histórias evolutivas armazenadas em organelas, cromossomos, genes e organismos simbiontes. Nos próximos capítulos: (1) revelamos pela primeira vez o genoma plastidial de uma *Opuntia*, que possui característica peculiar dentro das Angiospermas, investigamos como sua estrutura está relacionada com outros membros da subfamília Opuntioideae, e exploramos as sequências do plastoma para resolver as relações filogenéticas dentro de Opuntioideae; e (2) utilizamos dados do sequenciamento de nova geração para identificar, de maneira inédita, novas linhagens de Geminivírus associados à *Opuntia*.





INSIGHTS INTO CHLOROPLAST GENOME EVOLUTION ACROSS Opuntioideae (Cactaceae) REVEALS ROBUST YET SOMETIMES CONFLICTING PHYLOGENETIC TOPOLOGIES

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Insights Into Chloroplast Genome Evolution Across Opuntioideae (Cactaceae) Reveals Robust Yet Sometimes Conflicting Phylogenetic Topologies

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OPEN ACCESS

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ABSTRACT

Chloroplast genomes (plastomes) are frequently treated as highly conserved among land plants. However, many lineages of vascular plants have experienced extensive structural rearrangements, including inversions and modifications to the size and content of genes. Cacti are one of these lineages, containing the smallest plastome known for an obligately photosynthetic angiosperm, including the loss of one copy of the inverted repeat (~25 kb) and the *ndh* gene suite, but only a few cacti from the subfamily Cactoideae have been sufficiently characterized. Here, we investigated the variation of plastome sequences across the second-major lineage of the Cactaceae, the subfamily Opuntioideae, to address (1) how variable is the content and arrangement of chloroplast genome sequences across the subfamily, and (2) how phylogenetically informative are the plastome sequences for resolving major relationships among the clades of Opuntioideae. Our *de novo* assembly of the *Opuntia quimilo* plastome recovered an organelle of 150,347 bp in length with both copies of the inverted repeat and the presence of all the *ndh* gene suite. An expansion of the large single copy unit and a reduction of the small single copy unit was observed, including translocations and inversion of genes, as well as the putative pseudogenization of some loci. Comparative analyses among all clades within Opuntioideae suggested that plastome structure and content vary across taxa of this subfamily, with putative independent losses of the *ndh* gene suite and pseudogenization of genes across disparate lineages, further demonstrating the dynamic nature of plastomes in Cactaceae. Our plastome dataset was robust in resolving three tribes with high support within Opuntioideae: Cylindropuntieae, Tephrocacteae and Opuntieae. However, conflicting topologies were recovered among major clades when exploring different assemblies of markers. A plastome-wide survey for highly informative phylogenetic markers revealed previously unused regions for future use in Sanger-based studies, presenting a valuable dataset with primers designed for continued evolutionary studies across Cactaceae. These results bring new insights into the evolution of plastomes in cacti, suggesting that further analyses should be carried out to address how ecological drivers, physiological constraints and morphological traits of cacti may be related with the common rearrangements in plastomes that have been reported across the family.

Keywords – cacti, de novo assembly, *Opuntia*, plastid structural rearrangements, plastome, pseudogenization, reference-guided assembly

INTRODUCTION

Cacti comprise one of the most charismatic plant clades of the world, exhibiting an enormous variety of growth forms, morphology and intriguing niche occupancy across the Americas (Britton and Rose, 1919; Anderson, 2001; Hunt et al., 2006; Hernández-Hernández et al., 2011). This diversity is reflected in a high number of species and heterogeneous diversification rates across the clade (Arakaki et al., 2011; Hernández-Hernández et al., 2014). Some uncommon features in most angiosperms, such as succulent tissues, Crassulacean acid metabolism (CAM), betalain pigments and the reduction of or absence of leaves are typical characters of cacti that have long captured the attention of plant biologists and have been suggested as adaptations to allow survival in harsh environments (Mooney et al., 1977; Mauseth, 1999; Landrum, 2002; Nobel, 2002). Indeed, members of the family are conspicuous elements of the arid and semiarid succulent biome of the New World, but they are also found in subtropical and tropical forests, especially as epiphytes (Taylor and Zappi, 2004; Hunt et al., 2006). Besides major morphological and physiological adaptations, genetic and genomic-level changes are also expected. For example, whole genome duplication events have long been suggested to be associated with adaptations to extreme environments (e.g., Stebbins, 1971; Soltis and Soltis, 2000; Brochmann et al., 2004), and significant gene family expansion in genes related to stress adaptation, as well as more restricted events of gene duplications were reported in lineages of Caryophyllales adapted to severe environments including in cacti (Wang et al., 2019).

Although gene content, structural organization and size of the chloroplast genome (plastomes) of land plants is often considered highly conserved (Raubeson and Jansen, 2005; Chumley et al., 2006; Wicke et al., 2011), deviations have been increasingly reported in some clades and have challenged the generality of this phenomenon (Daniell et al., 2016; Mower and Vickrey, 2018; Ruhlman and Jansen, 2018). Astonishing variety of size have been observed across land plants, from 19 kb in a non-photosynthetic *Epipogium roseum* (D. Don) Lindl. (Orchidaceae) to giant plastomes with 217 kb, as in *Pelargonium* × *hortorum* L. H. Bailey (Geraniaceae) (Chumley et al., 2006; Schelkunov et al., 2015), reflected by expansions or contraction of the inverted repeat (IR), large single copy (LSC) or even small single copy (SSC) units. Also, the independent losses of one copy of the inverted repeat region (~25 kb in size) have been identified across disparate clades, such as Fabaceae, Geraniaceae, Orobanchaceae, and Cactaceae (Cai et al., 2008; Ruhlman and Jansen, 2014; Sanderson et al., 2015), and a variety of taxa have lost particular genes (e.g., *ndh* genes

in parasites, carnivorous plants and xerophytes) (Braukmann et al., 2009; Wicke et al., 2011; Iles et al., 2013; Peredo et al., 2013; Ruhlman et al., 2015; Sanderson et al., 2015).

Members of Cactaceae also have experienced different alterations in their chloroplast genome. A conserved inversion of ~6 kb on the large single copy unit comprising the *trnM-rbcL* genes have long been suggested (Wallace, 1995) and more recently confirmed (Sanderson et al., 2015; Majure et al., 2019; Solórzano et al., 2019). Besides that, the first cactus plastome assembled from the saguaro cactus [*Carnegiea gigantea* (Engelm.) Britton & Rose] exhibited an exceptional reduction in size (113 kb) and gene content, including the loss of one of the two inverted repeat regions and nine of the 11 *ndh* genes (Sanderson et al., 2015). More recently, newly assembled plastomes of seven species of the short-globose cacti of *Mammillaria* Haw. revealed three different plastome structures across the genus, all with two copies of a divergent inverted repeat, including (i) an extreme reduction in size of IRs [<1 kb, typically ranging from 15 to 30 kb in land plants (Zhu et al., 2016)]; (ii) an intermediate reduction of IR (~7 kb) with translocation of some typical LSC genes to the IR; and (iii) a structure with a divergent IR structure and a surprisingly reduced plastome (~107 kb), being now the putative smallest plastome known for an obligately photosynthetic angiosperm (Solórzano et al., 2019). However, considering these dissimilar patterns between the few described plastomes of cacti, a broad sampling including other lineages may shed new insights into chloroplast genome evolution across the family.

The classification of Cactaceae has been long proposed based on morphological characters (Schumann, 1899; Britton and Rose, 1919; Backeberg, 1958; Hunt et al., 2006), and further tested with the aid of molecular phylogenies (Nyffeler, 2002; Bárcenas et al., 2011; Hernández-Hernández et al., 2011). Three major well-supported clades are currently circumscribed as subfamilies: Opuntioideae, Maihenioideae and Cactoideae, while the traditional "Pereskioideae" has been revealed as a basal grade including the two leafy lineages of the cacti, which are subsequent sisters to the rest, i.e., *Leuenbergeria* Lodé and *Pereskia* Mill. (Edwards et al., 2005, reviewed in Guerrero et al., 2019). Opuntioideae (~350 spp.) is the most widespread subfamily with members occurring from southern South America (Argentina) to northern North America (Canada) (Britton and Rose, 1919; Anderson, 2001; Hunt et al., 2006; Ritz et al., 2012; Majure and Puente, 2014; Majure et al., 2019). The group shows interesting morphological synapomorphies, such as the small brushlike, barbed spines (i.e., glochids) and a bony aril surrounding a campylotropous ovule (Stuppy, 2002; Taylor et al., 2002). However, the delimitation of taxa within Opuntioideae is still not settled, and the controversy is observed across different taxonomic levels, from species to tribes (Schumann, 1899; Britton and Rose, 1919; Hunt, 2002; Stuppy, 2002; Taylor et al., 2002). Traditional

classifications based on general morphology – such as growth form, stem and leaf morphology, as well as floral, fruit, pollen, and seed characters – were used to divide the subfamily from few to up to 20 smaller genera (Britton and Rose, 1919; Stuppy, 2002; Hunt et al., 2006; Griffith and Porter, 2009). Nonetheless, molecular phylogenetic studies, mainly based on chloroplast (*rpl16* intron and *trnL-trnF* region) and nuclear ribosomal ITS sequences, revealed that the most comprehensive genus, *Opuntia* s.l. (L.) Mill., was paraphyletic, which reinforced the recognition of numerous smaller genera corresponding to well-supported clades (Stuppy, 2002; Taylor et al., 2002; Wallace and Dickie, 2002; Griffith and Porter, 2009; Majure et al., 2012; Ritz et al., 2012; Majure and Puente, 2014). Likewise, the tribal classification of Opuntioideae has been controversial based on different approaches, with up to six tribes proposed (Hunt, 2011). While Doweld (1999) and Wallace and Dickie (2002) proposed five tribes, with different circumscriptions from each other — four were recognized as monophyletic in the last comprehensive molecular study of Opuntioideae (Griffith and Porter, 2009). Despite great improvement in our phylogenetic understanding in Opuntioideae (Griffith and Porter, 2009; Ritz et al., 2012; Majure et al., 2019), support for the relationships among those clades, as well as a better taxon sampling with more molecular markers, still needs to be strengthened.

Apart from the external and internal transcribed spacer (*ETS* and *ITS*) of the nuclear ribosomal repeats (NRR) and ppc marker, most molecular phylogenies of cacti have been historically based on a few plastid markers (*trnL-trnF*, *rpl16*, *trnK*, and *matK*) (Nyffeler, 2002; Edwards et al., 2005; Korotkova et al., 2010; Arakaki et al., 2011; Bárcenas et al., 2011; Demaio et al., 2011; Hernández-Hernández et al., 2011, 2014; Ritz et al., 2012; Bárcenas, 2016; Vargas-Luna et al., 2018). While these markers have shown to be potentially able to resolve some clades, some relationships are still lacking support (Nyffeler, 2002; Griffith and Porter, 2009; Bárcenas et al., 2011; Hernández-Hernández et al., 2011). In this case, next-generation sequencing (NGS) could be a useful tool, since it has transformed the study of non-model plant taxa in phylogenetic inferences with high throughput data allowing deep resolution across major plant clades (Xi et al., 2012; Ma et al., 2014; Gardner et al., 2016; Zong et al., 2019). NGS data are also showing to be extremely useful for discovering informative regions across genomes, for marker development (Wu et al., 2010; Dong et al., 2012; Ripma et al., 2014; Reginato et al., 2016; Abdullah et al., 2019), as well as to investigate chloroplast genome evolution (Dong et al., 2013; Mower and Vickrey, 2018; Yao et al., 2019). Nevertheless, this approach is still in its infancy across Cactaceae (Majure et al., 2019) and remains a path to be explored.

Here, we investigate the use of next-generation sequencing across Opuntioideae to address two major questions: (1) how homogenous is the content and arrangement of chloroplast genomes across the subfamily? and (2) how phylogenetically informative are chloroplast genome sequences for resolving major relationships among the clades of Opuntioideae? We used a combination of *de novo* and reference-guided assemblies to process genome skimming data: (i) assembling and characterizing the first chloroplast genome of an *Opuntia* species, *O. quimilo* K. Schum., (ii) investigating overall patterns of reference-guided assemblies and comparative chloroplast genome sequence analyses across the subfamily, (iii) inferring phylogenetic relationships with assembled sequences and (iv) surveying plastomes for highly informative phylogenetic markers for Sanger-based studies for future use.

MATERIALS AND METHODS

Taxon sampling, DNA extraction, and sequencing

All currently recognized genera in Opuntioideae (sensu Hunt et al., 2006, plus Majure et al., 2019 for *Grusonia* s.l.), with the exception of *Punotia* (see Ritz et al., 2012), were sampled with one accession per genus, resulting in a dataset of 17 taxa, which were sequenced via genome-skimming (Straub et al., 2012; Majure et al., 2019). All seven genera of tribe Opuntieae were included; five of the six genera in Tephrocacteae were sampled, and all five genera in Cylindropuntieae were included in our analyses. Three additional samples were selected as outgroup taxa [Cactoideae: *Parodia magnifica* (F. Ritter) F. H. Brandt and *Coryphantha macromeris* (Engelm.) Lem.; and *Pereskia*: *Pereskia sacharosa* Griseb.] based on previous studies (Arakaki et al., 2011; Hernández-Hernández et al., 2014). Plant materials were from wild collections or from the Desert Botanical Garden's living collection (see Supplementary Table S1 for details). DNA was extracted from silica-dried or fresh epidermal tissues using a standard CTAB incubation (Doyle and Doyle, 1987) followed by chloroform/isoamyl alcohol precipitation and silica column-based purification steps, as described in Neubig et al. (2014) and Majure et al. (2019). Whole genomic DNAs were quantified using the Qubit dsDNA BR Assay Kit and Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA, United States); high-molecular-weight DNA (>15 kb) samples showing no degradation were considered suitable and sent to Rapid Genomics LLC (Gainesville, FL, United States) for library preparation and high-throughput sequencing using the Illumina HiSeq X platform with 150 bp paired-end reads. A total of sixty samples were included per lane for sequencing.

De novo assembly and data processing for chloroplast genome sequences

Raw reads were imported into Geneious 11.1.5 (Biomatters, Auckland, New Zealand), and paired reads were set with an expected insert size of 300 bp calculated with BBMap using default setting (Bushnell, 2016). Low quality bases (Q < 20) were trimmed, and all reads shorter than 20 bp were discarded using BBDuk for quality control (Bushnell, 2016). Different methods were employed to assemble the chloroplast genome of the diploid *Opuntia quimilo*. First, a *de novo* assembly was performed with 40% of the reads using the Geneious *de novo* assembler (low/fast sensitivity option, plus default settings). A consensus sequence (with a majority threshold for sequence matching – fewest ambiguities) of each contig greater than 1,000 bp in length was saved. Considering that the Cactaceae plastomes already published have unusual rearrangements, we looked for plastid contigs searching those saved contigs against the *Portulaca oleracea* L. plastome (Portulacaceae, one of the closest relatives of Cactaceae; see Walker et al., 2018) (GenBank accession KY490694, Liu et al., 2017) using MegaBLAST (following parameters proposed from Ripma et al., 2014). Additional chloroplast genome *de novo* assemblies of *O. quimilo* were performed using a set of different pipelines, such as GetOrganelle (Jin et al., 2019) and NOVOPlasty (Dierckxsens et al., 2017) to cross-validate and compare among the assemblies. After checking convergence of the assemblies from the different pipelines and the plastid contig recovered from the Geneious *de novo* assembly, we used the NOVOPlasty circular contig for downstream analyses. Annotations were performed with GeSeq (Tillich et al., 2017), using default parameters to predict protein-coding genes by HMMER profile search and ARAGORN v1.2.38 (Laslett and Canback, 2004); tRNA genes were annotated with tRNAscan-SE v2.0 (Lowe and Chan, 2016), and BLAST searches were used to annotate ribosomal RNA (rRNA), tRNA, and DNA genes conserved in embryophyte plastomes (Wommack et al., 2008). All annotations were cross checked with the "Annotate from" feature in Geneious, transferring annotations with a 50% or greater similarity from the *P. oleracea* plastome, and eventual start/stop codons were manually adjusted with the "Open Read Frame (ORF)" feature from Geneious. The genes that had their structures affected by the insertion of internal stop codons and/or a small ORF, thus did not form their respective full coding sequence (CDS), were annotated as putative pseudogenes. The graphical representation of the *O. quimilo* circular annotated plastome was created in OGDRAW (Lohse et al., 2013; Greiner et al., 2019). To visualize changes in gene order and content, we compared the *O. quimilo* assembly with the canonical gene order of the *P. oleracea* plastome via multiple whole genome alignments

using MAUVE (default options, assuming colinearity; Darling et al., 2004). Boundaries between the IRa, IRb, LSC, SSC and putative inversions were visually checked in Geneious using an *in silico* approach adapted from Oliver et al. (2010).

Comparative chloroplast genome sequence analyses across Opuntioideae

The newly annotated plastome of *Opuntia quimilo*, with one of the inverted repeats (IRa) manually stripped to avoid data duplication, was then used for a reference guided assembly on the trimmed reads from all other taxa using Geneious mapper with a medium-low sensitivity iterating up to five times (adapted from Ripma et al., 2014). Each of the assemblies mapped had a majority threshold consensus sequence generated and annotations transferred from the *O. quimilo* reference, and manually adjusted. To identify highly variable regions across the subfamily, the 17 assembled Opuntioideae chloroplast genome sequences were compared using mVista (Frazer et al., 2004) in Shuffle-LAGAN alignment mode (Brudno et al., 2003) using the annotated plastome of *O. quimilo* as a reference. We also used the full chloroplast genome sequence alignment (see below) to calculate nucleotide diversity values (π) to detect highly variable sites among Opuntioideae chloroplast genome sequences. DNA polymorphism analysis was performed on DnaSP v.6.10 (Rozas et al., 2017) using the sliding window analysis with a step size of 200 bp and window length of 800 bp. Assembly maps of raw read coverages from Geneious mapper of each taxon to the *O. quimilo* plastome were also used to visualize and compare the gene content of the chloroplast genome sequences across the subfamily.

Phylogenetic analyses and informative regions

The assembled chloroplast genome sequences, obtained as described in the previous section, were aligned using MAFFT v. 7 with an automatic strategy search for algorithm selection (Katoh and Standley, 2013), using 200PAM scoring matrix and an open gap penalty of 1.53 (offset value 0.123). The alignment was manually examined for misaligned areas following a similarity criterion (Simmons, 2004). Sequence portions that contained gaps and/or ambiguities across more than 80% of the taxa were stripped using the "Mask Alignments" feature in Geneious. Phylogenetic inference was performed using Maximum Likelihood implemented in RAxML 8.2.4 (Stamatakis, 2014) in the CIPRES Portal (Miller et al., 2010). As RAxML is mainly designed to implement generalized time-reversible molecular models (GTR), we employed the GTR + G model for the entire sequence, which have been suggested for topological reconstruction skipping model selection (Abadi et al., 2019), and GTR + I + G is not recommended by Stamatakis (see RAxML

v8.2 manual) given the potential interaction between the I and G parameters. Support values were estimated implementing 1,000 bootstrap pseudoreplicates.

To identify and rank highly phylogenetically informative regions in the Opuntioideae plastomes, we split the full plastome alignment into protein coding sequences (cpCDS – pseudogenes were included here), non-coding sequences (cpNCDS) and intergenic spacers (cpIGS) using the annotated *O. quimilo* plastome. Each individual marker (cpCDS, cpNCDS, cpIGS) was extracted from the above-mentioned alignment, and a Maximum Likelihood tree was inferred with RAxML using GTR + G model (see reasons above) and 100 bootstrap replicates. For each marker, we report the number of variable sites, number of parsimony informative sites (PIS), mean sequence distance (under K80 model), alignment length, mean sequence length, mean bootstrap support and distance to the full chloroplast genome sequence tree (RF distance; Robinson and Foulds, 1981). The metrics were retrieved using functions of the R packages *ape* and *phangorn* (Paradis et al., 2004; Schliep, 2011). Markers were ranked by phylogenetic information using a weighted mean of relative values of the following metrics: number of variable sites (weight = 1), mean bootstrap (weight = 2) and distance to the full plastid tree (weight = 3). We designed primer pairs for the top five markers identified in the previous step with suitable size for PCR amplification (< ~900 bp). Primers flanking the target regions were designed with Primer3, using the default settings (Rozen and Skaletsky, 2000). All metrics reported, as well as primer design, were considered only for the ingroup (the 17 Opuntioideae chloroplast genome sequences). Further phylogenetic inferences (RAxML, GTR + G, 1000 bootstrap), were performed for a dataset concatenating: (1) the top five markers, (2) the top 10 markers, and (3) the five markers which have primers designed.

RESULTS

DNA Sequencing

Runs on Illumina HiSeq X resulted in 227,003,814 reads from 20 samples (17 Opuntioideae and three outgroups), between 5,624,110 and 20,219,350 reads per sample, for a mean read number of 11,350,190 sequences. Reads per sample following quality control were between 5,360,990 and 19,863,298 with a mean post-quality control read pool number of 11,084,834. The GC content of the raw reads ranged from 37.4 to 40.6% with a mean of 38.45% and following quality control

were between 36.9 and 40% with a mean of 38%. Detailed results with the number of raw reads, post-quality control and %GC content per taxa are presented in Supplementary Table S1.

Opuntia quimilo plastome

The complete chloroplast genome of *Opuntia quimilo* was sequenced, assembled, annotated and deposited in GenBank (accession number MN114084). The length of the *Opuntia quimilo* plastome is 150,374 bp, including a 101,475 bp LSC region, a 4,115 bp SSC region and 22,392 bp of two IR (IRa and IRb) regions (Figure 1 and Table 1). A total of 701,318 reads were assembled, with an average organelle depth of 844x. The GC content varies from 33% in the SSC, to 35.5% in LSC and 39.6% in the IR regions, while 38% in coding regions (CDS) and 35.6% in non-coding regions (Table 1).

The *de novo* assembly of the Geneious assembler produced 1,000 contigs; of these, 988 were higher than 1,000 bp in length from a minimum length of 1,026 bp to a maximum of 283,150 bp. MegaBLAST search found one consensus plastid contig of 128,909 bp that included the full chloroplast sequence with two putative inverted repeats assembled as a single IR unit (~22 kb). The GetOrganele and NOVOPlasty pipelines both yielded one plastid contig of 150,374 bp with the same gene content, order, and structure as the plastid contig of the Geneious assembler, except for the two inverted repeats that were interleaved by the LSC and SSC on the first ones, while in Geneious these were merged as one IR.

The *Opuntia quimilo* plastome encodes 87 protein-coding genes (CDS), 35 transfer RNA genes (tRNA) and eight ribosomal RNA (rRNA) genes, totaling 130 genes (Tables 1, 2). Three canonical CDS from angiosperm chloroplast genomes were annotated as putative pseudogenes (Ψ) based on their structure: *accD*, *ycf1*, and *ycf2*. Two of them (*accD* and *ycf2*) are in the LSC, and *ycf1* in the IRs. Duplicated CDS in the IRs included *ndhA*, *ndhF*, *ndhG*, *ndhH*, *ndhI*, *rpl32*, *ycf1*(Ψ), and *rps15*; and all four rRNA genes and five of the 35 tRNAs were duplicated in the IR regions. The *O. quimilo* plastome includes 16 intron-containing genes, of which 15 contain one intron (*atpF*, *ndhA*, *ndhB*, *petB*, *petD, rpl16, rpoC1, rps12*, *rps16, trnA*^{UGC}, *trnG*^{UCC}, *trnI*^{GAU}, *trnK*^{UUU}, *trnL*^{UAA}, *trnV*^{UAC}), while one gene contains two introns (*ycf3*), and the *clpP* gene has lost its two introns, reduced to an exon of 609 bp.

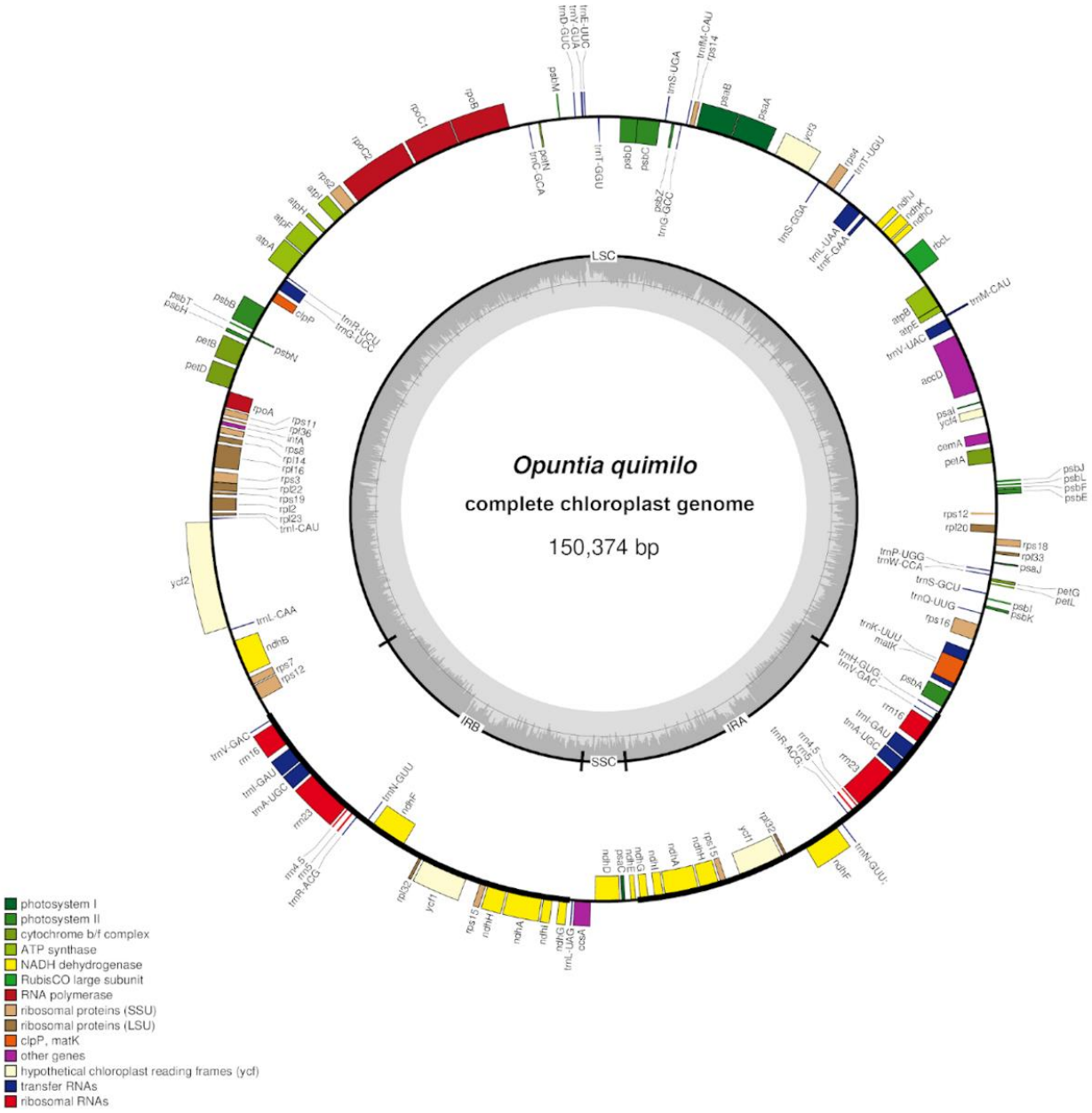

Figura 9 (Figure 1). Circular map of chloroplast genome of Opuntia quimilo with annotated genes. The genes transcribed clockwise are shown inside of the circle, whereas genes transcribed counter clockwise are shown outside of the circle. The borders of chloroplast genome are defined with LSC, SSC, IRa, and IRb. The dashed gray color of inner circle shows the GC content.

The LSC of the *Opuntia quimilo* plastome appears to have experienced an expansion, with surprisingly 101 kb, while the SSC was shown to have exceptional reduction (4 kb). The LSC contains 24 tRNA genes and 67 CDS, and the SSC contains a unique tRNA gene (*trnL*^{UAG}), and four CDS: *ccsA*, *ndhE*, *ndhD* and *psaC* (Figures 1, 2 and Tables 1, 2). A total of eight genes (*ndhB*, *rpl2*, *rpl23*, *rps7*, *rps19*, *trnI*^{CAU}, *trnL*^{CAA}, and *ycf2*) that are usually reported occurring in the IR regions of canonical angiosperm plastomes are apparently present as unique genes – not repeated – in the LSC region of the *O. quimilo* plastome (Figure 2, region V). On the contrary, seven genes (*ndhA*, *ndhF*, *ndhG*, *ndhH*, *ndhI*, *rpl32*, and *rps15*), usually from the SSC, are duplicated into the IR regions of the *O. quimilo* plastome (Figure 2, orange genes).

Tabela 2 (Table 1). Chloroplast genome composition of *Opuntia quimilo*.

Region	Size (bp)	GC (%)	Genes	CDS	tRNA	rRNA
Genome	150.374	36.6	130 (3)	87 (3)	35	8
LSC	101.475	35.5	91 (2)	67 (2)	24	0
SSC	4.115	33	5	4	1	0
IRa	22.392	39.6	17 (1)	8 (1)	5	4
IRb	22.392	39.6	17 (1)	8 (1)	5	4

The number in parentheses indicates pseudogenes (Ψ) identified.

When compared to the canonical angiosperm chloroplast genome of *Portulaca oleracea*, two block translocations in the LSC are present in the *O. quimilo* plastome: the first (Figure 2, region II) is a simple colinear translocation of nine genes (Figure 2, region II); while the second one is a big block inversion and translocation comprising 50 genes within the *trnG*^{UCC}*-psbE* region (Figure 2, region III). Inside that block (region III), the putative synapomorphic inversion of cacti encompassing the *trnM-rbcL* genes is confirmed for Cactaceae, but in the *O. quimilo* plastome this inversion also encompassed the *trnV*^{UAC} gene (Figure 2, green bars). Further gene order is mainly colinear (Figure 2, regions I, IV, V, VI, VII), except for the rearrangement comprising the SSC genes that were transferred to the IR regions, including a double inversion on the *ycf1-rpl32* region, placing *ycf1* gene adjacent to *rpl32* (Figure 2, orange genes).

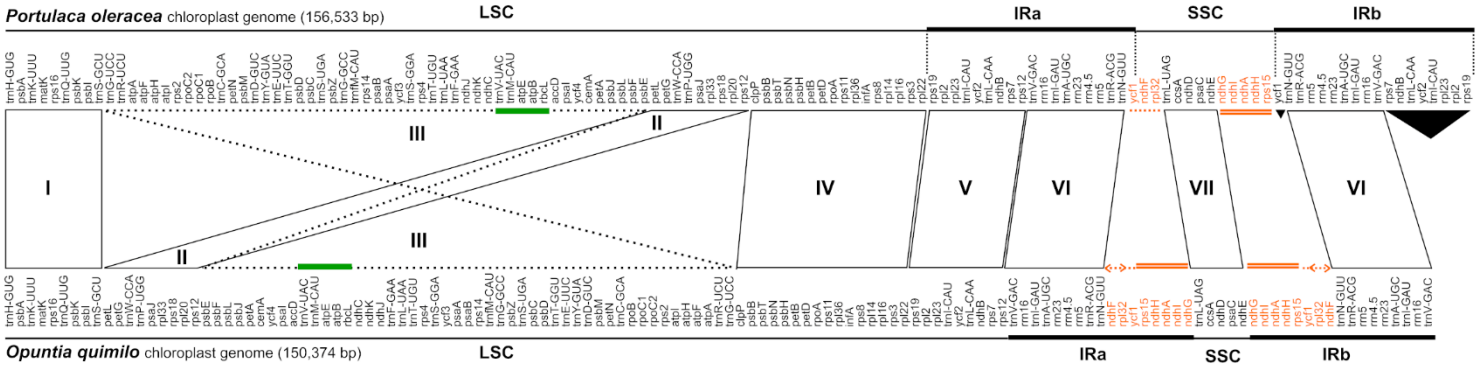

Figura 10 (Figure 2). Plastid genome structure and gene order *in Opuntia quimilo* compared with purslane (*Portulaca oleracea*). Purslane has the canonical order typical of most angiosperms. For simplicity, the circular map has been linearized. Green line highlights the *trnM*^{CAU}*-rbcL* synapomorphic inversion of Cactaceae, which in *O. quimilo* also includes the *trnV*^{UAC} gene. Regions I, IV, V, VI, and VII are colinear in both plastomes. Region II is colinear but is translocated in the *O. quimilo* plastome, while region III is inverted and translocated. Region V comprise the genes that are typically in the IR region but are translocated to the large single copy in *O. quimilo*. Genes highlighted in orange are those typically found in the SSC but transferred to the IR region in *O. quimilo*. Orange dashed line indicate the double inversion on the *ycf1-rpl32* genes, placing *ycf1* gene adjacent to *rpl32*. Black triangles represent duplicated genes present in purslane but absent in *O. quimilo*; LSC, large single-copy region; SSC, small single-copy region; IR, Inverted repeat.

Tabela 3 (Table 2). Structural and functional gene composition of *Opuntia quimilo* chloroplast genome

Gene type	Region	Genes
1. Ribosomal RNA (rrn)	IRa & IRb	*rrn4.5, rrn5, rrn16, rrn23*
2. Transfer RNA (trn)	LSC	*trnC*^{GCA}, *trnD*^{GUC}, *trnE*^{UUC}, *trnF*^{GAA}, *trnfM*^{CAU}, *trnG*^{GCC}, *trnG*^{UCC}*, *trnH*^{GUG}, *trnI*^{CAU}, *trnK*^{UUU}*, *trnL*^{CAA}, *trnL*^{UAA}*, *trnM*^{CAU}, *trnP*^{UGG}, *trnQ*^{UUG}, *trnR*^{UCU}, *trnS*^{GCU}, *trnS*^{GGA}, *trnS*^{UGA}, *trnT*^{GGU}, *trnT*^{UGU}, *trnV*^{UAC}*, *trnW*^{CCA}, *trnY*^{GUA}
	SSC	*trnL*^{UAG}
	IRa & IRb	*trnA*^{UGC}*, *trnI*^{GAU}*, *trnN*^{GUU}, *trnR*^{ACG}, *trnV*^{GAC}
3. Proteins of small subunits of the ribosome (rps)	LSC	*rps2, 3, 4, 7, 8, 11, 12*, 14, 16*, 18, 19*
	IRa & IRb	*rps15*
4. Proteins of large subunits of the ribosome (rpl)	LSC	*rpl2, 14, 16*, 20, 22, 23, 33, 36*
	IRa & IRb	*rpl32*
5. DNA dependente RNA polymerase (rpo)	LSC	*rpoA, B, C1*, C2*
6. NADH dehydrogenase (*ndh*)	LSC	*ndhB*, C, J, K*
	SSC	*ndhD, E*
	IRa & IRb	*ndhA*, F, G, H, I*
7. Photosystem I (psa)	LSC	*psaA, B, I, J*
	SSC	*psaC*
8. Photosystem II (psb)	LSC	*psbA, B, C, D, E, F, H, I, J, K, L, M, N, T, Z*
9. Cytochrome b/f complex (pet)	LSC	*petA, B*, D*, G, L, N*
10. ATP synthase (atp)	LSC	*atpA, B, E, F*, H, I*
11. Rubisco (rbc)	LSC	*rbcL*

12. Maturase K	LSC	*matK*
13. Protease (clp)	LSC	*clpP*
14. Envelope membrane protein (cem)	LSC	*cemA*
15. Subunit of acetil-CoA-carboxylase (acc)	LSC	*accD*(Ψ)
16. C-type cytochrome synthesis (ccs)	SSC	*ccsA*
17. Translational initiation factor (inf)	LSC	*infA*
18. Hypothetical chloroplast reading frames (ycf)	LSC	*ycf2*(Ψ), *3***, *4*
	IRa & IRb	*ycf1*(Ψ)

(Ψ) Putative pseudogenes. *Gene containing one intron. **Gene containing two introns.

Reference-Guided Assemblies and Comparative Chloroplast Sequence Analyses

The reference-guided assembles of the remaining Opuntioideae and outgroup taxa to the *Opuntia quimilo* plastome (one inverted repeat stripped) mapped an average of 616,615 reads with a mean genome depth of 721x (Supplementary Table S2). The consensus sequence length varied between 126,925 bp [*Pereskiopsis diguetii* (F.A.C. Weber) Britton & Rose] to 129,181 bp [*Tacinga palmadora* (Britton & Rose) N.P. Taylor & Stuppy] and the GC content between 35.8% (*Pterocactus gonjianii* R. Kiesling) to 36.3% [*Austrocylindropuntia cylindrica* (Lam.) Backeb. and *Cylindropuntia bigelovii*] (Supplementary Table S2).

Pairwise comparison of divergent regions within the Opuntioideae chloroplast genome sequences using mVISTA with *O. quimilo* as a reference revealed both strikingly conserved and divergent regions across the chloroplast genome sequences (Figure 3). Overall, the alignment uncovered sequence divergence across assemblies, suggesting that chloroplast genome sequences are not conserved. Divergences were observed both in non-coding regions and coding regions. Among coding regions (CDS), non-conserved regions were frequent on genes of the *ndh* gene suite (i.e., *ndhA, ndhD, ndhE*, *ndhF*, *ndhG*, *ndhH, ndhI*, *ndhJ*) as well *clpP*, *ycf3* and particularly highlighted on *ycf1*, *ycf2*, and *accD* genes (Figure 3). Ten non-coding regions show substantial divergence, being

all intergenic spacers: *ndhE-psaC, rpl32-ndhF, trnV*^{GAC}*-rps12, psbB-clpP, rpoB-trnC*^{GCA}*, psbM-trnD*^{GUC}*, trnT*^{GGU}*-psbD, psbE-rpl20, ndhC-rbcL* (Figure 3).

The nucleotide diversity values (π) within the 17 Opuntioideae chloroplast genome sequences ranged from 0.00191 to 0.18551, with a mean value of 0.02201, indicating the sequences as highly variable. Three major regions were identified as hypervariable (π > 0.1), which comprises *ycf1* and *accD* genes and an intergenic spacer *rpl32-ndhF* (Figure 4); while six regions were observed as moderately-variable (π > 0.05), those being four genes: *ycf2*, *ccsA*, *clpP* and *trnL*^{UAA}; and two intergenic spacers *rps18-rpl33* and *trnF*^{GAA}*-ndhJ* (Figure 4).

Reference-guided assembled maps of Opuntioideae and outgroups to the *Opuntia quimilo* chloroplast genome as a reference revealed regions with extremely low coverage or even gaps across different taxa (Figure 5). The regions highlighted with this feature are related with genes of the *ndh* suite, *ycf1*, *ycf2* and *accD*, suggesting gene loss, transfer to nuclear genomes and/or pseudogenization (Figure 5). Several members of Opuntioideae appear to have missing *ndh* genes in their chloroplast genome (*Micropuntia, Maihueniopsis, Pterocactus, Tephrocactus*), especially in the Tephrocacteae clade, but without a clear pattern across lineages.

Phylogenetic Analyses and Informative Regions

The full chloroplast genome sequences resulted in an alignment of 118,930 bp with 86,484 identical sites (72.7%), a pairwise identity of 94.5% and 8,694 distinct alignment patterns. There were 8,922 parsimony informative sites (PIS) and 11,509 sites with gaps. Maximum Likelihood analyses resolved a well-supported Opuntioideae (bs = 100), with three major subclades (those currently circumscribed as tribes), Opuntieae, Cylindropuntieae and Tephrocacteae (Figure 6A). Opuntieae, consisting of the seven genera *Consolea*, *Brasiliopuntia*, *Tacinga*, *Opuntia, Miqueliopuntia*, *Salmonopuntia* and *Tunilla*, was resolved as sister to a Tephrocacteae (*Tephrocactus*, *Maihueniopsis*, *Pterocactus*, *Cumulopuntia*, and *Austrocylindropuntia*) + Cylindropuntieae (*Quiabentia*, *Pereskiopsis*, *Micropuntia*, *Grusonia*, and *Cylindropuntia*) clade. All nodes had full bootstrap support values (bs = 100), except at two nodes, which were still higher than 90% (Figure 6A).

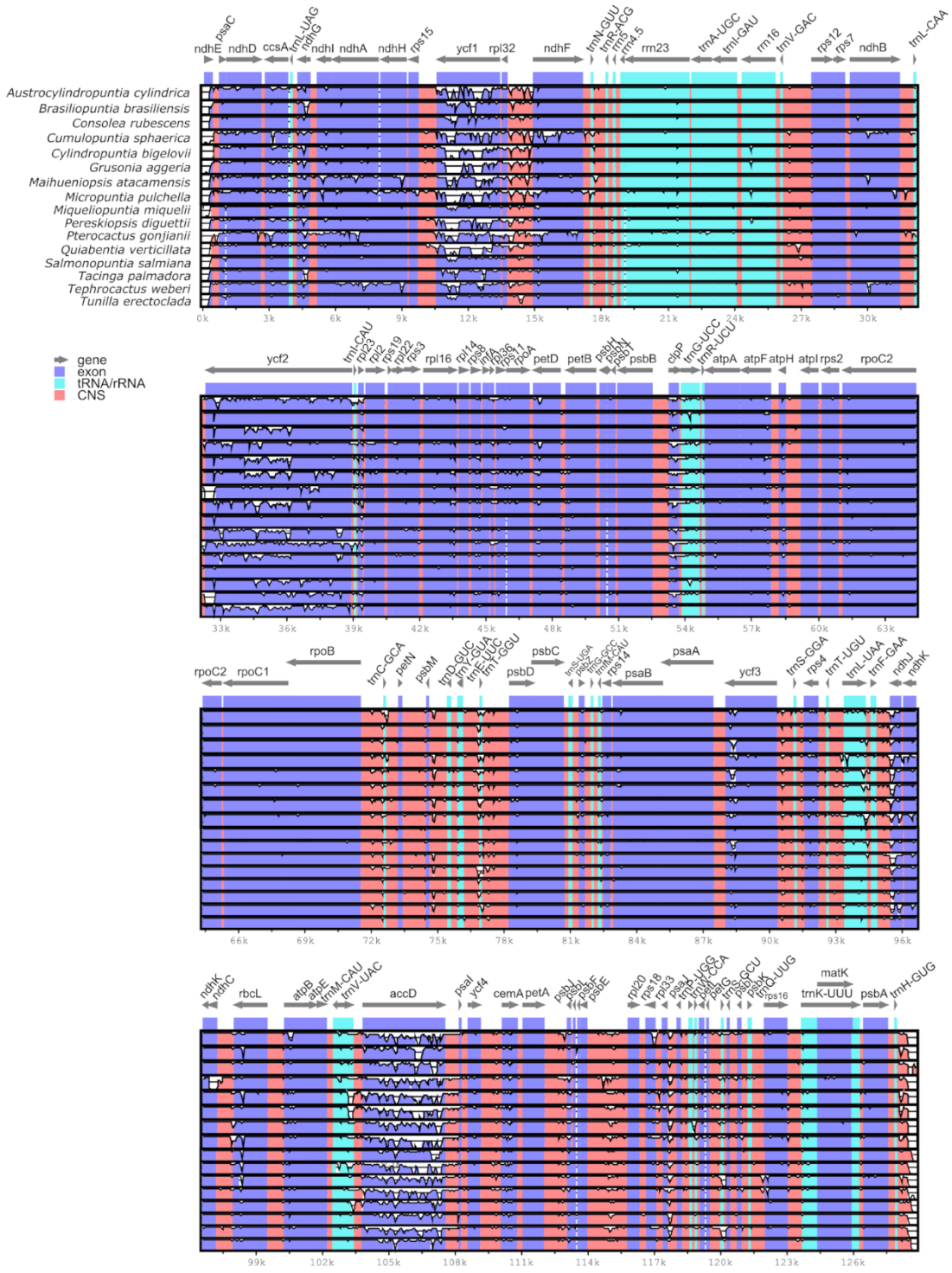

Figura 11 (Figure 3). Visualized alignment of the Opuntioideae chloroplast genome sequences (one IR stripped) with annotations using mVISTA. Each horizontal lane shows the graph for the sequence pairwise identity with Opuntia quimilo as reference. The x-axis represents the base sequence of the alignment and the y-axis represents the pairwise percent identity within 50–100%. Gray arrows represent the genes and their orientations. Dark-blue boxes represent exon regions; light-blue boxes represent tRNA and rRNA regions; red boxes represent non-coding sequence (CNS) regions.

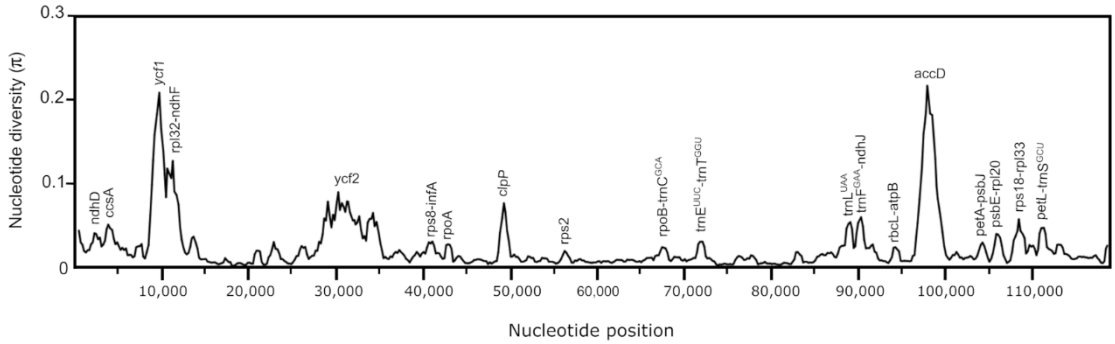

Figura 12 (Figure 4). Nucleotide diversity graphs of the 17 Opuntioideae chloroplast genome sequences from the sliding windows analysis performed in DnaSP (windows length: 800 bp, step size: 200 bp). The x-axis represents the base sequence of the alignment, and the y-axis represents the nucleotide diversity (π value). Each variation hotspot for the chloroplast genome sequences of the Opuntioideae alignment is annotated on the graph.

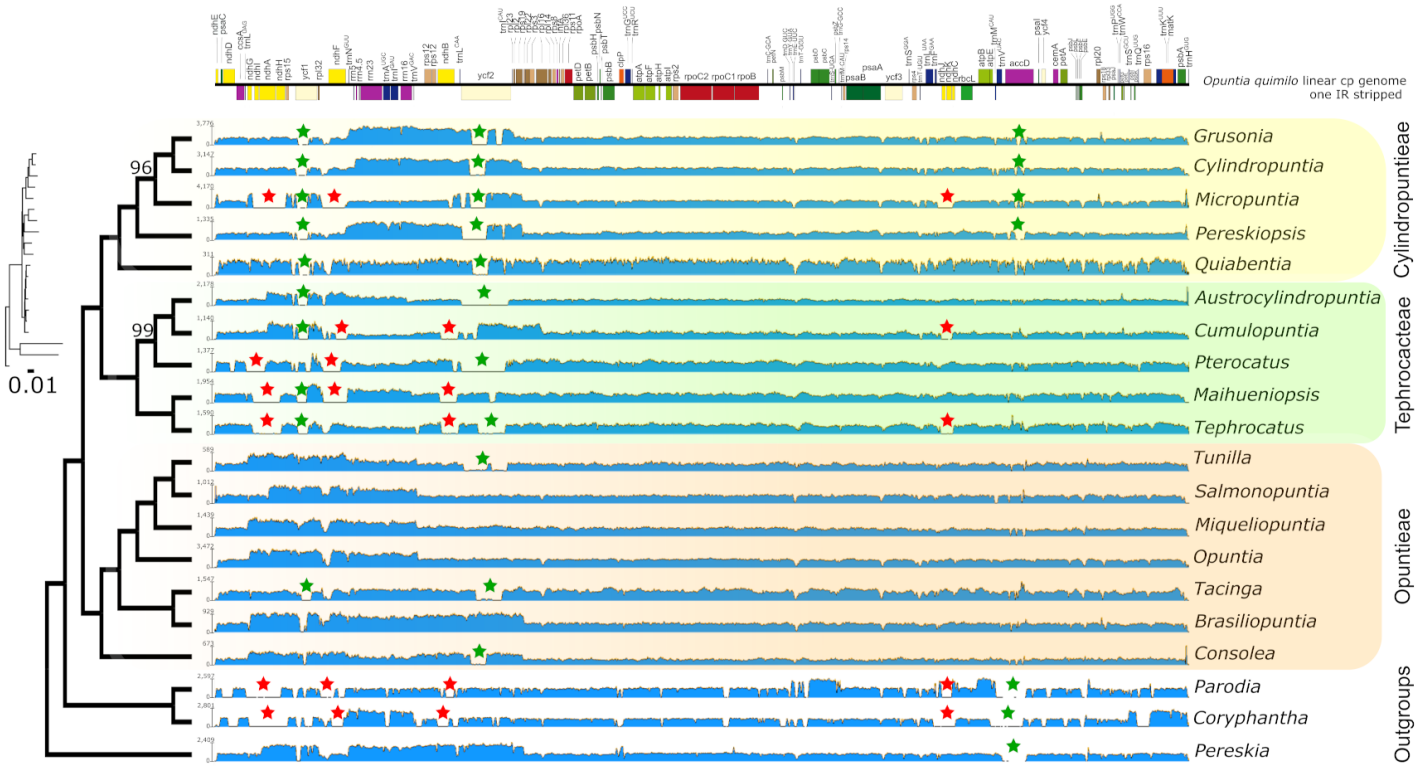

Figura 13 (Figure 5). Maximum likelihood phylogenetic tree from RAxML analysis transformed in cladogram with the phylogram represented in small size with substitution rate scaled. All nodes have total bootstrap values (bs = 100) with exception for those that are shown above the branch. Each tip is represented with the assembly map of raw read coverages from Geneious mapper to the *Opuntia quimilo* chloroplast genome (one IR stripped, represented on the top with annotated genes). Red stars represent low coverage mapping and putative losses associated with the ndh gene suite; green stars represent partial low coverages associated with putative pseudogenization of *ycf1*, *ycf2*, and *accD* genes. Tribe Opuntieae is highlighted in orange, Tephrocacteae in green and Cylindropuntieae in yellow.

The summary statistics for all markers (cpCDS, cpNCDS, cpIGS) are presented in Supplementary Table S3. A list of the top 10 markers ranked by phylogenetic information considering topological distance to the plastome tree, mean bootstrap support and number of parsimony informative sites is given in Table 3. All single marker phylogenies presented some disagreement to the plastome tree (RF tree distance ranging from 6 to 28), with bootstrap support ranging from 0 to 89 (mean = 37), and number of PIS from 0 to 619 (mean = 25), revealing many markers as not useful for phylogenetic inference (Supplementary Table S3). Phylogenetic trees of the top 10 individual markers are shown in Supplementary Figures S1, S2, and all trees are available as Supplementary Material. Primer pair sequences for PCR amplification are provided for the top five markers with suitable Sanger sequencing size (maximum ~900 bp) in Table 4.

Tabela 4 (Table 3). Summary statistics for the top 10 markers. Markers are ranked by phylogenetic information based on a weighed mean of relative values of number of variable sites (weight = 1), mean bootstrap (weight = 2) and distance to the full plastid tree (weight = 3).

	Bp	Aligned (bp)	Variable	PIS	Sites with gaps	Tree distance	Bootstrap (mean)
1. accD (cpCDS)	1876 [1489-1927]	1953	966	586	616	10	88
2. ycf1 (cpCDS)	1565 [1414-1615]	1650	958	429	456	8	76
3. ndhD (cpCDS)	1421 [1410-1421]	1421	210	52	11	6	79
4. trnK^{UUU} (cpNCDS)	2570 [2564-2572]	2573	173	45	15	8	82
5. psbE-rpl20 (cpIGS)	1731 [1714-1736]	1739	242	68	83	8	77
6. petD (cpCDS)	1265 [1257-1272]	1274	69	27	27	8	75
7. ccsA (cpCDS)	1008 [1007-1011]	1011	110	49	4	8	73
8. clpP (cpCDS)	359 [356-362]	362	112	64	6	8	70
9. rpoC2 (cpCDS)	4101 [4101-4101]	4101	165	47	0	8	69
10. rpoC1 (cpCDS)	2468 [2467-2469]	2469	86	35	4	8	69

Tabela 5 (Table 4). Primer pair sequences for the identified top 5 highly informative markers across the 17 chloroplast genome sequences of Opuntioideae.

Marker	Primer forward (5'–3')	Primer reverse (5'–3')	T_a (°C)	Expected product size (bp)
psbB/clpP	ACCAAGGCAAACCCATGGAA	TCCCCTTCTTACCAGCATCA	60	931
ycf4-cemA	GTCCTATTTCCTGCGTGTACCA	TGATAGAGAGATCCACCAGGGT	60	864
rps2	TTGAGATTCAGGAATAGTAACCGA	GTGTATCAATGGCCAATCCGC	57	885
rbcL-atpB	CAAAACAACAAGGTCTACTCGACA	GGAAACCCCAGGACCAGAAG	60	830
petA	ACGATTGATTGGACCATGCA	TCGGACAATTGAACCTTCTCGA	60	965

Phylogenetic inferences from the top 5 and top 10 markers concatenated yield similar topologies compared with the plastome tree, supporting three tribes (bs = 100) and Opuntieae as sister to (Tephrocacteae + Cylindropuntieae), although, there were minor incongruences within Tephrocacteae and Opuntieae (Figures 6B,C). Contrarily, phylogenetic inference from the five markers, which had primers designed (< ~900 bp) revealed a conflicting topology, with Cylindropuntieae as sister to (Tephrocacteae + Opuntieae) with high support (bs = 92), and all three tribes with full support (bs = 100) (Figure 6D).

DISCUSSION

Insights from chloroplast genome assemblies in Opuntioideae and Cactaceae

The first chloroplast genome of a member of subfamily Opuntioideae and a species of *Opuntia* is here reported. Although the bulk of its gene content is not far from canonical angiosperm plastomes, it deviates in some cases from the typical chloroplast genome structure, showing: (i) an expansion of the LSC incorporating genes that are typically in the IRs; (ii) a reduction of the SSC translocating some common genes of the SSC into the IR region; and (iii) at least one massive translocation with an inversion of a block of genes in the LSC (Figure 2). Part of the content of the IRs in the *O. quimilo* plastome remained remarkably constant, including all four rRNA and five tRNA genes that are nearly universally reported in IRs of land plants (Mower and Vickrey, 2018). The GC content observed in the *O. quimilo* plastome is regular as expected based on other

chloroplast genomes, being an AT rich organelle, with differences observed between coding/non-coding regions, where selection may be acting to preserve GC content for amino acid coding (Raubeson and Jansen, 2005; Downie and Jansen, 2015; Daniell et al., 2016).

Successive expansion–contraction events or even multiple contractions have been recurrently reported as one of the main ways of developing structural changes across angiosperm plastomes (Downie and Jansen, 2015; Daniell et al., 2016; Fonseca and Lohmann, 2017; Weng et al., 2017; Mower and Vickrey, 2018) and may also be one way in which genes are translocated to different regions of the genome, as suggested in adzuki bean (Perry and Wolfe, 2002). The atypical reduction of the SSC (~4 kb), reported here for the *O. quimilo* plastome, has also been noticed in *Viviana marifolia* (Francoaceae, Geraniales), and a slightly similar reduced size for the SSC (~6 kb) have been inferred for the ancestral chloroplast genome of Geraniaceae (Weng et al., 2014). A partial deletion of the SSC region has also been reported in two hemiparasitic *Taxillus* (Loranthaceae) species resulting in a ~6 kb region with only two genes (Li et al., 2017), and the smallest SSC hitherto reported is for the hemiparasitic *Pedicularis ishidoyana* (Orobanchaceae), with only 27 bp (Cho et al., 2018). A model to explain the major rearrangements observed in the *Lamprocapnos spectabilis* (Papaveraceae) plastome, involving at least six IR boundary shifts and five inversions resulting in a SSC of just 1,645 bp with a partial *ndhF* gene, was recently provided by Park et al. (2018). The SSC contains most *ndh* genes, and previous studies have shown that boundary shifts of the IR and SSC regions are correlated with transformations of *ndhF* and *ycf1* genes (Logacheva et al., 2014; Kim et al., 2015; Li et al., 2017).

The *Opuntia quimilo* plastome reinforces some different putative structural synapomorphies that have been reported in Caryophyllales. The loss of the *rpl2* intron, previously suggested to be absent throughout the Centrospermae (Palmer et al., 1988), is supported in our study and other newly assembled plastomes in Caryophyllales (Yao et al., 2019). The *trnM-rbcL* inversion is again recovered in the *O. quimilo* plastome, although also involving the $trnV^{UAC}$ gene, as in *Cylindropuntia bigelovii* (Majure et al., 2019), providing further support for this inversion as a synapomorphy in the family. Additionally, Sanderson et al. (2015) and Solórzano et al. (2019), inspecting plastomes of Cactoideae, reported a gene orientation of *ycf2-*$trnL^{CAA}$*-ycf1* in the SSC as a synapomorphy of Cactoideae. Our results corroborate this observation, since this feature is not present in the *O. quimilo* plastome, strengthening this gene order as a putative synapomorphy for Cactoideae. On the other hand, the *ycf1-rpl32-ndhF* orientation, reported in the *Cylindropuntia bigelovii* chloroplast sequence (Majure et al., 2019), is recovered in the *O. quimilo* plastome and is here suggested as a putative synapomorphy for Opuntioideae.

Reference-guided assemblies and comparative analyses revealed insights for plastome rearrangements across disparate Opuntioideae. The differences of depth and coverage among specific chloroplast genes suggest that gene presence or structure may vary over species in Opuntioideae, as have been observed in other Cactaceae, specifically Cactoideae (Sanderson et al., 2015; Solórzano et al., 2019). The putative independent losses of several *ndh* genes in all Cactoideae plastomes assembled hitherto, such as the saguaro cactus and several *Mammillaria* species, can be also inferred for our Cactoideae outgroups sampled (*Parodia magnifica* and *Coryphantha macromeris;* Figure 5, red stars). Likewise, some members of Cylindropuntieae and Tephrocacteae (*Micropuntia*, *Cumulopuntia*, *Pterocactus*, *Maihueniopsis*, and *Tephrocactus*) also likely experienced independent losses of several genes of the *ndh* suite in their chloroplast genomes, although this was not so for tribe Opuntieae, where those genes were found to be intact (Figure 5, red stars), indicating putative homoplasious events. The putative loss of one of the inverted repeat regions in *Quiabentia* must be further investigated through rigorous *de novo* assemblies (Figure 5).

Loss of *ndh* genes or the *ndh* gene suite has been reported in both gymnosperms (Wakasugi et al., 1994; McCoy et al., 2008; Wu et al., 2009) and angiosperms, as well as some other photosynthetic organisms. The loss of such genes is well-known and is often associated with hemi- or holoparasitism where genes necessary for photosynthesis are often unessential (e.g., *Epifagus*, Orobanchaceae, De Pamphilis and Palmer, 1990; Santalales, Shin and Lee, 2018). However, a number of autotrophic plants have also shown a similar trend with losses or pseudogenization of *ndh* genes. For example, Lin et al. (2017) showed the repeated loss of *ndh* genes across several different autotrophic orchid species and suggested that those losses could have been a step toward heterotrophy. Ruhlman et al. (2015) suggested that the evolution and retention of the NDH (NADH dehydrogenase-like) complex was associated with the transition of plants to environmentally stressful environments, and that *ndh* gene loss may be associated with a relaxed reliance on the complex based on decreased environmental stressors (e.g., through reliance on host species for resources in parasites).

Contrastingly, there are numerous reports of *ndh* loss or pseudogenization in angiosperms associated with the presence of CAM photosynthesis, which has evolved as a response to water limited habitats (i.e., water stress), such as in desert or other edaphically arid areas where cacti occur or also associated with an epiphytic habit, for instance in orchids (Luo et al., 2014; Givnish et al., 2015; Sanderson et al., 2015). Whether or not the absence of those *ndh* genes in the chloroplast corresponds to their integration into the nuclear genome often remains to be determined, but there are some studies showing that those genes likewise, have not been

incorporated into the nucleus (Lin et al., 2017) and thus are totally lost. Certain species of Opuntioideae have been shown to be facultatively CAM species (Winter et al., 2011), whereas other species appear to be obligate CAM. Perhaps the putative loss or pseudogenization of *ndh* genes across members of Opuntioideae coincides with the transition to more water limited habitats and thus a stronger association with obligate CAM photosynthesis. Although assumed that most derived cacti (Cactoideae, Opuntioideae) are obligate CAM, there are actually very little data to show photosynthetic pathways across Cactaceae, and the retention of large leaves in Opuntioideae bring into question this assumption (Majure et al., 2019). Likewise, our knowledge of CAM photosynthesis is in a state of flux, and it is clear that there are taxa that do not clearly fit into basic photosynthetic pathways as traditionally defined (Edwards, 2019). The putative connection with *ndh* gene loss and CAM photosynthesis needs to be rigorously tested.

The major plastid regions marked by pseudogenization in the *Opuntia quimilo* plastome (*ycf1*, *ycf2*, and *accD*) are visually highlighted as non-conserved regions in reference-guided maps (Figure 5, green stars), as in the mVista alignment across Opuntioideae (Figure 3). These regions are also emphasized as with hyper or moderate variability regarding nucleotide diversity values (Figure 4). All genes here reported as pseudogenes in the *O. quimilo* plastome (*accD*, *ycf1*, and *ycf2*) have also been reported as pseudogenes in the *Mammillaria* plastomes (Solórzano et al., 2019), while the *accD* was described as a pseudogene in *Carnegiea gigantea* (Sanderson et al., 2015). Pseudogenization of these genes has been repeatedly reported across different angiosperm lineages, such as Malpighiales, Campanulales, Ericales, Poales, Solanales, Geraniales, Santalales, and Myrtales (Haberle et al., 2008; Fajardo et al., 2013; Harris et al., 2013; Weng et al., 2014; Li et al., 2017; Machado et al., 2017; Bedoya et al., 2019; Cui et al., 2019). Even though these genes have been identified with essential functions beyond photosynthesis and retained in the plastome of most embryophytes (Drescher et al., 2000; Kuroda and Maliga, 2003; Kode et al., 2005; Kikuchi et al., 2013; Parker et al., 2014; Dong et al., 2015), there are several other plants where these genes are missing from the chloroplast genome (Magee et al., 2010; Kim et al., 2014; Liu et al., 2016; Graham et al., 2017). The pseudogenization or loss of the *accD*, *rpl22* and several genes of the *ndh suite* from the plastids has been reported to be a consequence of them being transferred to the nuclear genome (Jansen et al., 2011; Jansen and Ruhlman, 2012; Sanderson et al., 2015; Liu et al., 2016; Cauz-Santos et al., 2017). Plastid gene transfer to the nucleus remains to be examined in *O. quimilo* and related Opuntioideae.

Several regions highlighted as hyper or moderately variable regarding the nucleotide diversity values across Opuntioideae chloroplast sequences (i.e., *accD, ycf1, clpP, petD, rpl32,* and *ccsA*) have

been reported to be putatively under positive selection in some lineages, such as Brassicaceae, Bignoniaceae, Rutaceae, Orchidaceae, Geraniaceae, and Poaceae (Carbonell-Caballero et al., 2015; Hu et al., 2015; Weng et al., 2016; Park et al., 2017; Dong et al., 2018; Piot et al., 2018; Ruhlman and Jansen, 2018; Thode and Lohmann, 2019). Positive selection may come into play in response to environmental changes (Piot et al., 2018). For example, the *accD* gene, which encodes the β-carboxyl transferase subunit of acetyl-CoA carboxylase, is an essential and required component for plant leaf development (Kode et al., 2005), and it is suggested to have played a pivotal role in the adaptive evolution of orchids (Dong et al., 2018). The signatures of positive selection in the *accD* gene observed in Brassicaceae and Campanulaceae have also indicated that this gene may have been repeatedly involved in the adaption to specific ecological niches during the radiation of eudicotyledonous plants (Rousseau-Gueutin et al., 2013; Hu et al., 2015). Considering the harsh environment that cacti occupy, their fitness already expressed in its peculiar morphology and physiology, further studies should be carried out to investigate the putative relation of chloroplast rearrangement – such as pseudogenization, loss of genes, translocation and inversion – with ecological features.

Phylogenetic Relationship of Opuntioideae Tribes

The plastome phylogeny of Opuntioideae strongly resolves three major and well-supported clades, the tribes Opuntieae (O), Tephrocacteae (T) and Cylindropuntieae (C) (Figure 6A). Three previously described tribes (Austrocylindropuntieae, Pterocacteae, and Pereskiopsideae) (Doweld, 1999; Wallace and Dickie, 2002), mainly representing lineages of a single genus, are nested within these more broadly circumscribed tribes, and thus have no real practical taxonomic use (Hunt, 2011).

In our phylogenomic analyses, Opuntieae was sister to a Tephrocacteae/Cylindropuntieae clade, as in Majure et al. (2019), who also used plastome data but with a reduced taxon sampling in Opuntieae and Tephrocacteae. This same topology [O + (T + C)] was further uncovered with high support using our top 10 and 5 phylogenetic informative markers concatenated (Figures 6B,C). On the other hand, Walker et al. (2018) and Wang et al. (2019), using transcriptome data, revealed Cylindropuntieae as sister to an Opuntieae/Tephrocacteae clade [C + (O + T)] yet with very limited taxon sampling. Likewise, this alternate topology [C + (O + T)] was recovered in our study when exploring our five markers concatenated, which have primer-pairs designed (Figure 6D), i.e., those best ranked markers with suitable size for PCR amplification (< ~900 bp) (see further discussion in the next section). Hernández-Hernández et al. (2011, 2014), as well as

Bárcenas et al. (2011), although recovering the same three tribes sampling few genera, did not resolve the relationships among them, while Nyffeler (2002) did not have sufficient taxon sampling to infer relationships within Opuntioideae. Thus, this recalcitrant relationship between the three tribes must be further investigated using more genealogies, such as nuclear, plastome and mitochondrial datasets.

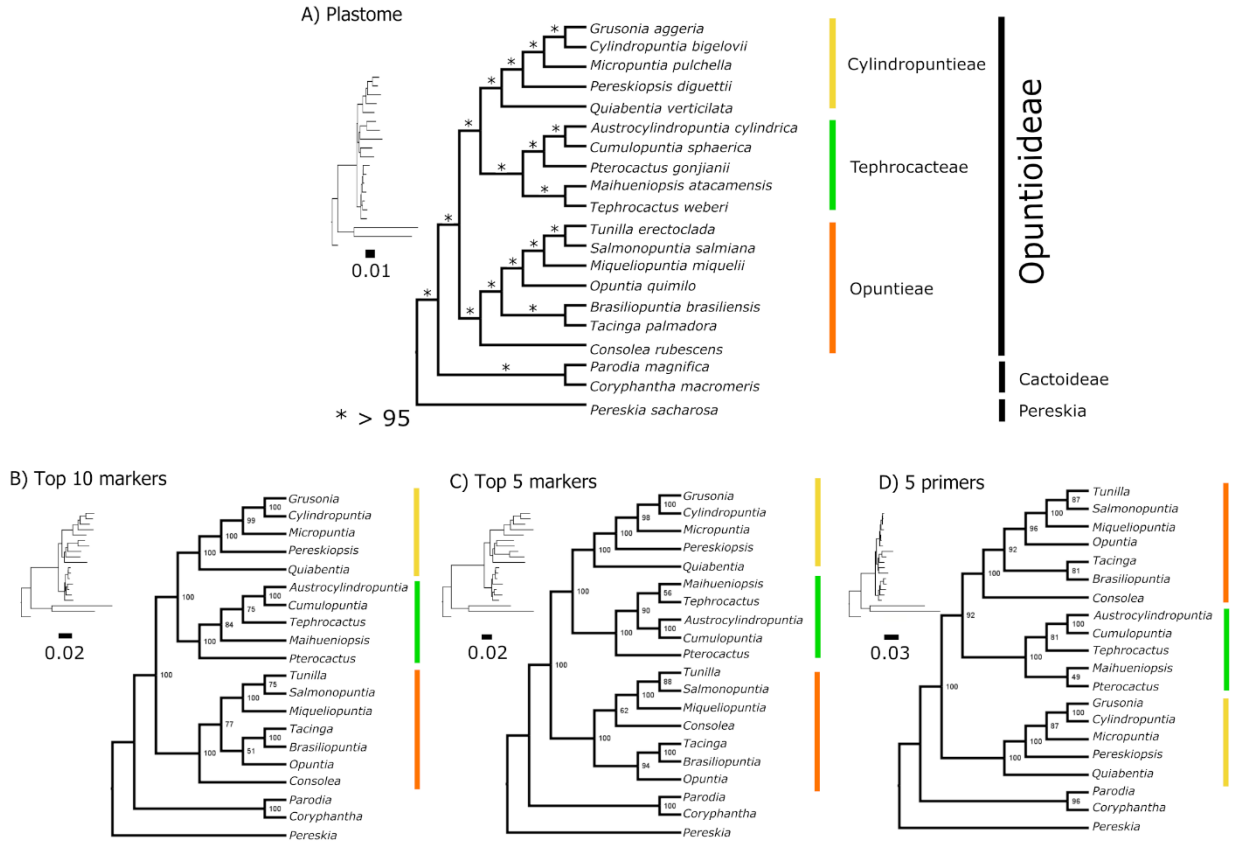

Figura 14 (Figure 6). Topological comparisons of different datasets based on ML analyses. **(A)** Plastome dataset topology, **(B)** top 10 marker dataset topology, **(C)** top five marker dataset topology, and **(D)** five marker dataset topology for which primers were designed. The Cylindropuntieae sister to Tephrocacteae + Opuntieae topology was recovered only in the five-marker primer dataset **(D)**. Generic relationships are highly variable in Tephrocacteae among the datasets used. *bootstrap support.

Griffith and Porter (2009) previously tackled relationships within Opuntioideae using DNA sequence data with a comprehensive sampling, yet based only on nrITS and *trnL-trnF* data. Our results partially recovered their topology, with a "flat-stemmed" and a "terete-stemmed" clade, moderately equivalent to our Opuntieae and Cylindropuntieae tribes, respectively. However, many members of Tephrocacteae recovered here were nested within their "terete-stemmed" clade, such as *Austrocylindropuntia, Cumulopuntia* and *Tephrocactus*. Likewise, the Griffith and Porter (2009) topology revealed a grade of two clades (*Pterocactus* and *Maihueniopsis*), which were sister to the rest of Opuntioideae but that was not recovered in our study. However, as our study is still based on one sample per genus, future studies including a wider sampling should be carried out across the subfamily to further test the relationships here recovered and are currently underway (Majure et al., in preparation).

Tribe Opuntieae is the most diverse and widespread clade among Opuntioideae, consisting of seven accepted genera and around 230 species (Majure and Puente, 2014; Guerrero et al., 2019). *Consolea* Lem., an endemic tree-like cactus of the Caribbean Islands and neighboring areas (Majure et al., submitted), is sister to the rest of Opuntieae, which consists of two subclades: (i) one comprising *Brasiliopuntia* (K. Schum.) A. Berger + *Tacinga* Britton & Rose; and the other comprising (ii) *Opuntia* (L.) Mill. + [*Miqueliopuntia* Friè ex F. Ritter + (*Salmonopuntia* P.V. Heath + *Tunilla* D.R. Hunt & Iliff.)]. Previous analyses did not resolve this position for *Consolea*, mostly based on the lack of data/data type (Griffith and Porter, 2009) and/or outgroup taxon sampling (Majure et al., 2012; Majure and Puente, 2014). Likewise, the sister relationship of *Opuntia* with the (*Salmonopuntia* + *Tunilla*) + *Miqueliopuntia* clade had not been recovered in previous analyses (Majure et al., 2012).

Based on our plastome analysis, Cylindropuntieae and Tephrocacteae are sister tribes, comprised of five and six genera, respectively (Figure 6A). Cylindropuntieae are primarily represented by genera that occur in the western North American desert regions [*Cylindropuntia* (Engelm.) F.M. Knuth, *Grusonia* F. Rchb. & K. Schum. and *Micropuntia* Daston], which formed a well-supported subclade, but they also contain two genera that are found in tropical dry forest of Mexico/Northern Central America (*Pereskiopsis* Britton & Rose) and Tropical Dry Forest and Chaco of South America (*Quiabentia* Britton & Rose). Tribe Pereskiopsideae (Doweld, 1999), previous described to only accommodate the leafy *Pereskiopsis*, is nested within Cylindropuntieae and is redundant, and thus unnecessary. Deeper relationships within Cylindropuntieae were recently untangled using a phylogenomic approach and dense sampling, revealing biogeographic patterns as well as characters evolution (Majure et al., 2019). Our plastome phylogeny here revealed

an identical phylogenetic pattern among genera (Figure 6A) of Majure et al. (2019), and equivalent to Bárcenas (2016).

Tephrocacteae is a South American clade adapted to diverse climatic conditions over a wide area of the southern Andes and adjacent lowlands (Ritz et al., 2012; Guerrero et al., 2019; Las Peñas et al., 2019). The tribe includes morphologically diverse species from geophytes and cushion-plants to dwarf shrubs, shrubs or small trees (Anderson, 2001); and probably geophytes and cushion-forming species evolved several times from shrubby-like precursors (Ritz et al., 2012). Tribes Austrocylindropuntieae and Pterocacteae (Wallace and Dickie, 2002) were described to circumscribe *Austrocylindropuntia* + *Cumulopuntia* and *Pterocactus*, respectively, and both are nested within the Tephrocacteae as amplified by Hunt (2011). So, as shown here, their use is mostly redundant. Although our plastome data recovered *Maihueniopsis* and T*ephrocactus* as sister to *Pterocactus* + (*Austrocylindropuntia* + *Cumulopuntia*), the phylogenetic topology among genera of the tribe are highly variable when using different datasets (Figures 6A–D). It is probable that increased taxon sampling may ameliorate this topological variability, as we still lack whole plastome data for the monospecific genus *Punotia*. Greater taxon and data sampling will be necessary to fully test these relationships.

Phylogenetically Informative Regions

Our plastome survey for phylogenetically informative markers revealed a list of potentially highly informative plastid markers for Sanger-based phylogenetic studies in Opuntioideae (Supplementary Table S3). The top 10 markers in our cpCDS dataset are: *accD, ycf1*, *ndhD, petD, ccsA, clpP, rpoC1, rpoC2*, including just one intron (the *trnK* intron comprising the *matK* gene – *trnK*/*matK*) and one intergenic spacer (*psbE-rpl20*) (Table 3). However, two of the better ranked markers (*accD* and *ycf1*) are putative pseudogenes and must be treated apart from traditional protein coding genes. The impact and utility of pseudogenes as markers for phylogenetic inferences must be further investigated (see below).

From the top 10 markers ranked in our list, just one (*trnK*/*matK*) has been used in more than one phylogenetic study in cacti (Nyffeler, 2002; Edwards et al., 2005; Korotkova et al., 2010; Arakaki et al., 2011; Bárcenas et al., 2011; Demaio et al., 2011; Hernández-Hernández et al., 2011, 2014; Ritz et al., 2012; Bárcenas, 2016; Vargas-Luna et al., 2018); while Majure et al. (2012) and Franck et al. (2013) used partial sequences of the *ycf1* gene. The other top 10 markers have been previously used under phylogenomic approaches in cacti (Arakaki et al., 2011; Majure et al., 2019).

Although the majority of the top 10 markers here reported have not been used in phylogenetic studies of cacti, the relationship of several other groups has been inferred with some of these markers. For example, the *accD* gene, combined with other plastid regions including *rpoC1*, was employed for phylogenetic inference of *Crocus* (Iridaceae), *Coptis* (Ranunculaceae) and Orchidaceae genera (Petersen et al., 2008; Guo et al., 2012; He et al., 2014). However, *accD* intergenic spacers, such as *rbcL-accD* and *accD-psaI*, have been much more widely used across disparate groups (Barfuss et al., 2005; Miikeda et al., 2006; Reginato et al., 2010; Sun et al., 2012; Michelangeli et al., 2013). The *ycf1* gene appears to be moderately used (Gernandt et al., 2009; Guo et al., 2012; Majure et al., 2012; Shi et al., 2013; Whitten et al., 2013; Dastpak et al., 2018), and increasingly reported to be a useful marker in phylogenetic inferences (Neubig et al., 2009; Neubig and Abbott, 2010; Dong et al., 2012; Thomson et al., 2018), and the most promising plastid DNA barcode of land plants (Dong et al., 2015). The *petD* intron has been used (Löhne et al., 2007; Worberg et al., 2007; Borsch et al., 2009; Scataglini et al., 2014), but in our analysis the entire gene was used (exon + intron) showing phylogenetic utility. The *ccsA* gene seems to be underexplored as a phylogenetic marker (Marx et al., 2010; Peterson et al., 2012) but was already suggested as convenient for phylogenetic inferences (Logacheva et al., 2007). The *rpoC1* and *rpoC2* genes have been occasionally used together as markers (Liston and Wheeler, 1994; Kulshreshtha et al., 2004) or combined with other markers (Downie et al., 2000; GPWS, 2001; Zhang et al., 2011; Guo et al., 2012) yielding satisfactory results. The *rpoC2* gene was recently found as the best performing marker to recover, with high levels of concordance, the "accepted tree" of the angiosperm phylogeny (Walker et al., 2019). The *ndhD* gene seems to be scarcely used for phylogenetic inference (Panero and Funk, 2002), while the intergenic spacer of *psbE-rpl20* genes has never been used individually to our knowledge.

Eight of the top 10 markers are more than 900 bp, indicating that longer genes are superior for phylogenetic reconstruction, as previous suggested by Walker et al. (2019), although they may require internal primer designing for complete Sanger sequencing. A list of the top 10 markers with less than 900 bp is reported (Supplementary Table S4), and primer pair design for the top five is provided in Table 4. Our phylogenetic inference from the top five markers concatenated, which had primers designed (Figure 6D) recovered a conflicting topology compared with the plastome tree (Figure 6A). The topology with Cylindropuntieae as sister to Tephrocacteae + Opuntieae has also been recovered based on transcriptome data (Walker et al., 2018; Wang et al., 2019). Curiously, we obtained this same topology, although not strongly supported, using the top 10 marker dataset

concatenated, when stripping the two pseudogenes *accD* and *ycf1* (Supplementary Figure S3), suggesting that functional constraints of these pseudogenes may influence the underlying topology.

Our top five markers contained intergenic spacers, which influence our alignment, wherein the incorporation of gaps is necessary. Duvall et al. (2020) found that as gaps increased in their alignment of plastomes across Poaceae, differing topologies were increasingly supported. This may also play a role in the incongruent topologies recovered in our analyses. Perhaps a higher level of homoplasy across datasets including gaps may reduce their suitability for resolving deep phylogenetic relationships, however, those same regions (i.e., intergenic spacers) are likely more appropriate for resolving species relationships among closely-related species (Shaw et al., 2005). Likewise, selective pressures on the genes in both our reduced 10 marker datasets, as well as in previously published transcriptome data (Walker et al., 2018), may likewise influence topology. Homoplasy in these reduced datasets may also be a factor leading to conflicting topologies. More research should be focused on the level of utility of specific gene regions (e.g., coding genes, intergenic spacers) across clades.

Chloroplast markers have been used for testing evolutionary relationships among plants for the past 30 years (Gitzendanner et al., 2018). While the assumption that these markers are evolving as a single unit without recombination, routine analyses have used concatenated data producing highly supported phylogenies that have been underlying the current classification of angiosperms (APG, 2016). However, as here reported, no marker as a single unit (gene tree) recovered the same topology of the plastome inference (concatenated tree), and even within the top 10 markers listed, some showed high values of discordance (Table 3, Supplementary Table S4, and Supplementary Figures S1, S2). Such results discourage and call attention to phylogenetic approaches based on one or few markers. While the full chloroplast sequences showed to be the most robust dataset to resolve relationships within Opuntioideae, phylogenies from the top 10 and 5 markers concatenated resolved many relationships with high bootstrap values and few nodes with low support (Figures 6B,C). Although we cannot test how effective these datasets would work in determining closely related species relationships, based on our limited taxon sampling here, it is significant that these smaller datasets resolve relationships among these clades and genera that have not been resolved previously using a similar number of loci (e.g., Hernández-Hernández et al., 2011). Thus, we would expect that using these more highly variable loci, although few, should greatly increase resolution across many subclades in Cactaceae. We also encourage their use across subclades within Cactoideae to test their broader utility.

Recent studies have explored gene tree conflict in plastome-inferred phylogenies and incongruence between gene trees and species trees in plastid genes (Gonçalves et al., 2019; Walker et al., 2019). Gonçalves et al. (2019) emphasized the importance of considering variation in phylogenetic signal across plastid genes and the exploration of plastome data to increase accuracy of estimating relationships; they also revealed that phylogenies inferred with multispecies coalescent (MSC) methods are accurate with plastome matrices and should be considered in future phylogenomic investigations. Walker et al. (2019) highlighted that most plastid genes are largely uninformative and are unlikely to misguide plant systematics. However, the concatenating of plastid genes without some level of scrutiny can mislead branch length estimation (Walker et al., 2019). The causes of discordant topologies across gene trees from chloroplast genome still needs to be better investigated. The main explanations include systematic errors (e.g., poor modeling, stochastic events) or more biologically meaningful processes, such as heteroplasmic recombination that have been invoked to explain discordance in disparate plant clades (Huang et al., 2001; Marshall et al., 2001; Bouillé et al., 2011; Walker et al., 2019).

CONCLUSION

Chloroplast genomes have long been considered conserved among land plants, but recent generation of 1000s of plastomes through NGS has illuminated that this is not always the case. Cactaceae are no exception to variation that has been observed in other clades. Previous plastomes of cacti have shown to have lost one copy of the inverted repeat regions and several genes of the *ndh* gene suite, as well as to possess divergent inverted repeat regions and the smallest chloroplast genome known for an obligately photosynthetic angiosperm. We showed that the *Opuntia quimilo* plastome also presents deviations of canonical angiosperm plastomes with an expansion of the LSC incorporating genes that are typically in the IRs, a reduction of the SSC translocating some common genes of the SSC into the IR region, and one massive translocation with an inversion of a block of genes in the LSC. Strikingly different from other cacti, two copies of the inverted repeat region were recovered in the *Opuntia quimilo* plastome. Our reference-guided assemblies across Opuntioideae allowed us to infer putative independent losses of some *ndh* genes across disparate taxa of the subfamily. We did not find synapomorphic plastome features within Opuntioideae clades, thus, we hypothesize that putative rearrangements across the subfamily are from homoplasious events. Further analyses should be carried out to address how ecological drivers and morphological traits of cacti may be related with positive selection of genes and the common

rearrangements in chloroplast genomes that have been reported in the family. Phylogenetic analyses of chloroplast genome sequences strongly support Opuntioideae and its three tribes: Opuntieae, Cylindropuntieae, and Tephrocacteae. As computational and budget limitations are still a bottleneck to deal with high throughput data, especially in developing countries, a list of highly informative plastid markers is presented for future use, and several top ranked markers have not been used in phylogenetic studies of cacti. However, conflicting topologies were recovered among major clades when exploring different assemblies of markers, revealing that gene tree discordance among markers must be carefully considered while inferring phylogenies in this remarkable group of plants, especially considering the occurrence of putative pseudogenes. Even so, topological incongruences may actually signal deeper phylogenetic patterns underlying biologically relevant evolutionary history and should be further explored using both nuclear and plastome datasets.

Data Availability Statement

The datasets generated for this study can be found in the GenBank: MN114084 and MT369928–MT369946.

Author Contributions

MK: conceptualization, methodology, validation, formal analysis, investigation, data curation, writing – original draft, writing – review and editing, visualization, project administration, and funding acquisition. MR: methodology, software, validation, formal analysis, and writing – review and editing. TS-C: writing – review and editing. LM: methodology, validation, investigation, resources, data curation, and writing – review and editing, visualization, supervision, project administration, and funding acquisition.

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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.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.00729/full#supplementary-material

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A NOVEL DIVERGENT GEMINIVIRUS IDENTIFIED IN ASYMPTOMATIC NEW WORLD CACTACEAE PLANTS

Esse artigo está publicado de forma editorada e diagramada no periódico *Viruses* 12: 398 (2020). Pode ser acessado pelo endereço: https://doi.org/10.3390/v12040398, o qual deve-se referir para citação. Esse trabalho faz parte do projeto liderado pela pesquisadora virologista Rafaela S. Fontenele (Arizona State University, USA). Fontenele tem usado dados de sequenciamento provenientes de colaboradores cactólogos, e tem revelado uma nova e inédita faceta da história evolutiva e ecológica de cactos, em especial *Opuntia*, relacionada com a comunidade viral e os ambientes áridos/desérticos. Esse trabalho é aqui introduzido para integração e visibilidae de uma área pouco explorada no tópico "evolução de plantas", mas extremamente relevante. O artigo é reproduzido no Apêndice I dessa tese.

viruses

Viruses **2020**, *12*, 398; doi:10.3390/v12040398

MDPI

Article

A Novel Divergent Geminivirus Identified in Asymptomatic New World Cactaceae Plants

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ABSTRACT

Cactaceae comprise a diverse and iconic group of flowering plants which are almost exclusively indigenous to the New World. The wide variety of growth forms found amongst the cacti have led to the trafficking of many species throughout the world as ornamentals. Despite the evolution and physiological properties of these plants having been extensively studied, little research has focused on cactus-associated viral communities. While only single-stranded RNA viruses had ever been reported in cacti, here we report the discovery of cactus-infecting single-stranded DNA viruses. These viruses all apparently belong to a single divergent species of the family *Geminiviridae* and have been tentatively named Opuntia virus 1 (OpV1). A total of 79 apparently complete OpV1 genomes were recovered from 31 different cactus plants (belonging to 20 different cactus species from both the Cactoideae and Opuntioideae clades) and from nine cactus-feeding cochineal insects (*Dactylopius* sp.) sampled in the USA and Mexico. These 79 OpV1 genomes all share > 78.4% nucleotide identity with one another and < 64.9% identity with previously characterized geminiviruses. Collectively, the OpV1 genomes display evidence of frequent recombination, with some genomes displaying up to five recombinant regions. In one case, recombinant regions span ~40% of the genome. We demonstrate that an infectious clone of an OpV1 genome can replicate in *Nicotiana benthamiana* and *Opuntia microdasys*. In addition to expanding the inventory of viruses that are known to infect cacti, the OpV1 group is so distantly related to other known geminiviruses that it likely represents a new geminivirus genus. It remains to be determined whether, like its cactus hosts, its geographical distribution spans the globe.

Keywords – geminivirus; Cactoideae; Opuntioideae; ssDNA virus; cochineal insects.

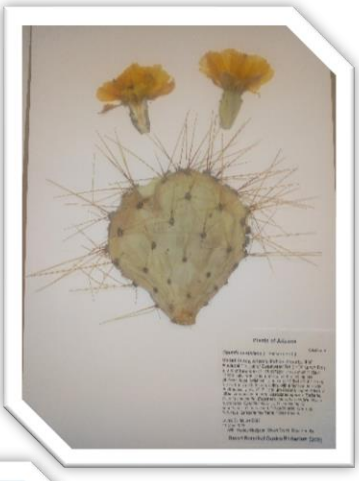
"Evolution… is the most powerful and the most comprehensive idea that has ever arisen on Earth."

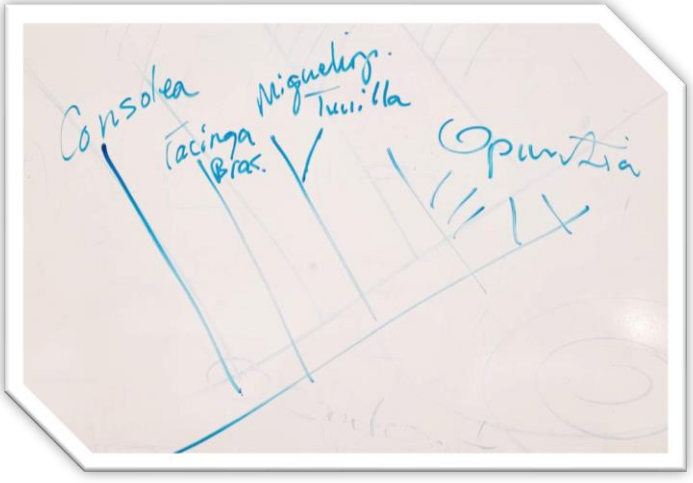
— Julian Huxley

PARTE III

DO CAMPO À GENÔMICA – SISTEMÁTICA, EVOLUÇÃO E DIVERSIFICAÇÃO

A integração de diferentes fontes da dados – do campo à genômica – permite ampliar o escopo de estudo, testando novas hipóteses, trazendo novas evidências, e preenchendo lacunas de conhecimento. Nos próximos capítulos: (1) utilizamos observações morfológicas e métodos de sistemática molecular para confirmar a ocorrência no Brasil e no Uruguai de uma espécie de cacto-palma previamente considerada endêmica da Argentina; e por meio de modelagem de nicho ecológico, investigamos o que poderia explicar esse padrão de distribuição geográfica mais amplo do que o previsto; (2) utilizamos dados moleculares, morfológicos e geográficos para investigar a história evolutiva dos cactos-palma da tribo Opuntieae, revelando uma rápida, complexa e recente diversificação do grupo; (3) apresentamos a hipótese de um novo evento de especiação híbrida em *Opuntia,* com base em dados morfológicos, moleculares e citogenéticos - sendo esse o primeiro putativo caso de hibridação entre uma espécie introduzida com uma *Opuntia* nativa do Sul da América do Sul*;* (4) propomos uma novidade nomenclatural, ao verificar que *Opuntia schickendantzii* está mais relacionada morfológica e molecularmente com a linhagem de *Salmonopuntia;* e (5) propomos um novo nome, na categoria de nova espécie, para acomodar um táxon já conhecido, mas que é historicamente associado com outro táxon de maneira equivocada.





BEYOND ENDEMISM, EXPANDING CONSERVATION EFFORTS: WHAT CAN NEW DISTRIBUTION RECORDS REVEAL?

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Beyond endemism, expanding conservation efforts: What can new distribution records reveal?

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HIGHLIGHTS

- Integrative tools support species identification in a problematic group.
- Previously considered endemic of Argentina, *Opuntia bonaerensis* has a broader distribution.
- Pleistocene events have impacted the distribution of cacti in southern South America.
- Update on distribution reveal new insights for the historical biogeography of the species.
- Knowledge regarding species distribution will affect its assessment for conservation.

ABSTRACT

Geographical range is one of the critical features for species conservation assessment. Nevertheless, species distribution is frequently unknown, undervalued or overlooked. During a broad taxonomic and floristic study of the southern South American prickly pear species (*Opuntia* spp.), new records of a species previously considered endemic to Argentina have been found in Uruguay and Brazil. Molecular phylogenetic inference was carried out to further evaluate the identity of the new records, and ecological niche models were implemented to test how the new records would fit in the previous known distribution of the species. Through molecular and morphological evidence, we confirmed the new records of *Opuntia bonaerensis* for Brazilian and Uruguayan floras and discussed its phylogenetic relationship and morphological similarities with closely related species. Our new records uncovered a distribution pattern congruent with the Neotropical Peripampasic Orogenic Arc, which must be further explored to better determine the biogeographic history of the species. Ecological niche models (ENM) revealed that *O. bonaerensis* likely had a putative ancient distribution across the grasslands and shrublands in the Pampean region largely congruent with the populations found in Brazil and Uruguay, suggesting relictual Pleistocene populations of the species and the role of glacial/interglacial cycles on the distribution of the species. In a prospective climate change scenario, ENM suggests that the species would in general be more restricted to the southernmost limits of the Pampa region and previous outlying records from Mendoza (Argentina) are a putative future refuge for *O. bonaerensis*. The importance of these new records for biodiversity and conservation assessment efforts that are ongoing at different scales in Brazil and neighboring areas is highlighted.

Keywords – Cactaceae; Ecological niche modelling; Molecular systematics; *Opuntia;* Pampa; Pleistocene

INTRODUCTION

Cacti are a conspicuous and diverse family of Angiosperms composed of roughly 1500 species distributed throughout the entire American continent (Britton and Rose, 1919; Anderson, 2001; Hunt et al., 2006; Guerrero et al., 2019). The clade is remarkable in showing intriguing modifications to survive in extremely adverse environments with drought and arid conditions usually exhibiting succulence accompanied by spines (Mauseth, 2006), but it also occurs in tropical and wet environments, especially as epiphytes (Anderson, 2001; Taylor and Zappi, 2004). The cactus lineage diverged from its closest relatives around 35 million years ago (Mya) (Arakaki et al., 2011; Hernández-Hernández et al., 2014). However, there is a time lag between the origin and extant diversification of Cactaceae, with the latter taking place mainly during the last 10 Mya, in which across disparate clades, the family has experienced high and differential rates of diversification (Arakaki et al., 2011; Hernández-Hernández et al., 2014). Impacts of the Pleistocene glacial/interglacial cycles have also been documented driving the diversification and distribution of some groups (Majure et al., 2012a, 2012b; Ornelas and Rodríguez-Gómez, 2015; Franco et al., 2017b; Silva et al., 2018a).

Evaluation of the conservation status of cacti has recently captured much attention, revealing the group as the fifth most endangered clade of any major taxonomic group, with 31 % of evaluated species under some categories of threat (Goettsch et al., 2015). Several threats are driven by human activities accompanying land conversion to agriculture, unscrupulous collection of live plants as biological resources for the horticultural trade and private ornamental collections, and residential and commercial development (Oldfield, 1997; Goettsch et al., 2015). However, although cacti are an iconic group and broadly call attention from scientists and cactus aficionados, reliable information regarding species limits and their geographic distribution throughout the family are frequently unavailable or deficient (Zappi et al., 2011). In fact, succulence, and especially spines, have made cacti an intimidating group for botanists to collect, resulting in herbaria with very deficient representation of cactus specimens or very poorly prepared specimens with many gaps to be filled (Reyes-Agüero et al., 2007; Hunt, 2014; Majure et al., 2017; Zappi et al., 2018).

Opuntia Mill. s.str. is the second most speciose genus of the family (after *Mammillaria* Haw.), containing around 180 species, with a broad distribution across all the Americas from Argentina to Canada, including Central America and Caribbean region (Anderson, 2001; Hunt et al., 2006; Majure et al., 2012a). The group has a putative origin in southern South America with subsequent

dispersal events of lineages to Northern South America, the Caribbean region, Central America and to the North American deserts (Majure et al., 2012a). Members of *Opuntia* share a combination of morphological traits, including sympodial shrubs or trees with flattened photosynthetic stems (cladodes), areoles with smooth or retrorsely barbed spines and glochids (small, hair-like spines), reduced and ephemeral leaves, radial, diurnal flowers with inferior ovaries and multilobed stigmas, stamens frequently thigmonastic, reticulate semitectate pollen, and seeds covered by a sclerified, funicular aril (Buxbaum, 1953; Anderson, 2001; Stuppy, 2002; Hunt et al., 2006; Majure and Puente, 2014; Majure et al., 2017). Eight major clades are recognized within *Opuntia* (Köhler et al., in prep.), which exhibit a variety of morphological characters such as dioecy, hummingbird pollination, dry fruit, epidermal and seed pubescence, as well as rhizomes and tuberous roots that are unique to some species in the genus (Britton and Rose, 1919; Majure et al., 2012a; Majure and Puente, 2014; Majure et al., 2017).

Conservation status assessments of the prickly pear cacti (*Opuntia* spp.) are relatively incipient (Goettsch et al., 2015) and are not a simple issue. Geographical range is a critical feature for conservation assessment of the International Union for the Conservation of Nature – IUCN (Criteria B and D2, IUCN, 2001), and it has been the most used criterion to assess threatened plant species in one of the categories of extinction risk (Collen et al., 2016). However, many *Opuntia* species are worldwide cultivated for different purposes (such as fruit and vegetable crops, forage and fodder for livestock and ornamentals (Inglese et al., 2002; Nefzaoui and Salem, 2002)), increasing the complexity of knowledge regarding the distribution of some species. So, the lack of data regarding the distribution of species has an immense impact on those evaluations. There are currently five of the 84 evaluated species under threat in some of the three criteria of extinction risk (EN, VU, CR) of the IUCN, and ten taxa are data deficient (DD) (IUCN, 2019), and many taxa remain unevaluated. Morphologically variable species, and frequent hybridization make species delimitation within *Opuntia* a problematic issue that also is reflected in their conservation and biodiversity management.

Ecological niche models (ENM), which are produced by combining species occurrence data with environmental data layers, have transformed evolutionary, systematics and conservation biodiversity studies across disparate organisms (Raxworthy et al., 2007; Peterson, 2001; Kozak et al., 2008). ENM have allowed scientists to develop more reliable hypotheses to describe, understand and predict geographic and environmental distributions of species to the present, as well as to the past and future scenarios (Peterson, 2006), becoming a powerful tool to infer local adaptations (Rolland et al., 2015), environmental drivers of diversity (Barros et al., 2015),

interglacial microrefugia in paleoenvironments (Bonatelli et al., 2014) and impacts of climate change on species distribution (Maguire et al., 2015). Besides being descriptive, novel approaches have used ENM to quantitatively test niche differences, such as niche overlap, niche equivalency and niche similarity (Warren et al., 2008; Broennimann et al., 2012; Dagnino et al., 2017), increasing the significance of niche modelling applications.

Opuntia bonaerensis Speg. is a species described for the Argentinean flora in the early 20th century (Spegazzini, 1901). When described, it was mentioned to be rare, since it was observed at only a few localities in the southern Buenos Aires province (Sierras of Curamalál and Tornquist). The taxon has a complicated taxonomic history being circumscribed in various ways in seemingly arbitrary taxonomic treatments (Spegazzini, 1905; Britton and Rose, 1919; Spegazzini, 1925; Leuenberger, 2002), which is not uncommon in the taxonomic history of *Opuntia* (Hunt, 2002). Just recently, in a taxonomic revision of the *Opuntia* series Elatae, *O. bonaerensis* was resurrected as an endemic species of the Argentinian pampean region and delimited using a set of diagnostic morphological characters that have been historically ignored for southern South American species, such as bud flower apices, stigma colors and color of the inner pericarpel tissue (Font, 2014; Las Peñas et al., 2017). The species was further included in a preliminary phylogenetic study of the southern South American species of *Opuntia*, confirming it assignment to this taxonomic series (Realini et al., 2014b).

During a broad floristic and taxonomic study of *Opuntia* in southern South America, unexpected populations of *O. bonaerensis* have been found outside of its previously known Argentinian distribution, in the Uruguayan and Brazilian pampean regions. Considering the general difficulty in identifying *Opuntia* species using morphological characters, we further tested the lineage identity of the groups under study using molecular phylogenetic inference of populations across the range of the taxon. To test how these unexpected records outside of Argentina could be explained in the natural history of the species, we generated four datasets of different distribution records in which we explored ENM with projections to past, current and future climate scenarios. Confirming the new records of *Opuntia bonaerensis* in Uruguay and Brazil using both morphological characters and molecular phylogenetics, as well as a putative relictual Pleistocene distribution in these regions, we highlight implications for conservation efforts in the regional floras of Brazil and neighboring areas considering the current global and local strategies for biodiversity conservation.

MATERIAL AND METHODS

Studied area and data collection

Extensive fieldwork was carried out in southern South America encompassing the main natural ecoregions to obtain data about natural populations of *Opuntia*. The region is represented by subtropical grasslands permeated by rocky outcrops that compose the Pampa biome or Río de la Plata grassland (Andrade et al., 2018) and the Chaco region (Pennington et al., 2000). The major herbaria from the region have been examined to check distribution records and specimen identification of *Opuntia*, including unidentified materials: BA, BAF, BCWL, CORD, CTES, HAS, ICN, LIL, LP, MBM, MCN, MVFA, MVJB, MVM, SI (acronyms according Thiers, 2019+, except BCWL, non-indexed herbarium of the Biological Control of Weeds Laboratory (FuEDEI), Hurlingham, Buenos Aires, Argentina). The digital database of Brazilian collections was also consulted through the SpeciesLink (2019) to check herbaria from disparate geographical regions.

Herbarium materials were examined to obtain data regarding morphological features as well as other important information, such as ecological, biological and distributional data (Vogel, 1987). Likewise, fieldwork was carried out to obtain morphological, as well as biological (e.g., phenology, pollinators) and ecological (e.g., soil and vegetation type) data across populations. Samples of materials collected in the field were dried in silica gel to keep tissues available for further molecular studies (Funk et al., 2017), and representative materials were collected as vouchers (see Table S2 for further information). Morphological characters were assessed based on commonly used characters for prickly pears species identification (e.g., cladode morphology, habit and growth form, spine production) (Pinkava, 2003; Majure et al., 2017), with a special focus on those reported by Font (2014) for the southern South American species, such as bud flower apices, stigma color, and inner pericarpel tissue color. The criteria for identification of southern South American species of Opuntia followed those proposed by Font (2014) and Las Peñas et al. (2017).

DNA sampling, sequence alignment and phylogenetic analyses

A small dataset was selected to test the hypothesis of whether the new Uruguayan and Brazilian records represented lineages of typical Opuntia bonaerensis from Argentina. One sample of each of those new records was selected for sequencing, and one sample of O. bonaerensis from the type locality in Argentina was also incorporated. We included a representative dataset of all southern South American species from Elatae series (sensu Font, 2014) to contextualize the

phylogenetic relationships between the taxa sampled. Additionally, two North American taxa (Opuntia macrorhiza Engelm. and Opuntia austrina Small) and a sample of Brasiliopuntia brasiliensis (Willd.) A. Berger were selected as outgroups according to previous phylogenetic studies in the group (Majure et al., 2012a; Majure and Puente, 2014). The complete information regarding the taxa sampled is presented in the supplemental Table S1.

Cladode epidermal tissue of selected taxa was used for DNA extraction using a standard CTAB incubation (Doyle and Doyle, 1987) followed by chloroform/isoamyl alcohol precipitation and silica column-based purification steps as described in Neubig et al. (2014) and Majure et al. (2019). Whole genomic DNAs were quantified using the Qubit dsDNA BR Assay Kit and Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, California, USA); high-molecular-weight DNA (> 15 kb) samples showing no degradation were considered suitable and then sent to Rapid Genomics LLC (http://rapidgenomics.com/home/; Gainesville, FL.) for library preparation and high-throughput sequencing using the Illumina HiSeq X platform with 150 bp paired-end reads.

Raw reads were imported into Geneious 11.1.5 (Biomatters, Auckland, New Zealand) and set paired reads with an expected insert size of 300 bp calculated with BBMap (Bushnell, 2016). Low quality bases (Q < 20) were trimmed and all reads shorter than 20 bp were discarded using BBDuk for quality control of the reads (Bushnell, 2016). Then, a reference guided assembly was carried out on the trimmed reads using selected phylogenetically informative regions from the chloroplast genome as a reference (see below). Based on previous molecular phylogenetic studies in Opuntia and related cacti (Arakaki et al., 2011; Hernández-Hernández et al. (2011), 2014; Majure et al., 2012a), we selected the chloroplast genes ccsA, rpl16, trnK including matK (trnK/matK) and the intergenic spacer trnL-trnF as markers, using those GeneBank sequences as references. The reference mapping pipeline was conducted using the Geneious mapper feature with a medium-low sensitivity, and we generated a majority consensus sequence from the reference-mapped raw reads.

Sequences of each marker from each taxon were concatenated as one sequence, and a multiple sequence alignment was performed across all samples using the MAFFT v. 7 (Katoh and Standley, 2016) plugin in Geneious with default settings, and then manually corrected. Phylogenetic inference was performed using the Maximum Likelihood (ML) approach implemented in RAxML 8.2.4 (Stamatakis, 2014) on the CIPRES Science Gateway Web Portal (Miller et al., 2010). As RAxML is mainly designed to implement generalized time-reversible molecular models (GTR), we employed the GTR+G model of molecular evolution for the concatenated sequence (unpartitioned), which have been demonstrated to be accurate when topological reconstructions

are the desired output skipping model selection (Abadi et al., 2019), and GTR+I+G is not recommended by Stamatakis (see RAxML v8.2 manual) given the potential interaction between the I and G parameters. Support values were estimated implementing 1000 bootstrap pseudoreplicates.

Building, assembling, projecting and exploring ecological niche models

As presence records, four approaches were considered, and are summarized in Fig. 1: (1) using data previously known as the natural distribution of the species, gathered from our herbarium studies, but segregating records from Mendoza (Argentina), which could be environmental outliers from a non-natural distribution (see Font, 2014) (henceforth, P data, 17 records); (2) summing data previously known (**P**) and new records from our field expeditions in Uruguay and Brazil (henceforth, **PN** data, 25 records); (3) as an alternate approach, we also clustered Mendoza and previously known records (henceforth, **PM** data, 19 records); and (4) the sum of previously known, new data and Mendoza records (henceforth, **PMN** data, 27 records). This segregation was important to track environmental contribution from each set, as well as to determinate whether they are more different than expected by chance.

Considering that little is known about the biology of Opuntia bonaerensis and its relationship with bioclimatic variables, we used PCAaxes as a proxy of ecological niche models, as they summarize species relationship with environment variables. We generated a correlation matrix of standardized variables, which was composed using all bioclimatic variables from WorldClim v.1.4 (30" resolution, Hijmans et al., 2005). In total, three axes were selected, which accounted for 89 % of the overall variation. We could not use more than three, since the use of more variables could result in overparameterization. Axes were projected to the past, current and future scenarios using ENMGadgets package (Barve and Barve, 2016) on R (R Core Team, 2017). Past scenarios comprised the Last Glacial Maximum (LGM: about 21,000 years ago - 21 kya) and Mid-Holocene (MID: about 6 kya), while future scenarios comprised the extreme scenarios RCP 2.6 and RCP 8.5 to the year of 2070 (IPCC, 2013). The extreme scenarios were selected to allow us to infer the putative minimal and maximal impacts of climate change on the species distribution. To keep comparability between scenarios and reduce uncertainties we used two General Circulation Models (CCSM4 and MIROC-ESM) from WorldClim v.1.4 (2.5 arcminutes resolution, Hijmans et al., 2005), which were the only two available to every scenario calculated.

Modeling domain comprised a 6-degree wide buffer in the region of the Río de La Plata grasslands/Pampa (Dixon et al., 2014; Andrade et al., 2018), which is the previously known

distribution of the species (Font, 2014), with a buffer that comprises Mendoza records. Models were generated using ten algorithms available in biomod2 R package, to be known: Artificial Neural Network, ANN, Classification Tree Analysis, CTA, Flexible Discriminant Analysis, FDA, Generalized Additive Model, GAM, Generalized Boosting Model, GBM, Generalized Linear Model, GLM, Surface Range Envelope, SRE, Multiple Adaptive Regression Splines, MARS, Random Forest, RF, and MaxEnt (Thuiller et al., 2016). Pseudoabsence selection was performed in groups following Barbet-Massin et al. (2012). The first group comprised GLM, GAM and MaxEnt, where we selected 1000 pseudoabsences disposed randomly in environmental space. Afterwards, we generated a two-degree wide buffer from each presence point and selected pseudoabsences for the other two groups, as follows: for the second group, which comprised FDA, MARS and ANN, we selected 100 pseudoabsences, while for the third group (CTA, BRT, RF and SRE) we selected the same number of presence records. This routine was applied to every dataset separately, as they differed in number and location of presence records. We made 10 independent runs of 4-fold cross-validation, keeping 75 % of data to build the models and 25 % to test them, summing a total of 400 models for each dataset. Ensemble models were built through a committee average approach, where projections are binarized using a TSS threshold and summed (see further cutoff values in Table 1), resulting in a map with both congruence and uncertainty. A cell with value 1 or 0 has 100 % congruence in models, predicting respectively a presence and an absence, while 0.5 represents cells where half of projections predict a presence, while the other half predict an absence. True skill statistics (TSS) and area under the receiver operating characteristic (ROC) values were calculated for each model, as well as a summary statistic for each of them. Models with both TSS and ROC values greater than the mean plus one standard deviation were kept to build the ensembles. This approach was pursued to evade subjective threshold adoptions.

We further explored the trends of climatic suitability values across past, current and future scenarios for each group of records (previously known, new and those from Mendoza). We extracted the suitability values for each presence record and calculated the average suitability in each group of records for each scenario. Values from the **PMN** dataset were used, as well as an average from the values considering all datasets separately ((**P** + **PN** + **PM** + **PMN**)/**4**). We compared both approaches and fitted a linear regression to make trends explicit.

Niche equivalency and niche similarity tests

Ensemble models were a proxy of niche similarity and niche equivalency tests using the ecospat R package (Warren et al., 2008; Broennimann et al., 2012, 2018). Thus, we calculated for each pair of datasets the Schoener's D statistics for niche overlap (Schoener, 1968) and a Hellinger distance-based metric (I) proposed by Warren et al. (2008). Both metrics range from 0 (no overlap) to 1 (indistinguishable) and are used to calculate similarity and equivalency p-values with simulated niches. The similarity test consists of trying to predict the niche of one dataset using the model generated for other dataset (Peterson et al., 1999) and has the assumption that niche conservatism is expected as a consequence of phylogenetic relationships and a finite rate of evolutionary divergence (Warren, 2008), thus, p-values lower than 0.05 represent niches that are more similar than expected by chance. This test returns two p-values for each pair of species based on the ensemble model built, e.g., for **P** will be used to predict **PN**, and ensemble model built for **PN** will be used to predict **P**. The equivalency test has an opposite view, where the assumption is that niches need to be indistinguishable to be equivalent, thus p-values greater than 0.95 represent niches that are more equivalent than expected by chance (Graham et al., 2004). This is reached through a random reallocation of occurrences of both datasets among their ranges. This routine was made through 100 simulated model niches (Dagnino et al., 2017), and for each simulated model niche we calculated the D niche overlap metric. We also explored a climatic space using the point localities values from the PCA-axes to discuss this topic.

RESULTS

New distribution records and morphological features

In total, 27 distribution records were confirmed as Opuntia bonaerensis based on herbarium specimens. Of these, 19 are previously known from Argentina, mostly from the Buenos Aires province (15), one is from La Pampa and one is from Entre Ríos provinces; and two of them are from Mendoza, previously considered as from a non-natural distribution (discussed below). Eight records are newly generated from our field work and encompass the first records for the region, three being from south Brazil (Rio Grande do Sul state) and five from Uruguay (Colónia and Montevideo departments). All records are part of the Pampa or Río de La Plata grassland ecoregion, except those from Mendoza that are in the Puneña, Altoandean and Monte ecoregions.

The full details regarding the distribution records reported here are presented in Fig. 1 and Table S2.

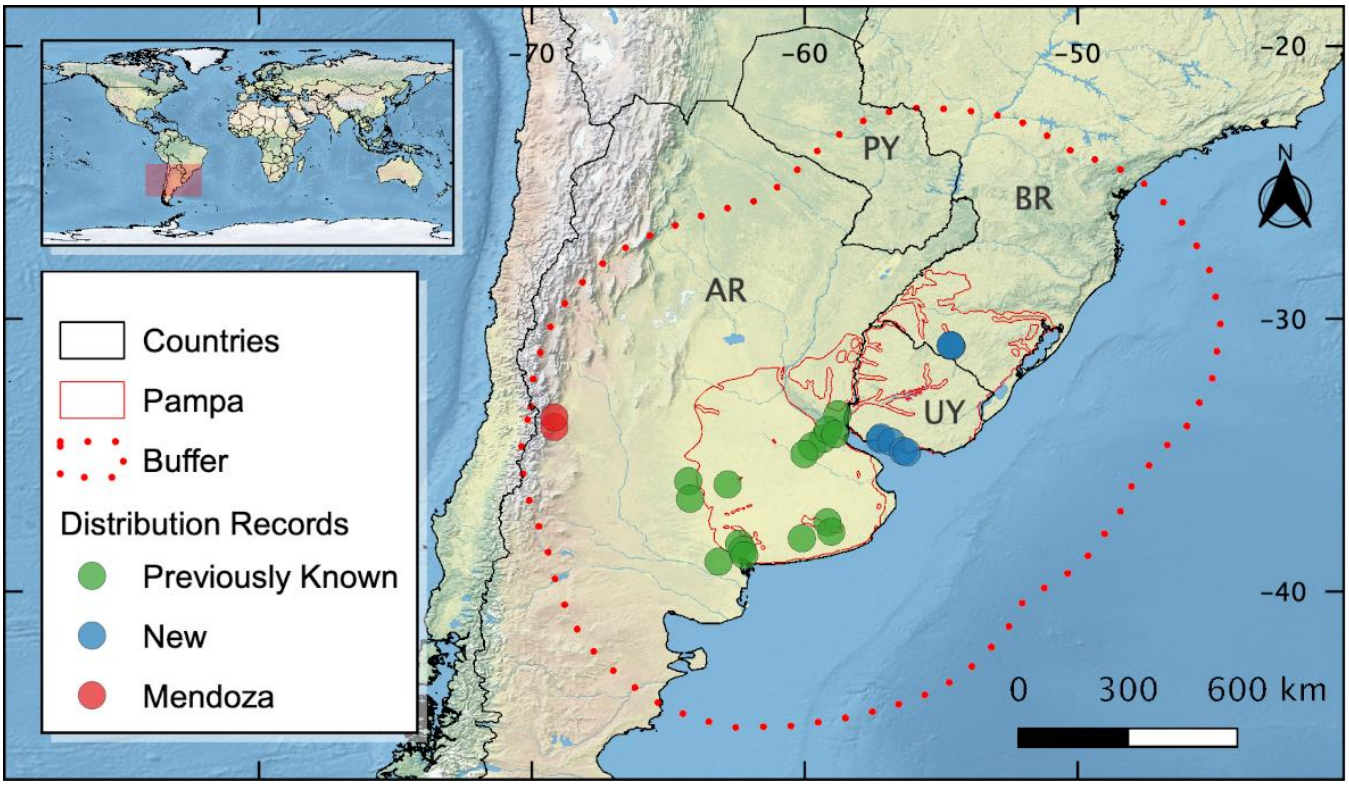

Figura 15 (Figure 1). Distribution records of *Opuntia bonaerensis* (Cactaceae). Green circles represent the previously known distribution of the species in Argentina (AR), while blue circles in Uruguay (UY) and Brazil (BR) are newly reported here. Red circles represent previously known records from Mendoza, which are environmentally distinct populations.

None of the unidentified materials previous deposited in the examined herbaria corresponded to *O. bonaerensis*, while three materials (UFP 24722, UFP 24,723 and MPUC 13,373) had misidentification as *O. bonaerensis* and were correctly assigned during our study as *O. elata* Salm-Dyck. Herbaria and field studies revealed morphological characters, such as bud flower apices (acute), stigma color (green) and inner pericarpel tissue color (vinaceous), as consistently useful to recognize *O. bonaerensis* (Fig. 4D,F–G) and separate that taxon from *O. elata* (rounded bud flower apices, creamy stigma color and green inner pericarpel tissue color). Cladode morphology (spathulate to long-elliptic) is consistent across the materials studied of *O. bonaerensis* (Fig. 4A-C,E). Spine production, although useful to recognize *O. bonaerensis*, was found to be a plastic character across populations, varying from spineless morphotypes (Fig. 4C,E) to 1–2 spine/areolearmed plants with dark-reddish developing spines (Fig. 4B,D).

Phylogenetic analysis

Our alignment was 5496 base pairs in length with 51 parsimony informative characters, 5399 constant characters and 30 sites with gaps. The maximum likelihood inference depicted a tree with all nodes resolved with total bootstrap support (bs=100) except for the position of *Opuntia rioplatense* Font recovered as sister to the clade that includes *O. elata* + *O. megapotamica* Speg. (Fig. 2, bs=52). The South American species of series Elatae formed a well-supported clade including all species proposed by Font (2014) except for *O. penicilligera* Speg., which was nested in the North American clade (Fig. 2). The three samples of *Opuntia bonaerensis*, including the records from Uruguay, Brazil and the specimen from the type locality in Argentina, formed a well-supported clade (bs=100) sister to a clade with *O. elata*, *O. megapotamica* and *O. rioplatense*.

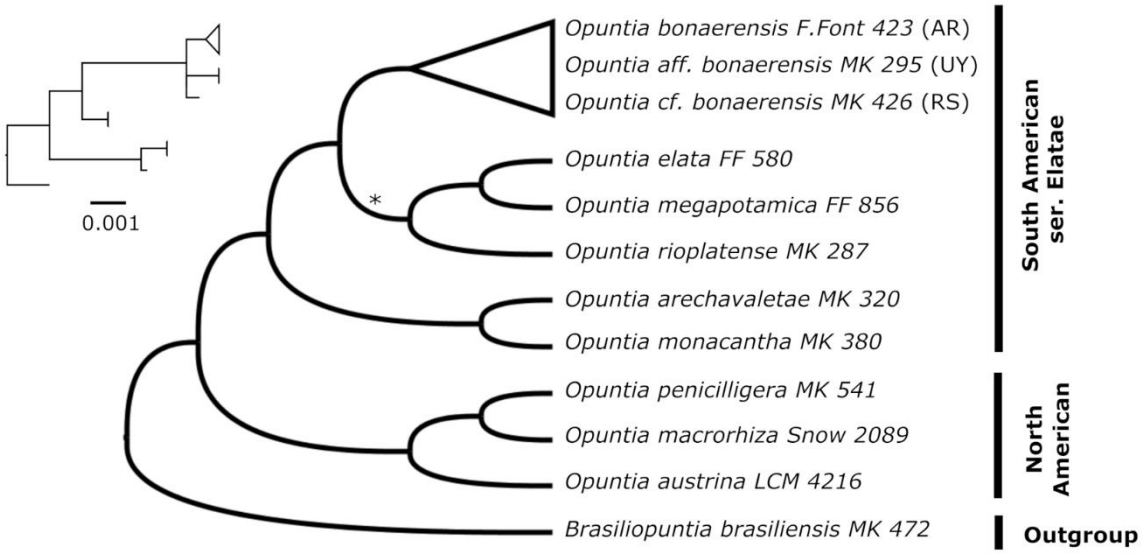

Figura 16 (Figure 2). Maximum likelihood phylogenetic tree from RAxML analysis transformed in cladogram with the phylogram represented in small size with substitution rate scaled. All nodes have total bootstrap values (bs=100) with exception to the asterisk denoting low bootstrap support (bs=52). *Opuntia bonaerensis* from Argentina (*F.Font 423*) forms a well-supported clade with the two new records from Uruguay and Brazil (*MK 426* and *MK 295*).

Ecological niche modelling

The models and projections for each climatic scenario and dataset generated are shown in Fig. 3. Statistics associated with ROC, TSS and number of models are summarized in Table 1. The dataset of the previous known distribution (P), excluding Mendoza records, projected a LGM (21 kya) distribution centered in Uruguay and adjacent areas as Entre Ríos province (Argentina) and Rio Grande do Sul (Brazil), occupying the continental shelf in the coast of Uruguay, shifting to centraleast Argentina (especially the Buenos Aires province) in the MID (Middle Holocene (6 kya)). This distribution (P/MID, Fig. 3) persisted until the current scenario, and future scenarios were predicted to keep suitability levels in the southern half of the distribution in the Buenos Aires province (RCP 2.6) and in the southernmost quarter of the distribution in the worst-case scenario (RCP 8.5). The previous known distribution plus the new records dataset (PN) projected a similar result for the LGM, but MID presented whole western Uruguay as a suitable area, as well as connected areas in the southern Brazilian region of Pampean grasslands (Rio Grande do Sul). This pattern persists in the current scenario, where suitability of the models is divided between areas of previously known distribution and areas of Pampean region from Uruguay to Rio Grande do Sul,

with an overall moderate suitability. Future scenario suitability shifts mostly to the coast in contrast to the P dataset, which presents a higher suitability in the inland. Despite the differences, both sets (P and PN) present a higher suitability along the coast of Argentina in the worst-case scenario (RCP 8.5). The third dataset (PM) shows a slightly different result, revealing the influence of the Mendoza records. Interior areas close to the piedmont of the Andes are more suitable than in other datasets. In the LGM, what is today called the Cuyo region in Argentina, had a greater suitability when comparing to aforementioned datasets. This effect persists through scenarios, increasing in RCP 2.6 and RCP 8.5, with lower suitability in southern Brazil. The last dataset (PMN), provides a different perspective, where the species was predicted to have had a wider distribution in the LGM, occupying a connected area ranging from southern Paraguay, northeast and pampean Argentina, occurring throughout Uruguay, reaching parts of Brazil and the continental shelf. Within the MID scenario, the climate in Paraguay was less suitable, southern Brazil became widely climatically suitable and an overall southern shift started reaching the San Matías Gulf (Argentina). The current scenario in the PMN dataset suggested range contraction towards the province of Buenos Aires, with relicts in Uruguay and southern Brazil. Future projections show a tendency towards a southern and coastal shift, accompanied by an increase in suitability in the piedmont of the Andes. Generally, projections of all models unveiled a north-south distribution shift, ranging from a wider pattern in LGM to a more restricted distribution in an extreme climate change scenario (RCP 8.5).

Projections unveiled an uneven response to climatic change across populations (Fig. S1). Previously known records (Fig. 1, green circles) had a higher mean climatic suitability in past and current scenarios, decaying in future scenarios, with values ranging from 0.55 and 0.8 in the LGM and MID, reaching a maximum in our current scenario, 0.93; decaying to 0.51 and 0.19 in RCP-2.6 and RCP-8.5. Likewise, new records (Fig. 1, blue circles) had a higher mean climatic suitability in past and current scenarios, decaying in future scenarios, with values ranging from 0.49 and 0.7 in LGM and MID, reaching a maximum in current scenarios, 0.54 (average from all datasets) and 0.9 (PMN dataset); decaying to 0.37−0.58 and 0.17−0.23 in RCP-2.6 and RCP-8.5. On the contrary, records from Mendoza had a lower mean climatic suitability in past scenarios, while increasing in current and future scenarios, with values ranging from 0.11 and 0.17−0.3 in LGM and MID, reaching a maximum in current scenario, 0.4−0.6, with a little decay to 0.3−0.4 and 0.2 in RCP-2.6 and RCP-8.5 (Fig. S1).

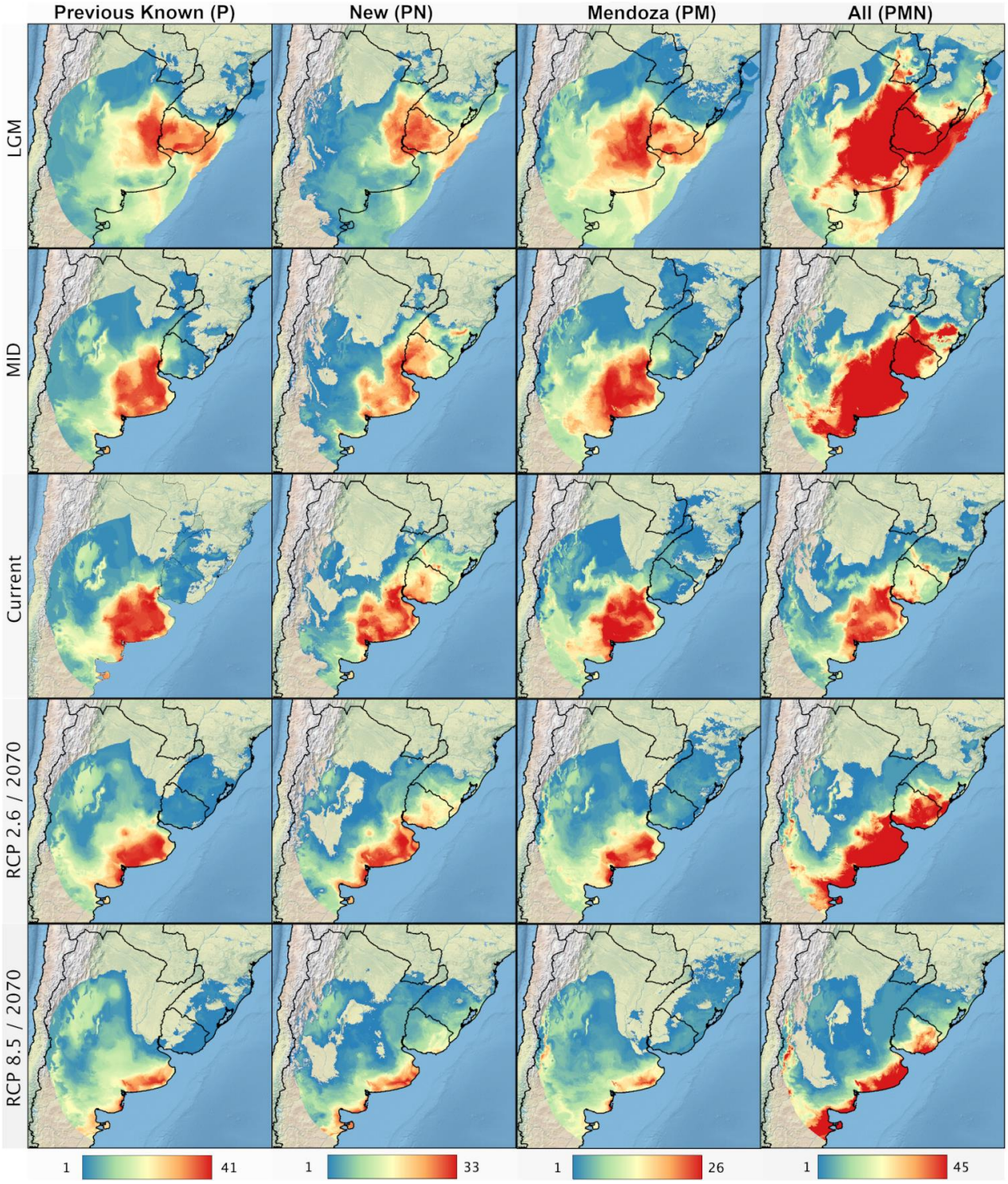

Figura 17 (Figure 3). Projections for the *Opuntia bonaerensis* distribution to multiple scenarios (rows) considering different datasets (columns) using ensemble ecological niche models. LGM (Last Glacial Maximum, 21 ka); MID (Mid-Holocene, 6 ka); RCP (Representative Concentration Pathway) of 2.6 and 8.0 for future projections in 2070. Color scale represent agreement between models' projections (cold colors represent low agreement; hot colors represent high agreement).

Niche equivalency and niche similarity

Niche equivalency tests returned higher p-values when comparing the P dataset with PN ($p > 0.97$) and PMN datasets ($p > 0.99$), which suggests that those sets have equivalent niches (Table 2). Contrarily, when comparing the PM dataset to every other set, as well as comparing the PMN set with the PN set, niche is less similar than expected by chance (Table 2). Climatic space showing the point localities from the PCA-axes also revealed that the Mendoza records add substantially different climatic information (Fig. S2). Despite that, niche similarity tests detected a small difference between sets, but with no statistical difference in any of them (Table 2).

Tabela 6 (Table 1). Ecological niche modelling results. Receiver Operating characteristic (ROC) and True Skill Statiscs (TSS) values. Cutoff values represent thresholds used to keep models in each dataset, summing a total of N models for each set.

	ROC			TSS			
Set	**Max-Min**	**Mean (SD)**	**Cutoff**	**Max-Min**	**Mean (SD)**	**Cutoff**	**N**
P	1-0.34	0.85 (0.12)	0.98	1-0	0.72 (0.23)	0.95	41
PN	1-0.41	0.84 (0.11)	0.95	1-0	0.67 (0.19)	0.86	33
PM	1-0.47	0.83 (0.11)	0.95	1-0	0.67 (0.21)	0.88	26
PMN	1-0.35	0.80 (0.11)	0.92	1-0	0.60 (0.20)	0.80	45

Tabela 7 (Table 2). Niche similarity and equivalence tests. Values in Similarity and Equivalency columns are p-values. Bold equivalency values are those with p-values lower than 0.95, thus indicating that niches are less equivalent than expected by chance.

Dataset Pair (A,B)	I	D	Similarity (A>B)	Similarity (B>A)	Equivalency
P, PN	0.697	0.490	0.0099	0.0099	0.9703
P, PM	0.833	0.694	0.0099	0.0099	**0.48515**
P, PMN	0.557	0.312	0.0297	0.0396	0.9901
PN, PM	0.752	0.645	0.0099	0.0099	**0.39604**
PN, PMN	0.801	0.642	0.0198	0.0099	**0.50495**
PM, PMN	0.674	0.469	0.0297	0.0396	**0.93069**

DISCUSSION

Morphology and phylogenetic relationship of Opuntia bonaerensis

Species delimitation within *Opuntia* is well-known to be problematic (Rebman and Pinkava, 2001; Hunt, 2002; Powell et al., 2004; Majure et al., 2017). Several issues have contributed to this as 1) high amounts of hybridization resulting in mosaics of morphological features expressed by hybrid progeny, 2) morphologically variable species, where morphological characters are oftentimes dependent upon environmental variables (Griffiths, 1906; Reyes-Agüero et al., 2007) 3) poor specimen preparation and the lack of general collecting of species throughout their ranges, 4) the lack of basic biological data (e.g. chromosome counts, phenology, pollinators and floral biology, geographical distribution), 5) the absence of detailed studies regarding morphology across the distribution of species, and 6) the deficiency of phylogenetic data (Majure and Puente, 2014). However, various efforts have been recently carried out to increase the knowledge of the group across all the range of distribution yielding valuable information to decisions regarding species delimitation (Powell et al., 2004; Majure et al., 2012a, 2012b, 2017; Font, 2014; Realini et al., 2014a, 2014b; Las Peñas et al., 2017; Köhler et al., 2018; Martínez-González et al., 2019; Majure et al., 2020). Here, we highlighted the use of multiple approaches, such as molecular data, morphological characters, herbarium and field studies across geographical distribution as powerful tools to reveal accurate species identification in a problematic group.

Opuntia bonaerensis is easily recognized by an array of morphological features, such as the bright dark-green spathulate to long-elliptic stem segments, the acute flower buds with inner orange tepals and green stigma, and the short to long obconic fruits with the purple-wine (vinaceous) inner pericarp tissue (Font, 2014; Las Peñas et al., 2017) (Fig. 4), whereas the morphologically similar *O. elata* has oblong stem segments, creamy-white stigma lobes and pyriform fruits with green inner pericarpel tissue. The original description of *O. bonaerensis* suggested it to be a spineless or rarely 1–2 spine/areole-armed plant. Our revision of living plants and herbarium specimens revealed this to be a putatively plastic character, as some morphotypes were observed to be growing erect to curved, dark-reddish developing spines (Fig. 4B). This feature has also been observed in some spineless morphotypes when grown under cultivation. Our extensive field work and herbaria examination suggested a transition of spineless morphotypes from southern Argentina to more spiny specimens in the newly reported populations. It is known that the spine color, as well the spine production, are in many instances phenotypically variable in *Opuntia* and

change through time but can also be extremely diagnostic at the species level (Pinkava, 2003; Powell et al., 2004; Majure et al., 2017). Spine production seems to be a character not useful alone for diagnosing this species, and further studies should be carried out to determine if ecological factors are leading to its development.

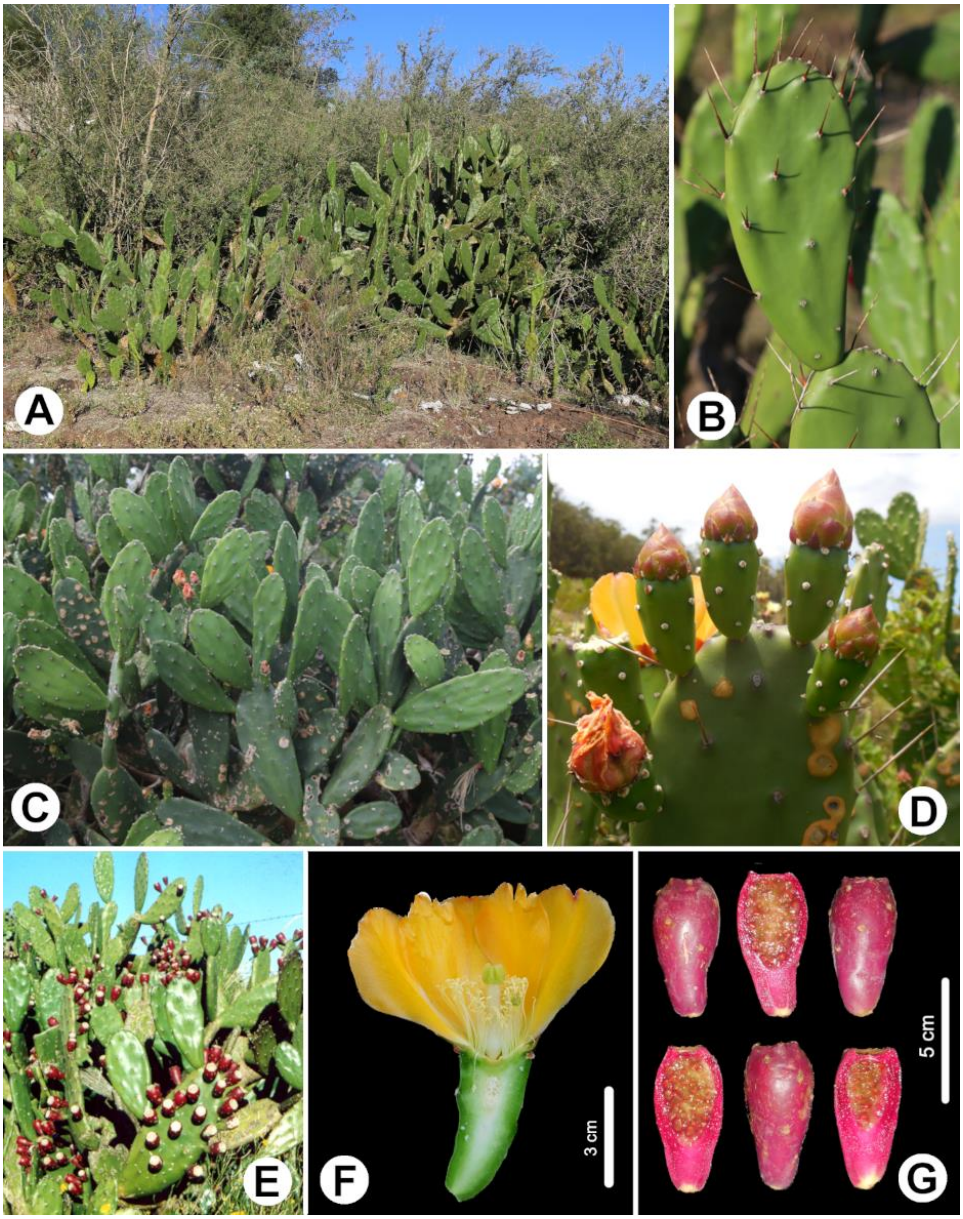

Figura 18 (Figure 4). Morphological aspects of *Opuntia bonaerensis*. **A.** Typical habitat of the species occurring on rocky outcrops of the Pampa shrubby-grasslands (*M. Köhler 426*) **B.** Details of the cladodes with dark purple-reddish new spines when present in prickly morphotypes (*M. Köhler 424*) **C.** Typical spineless morphotype with elliptic to elongated-spatulate cladodes (*M. Köhler 295*) **D.** Acute flower buds with the dark-reddish external tepals (*M. Köhler 295*) **E.** Specimen from the type locality in Argentina (*F. Font 423*) **F.** Flower in transverse section showing the green stigma and the obconic ovary (*M. Köhler 295*) **G.** Long to shortly obconic ripe fruits in transverse section showing the vinaceous pulp (*M. Köhler 424*).

Opuntia bonaerensis is a resurrected taxon proposed from an attentive revision of the southern South American species of the Elatae series based on analyses of protologues, field work, cultivation of specimens and examination of herbarium materials (Font, 2014). This circumscription was supported in previous molecular studies (Realini et al., 2014b), and the molecular analysis presented here reinforces the taxon as a distinct lineage. The species is closely related to *Opuntia elata*, with which it was previously synonymized, and it is nested in the clade of the other "orange-flowered" southern South American *Opuntia* species of the Elatae series, such as *O. megapotamica* and *O. rioplatense* (Fig. 2). The morphological characters that have been now adopted to delimit these species (e.g., bud flower apices, stigma and inner pericarpel tissue color) appear to be strongly informative and reflect those evolutionary lineages.

The inclusion of all species proposed by Font (2014) as part of *Opuntia* series Elatae Britton and Rose (= ser. Armatae K. Schum.) in our phylogenetic analysis revealed that the series is not appropriately circumscribed. Font (2014) included seven species in the series and made a tentative inclusion of *Opuntia penicilligera* Speg. to accommodate a taxon that has been historically controversial and with minor affinities to the rest of the southern South American *Opuntia*. Previously treated in the Sulphureae series (Britton and Rose, 1919), Las Peñas et al. (2017) maintained it in the series Elatae, but as suggested in our analysis, recent studies have shown that the species is phylogenetically nested in the North American Humifusa clade, and is likely derived from the *O. macrorhiza* species complex (Majure et al., 2020).

An ancient distribution and now relictual in Brazil and Uruguay

Exploring the P dataset, ensembled models and projections to the past (LGM) clearly indicate that the potential distribution of *Opuntia bonaerensis* is largely congruent with our new records of the species in the Uruguayan and Brazilian pampean region, which was previously unexpected from herbarium and literature reviews. Combining the previous known distribution with the new records (PN dataset), the ensembled models and projections maintain the suitability of the past distribution (LGM) in these regions with minimal differentiation, confirmed by the niche equivalent and niche similarity tests showing that newly discovered populations occupy similar environmental niches, compared to Buenos Aires populations (Table 2). This allowed us to suggest that the extant populations in these regions can be from a putative Pleistocene distribution that is relictual in the present.

During the climatic fluctuations of the Quaternary, the Southern Hemisphere was not as affected by extensive glaciations as in the Northern Hemisphere, but the impacts of the Pleistocene

have been increasingly documented in the South American flora and revealed as an important epoch for diversification both at the generic, as well as at the species level (Rull, 2008; Ramos-Fregonezi et al., 2015; Turchetto-Zolet et al., 2013; Ramírez-Barahona and Eguiarte, 2013). In cacti, several studies have uncovered Pleistocene events related to climatic oscillations and glacial/interglacial cycles as a decisive driver for disjunct distributions, microrefugia and diversification across disparate clades emphasizing Mesoamerica, as well as central, eastern and southeastern South American regions, primarily in the Atlantic forest and Cerrado biomes (Ornelas and Rodríguez-Gomez, 2015; Bonatelli et al., 2014; Franco et al., 2017a, 2017b; Silva et al., 2018a). However, diversification and distribution patterns in southern South American regions during the Pleistocene have been neglected, especially in the Pampas and Chaco regions. Just recently, studies revealed the impacts of climatic oscillations (e.g. glacial/interglacial cycles, sea level changes) as a driver of speciation and distribution in Solanaceae and Passifloraceae grassland species of the Pampa and Chaco domains (Mäder et al., 2013; Fregonezi et al., 2013; Ramos-Fregonezi et al., 2015; Moreno et al., 2018; Giudicelli et al., 2019).

Here, we demonstrate for the first time that events of the Pleistocene may have also impacted the distribution of cacti in southern South America. The Chaco-Pampa Plain is the southern part of the vast South American deposition trough. The present topography of the region was formed through the last regression of the Miocene Paranaense Sea, and in a great part of the Chaco-Pampa Plain Quaternary loess and loessoid deposits cover Pliocene fluvial sand (Kruck et al., 2011). Lithostratigraphical and paleoenvironmental interpretations based on fossils have suggested that during the 28 – 16 kya, an arid climate with very weak humid spells dominated the region favoring a xerophytic vegetation (Zarate and Fasano, 1989; Barreda et al., 2007; Quattrocchio et al., 2008; Kruck et al., 2011). This is congruent with other evidence that aridity has played an important role in cactus diversification and distribution (Hershkovitz and Zimmer, 1997; Ritz et al., 2007; Arakaki et al., 2011). Besides that, aridity in the Chaco-Pampa Plain was accompanied by a lowering mean temperature (Zarate and Fasano, 1989; Quattrocchio et al., 2008), which suggests that cold tolerance is an important feature for plants surviving in these environments. This feature can be easily related with extant populations of *Opuntia bonaerensis*, which have a remarkable presence in southern Buenos Aires province and in the subtropical grasslands of Uruguay and Rio Grande do Sul, where low temperatures are striking during the winter (Aliaga et al., 2017).

In Uruguay and Rio Grande do Sul (Brazil), some metamorphic and granitic formations were temporarily isolated during the Pleistocene and Holocene by marine ingressions, affecting population dynamics and leading to the diversification of plant species in these regions (Mäder et

al., 2013; Longo et al., 2014; Moreno et al., 2018). The higher parts of the orographic system of these regions are speculated to have been refugia during population expansions and retractions in the interglacial/glacial cycles and marine ingressions (Rambo, 1954), and could also had acted as orogenetic barriers for population containment. The putative participation of extinct large-size mammals (megafauna) on long-distance dispersal via migrating herbivores (Janzen, 1986) should not be neglected, since seeds of *Opuntia* have been found in wooly mammoth (*Mammuthus*) dung, and megafauna fossils have been richly recovered along many rivers in the Pampean region (Davis et al., 1984; Scanferla et al., 2013).

There are no fossils to aid in an absolute dating of the Cactaceae. Taxon sampling with representative fossils in outgroups has yielded estimates for the crown age of cacti to be around 28.6 (26.7–30.5) million years ago (Mya) (Arakaki et al., 2011), 26.88 (16.67–37.10) Mya (Hernández-Hernández et al. (2014)), 28.8 (15.08–48.15) Mya (Magallón et al., 2015) and 42.5 (54.5–26.5) Mya (Silva et al., 2018a). Although these can be understood as of moderate age, the subsequent divergence and diversification in the family was generated by significant radiations occurring more recently throughout the mid to late Miocene, into the Mid-Pliocene and more recently in the Pleistocene (Ritz et al., 2007; Arakaki et al., 2011; Hernández-Hernández et al., 2014; Bonatelli et al., 2014; Majure et al., 2019). In *Opuntia*, previous studies proposed that the clade started to diverge 5.6 Mya (+/- 1.9) in the Late Miocene, but all major extant clades diverged during the Pliocene with subsequent diversification and speciation fully nested into the Pleistocene (Majure et al., 2012a), which is largely congruent with our hypothesis of Pleistocene impacts in the geographical distribution of *O. bonaerensis* across southern South American areas. However, further studies should be carried out sampling more individuals per population and a larger set of molecular markers, including from the nuclear genome, to access a more precise history of the Pleistocene influences in cactus genetic differentiation and putative linkage to specific bioregions.

The records from Mendoza are not absolutely resolved yet. The region is not represented by the Pampean domain, but rather from an Andean-Patagonic domain characterized by the Puneña and Altoandean floras (Oyarzabal et al., 2018). These records were previously reported by Font (2014) with accurate species identification, who suggested that this seemingly anomalous distribution could be from non-natural dispersion, as minor ornamental uses of the species are known in homegardens in the capital of the province. However, our new records, combined with the previously known distribution, revealed a congruent distribution pattern that includes mountainous areas of southern Brazil, Uruguay and Argentina - known as the Neotropical Peripampasic Orogenic Arc – that has been reported for an array of animal taxa, such as spiders,

scorpions, harvestmen and moths (Ferretti et al., 2012; Silva et al., 2018b), suggesting new insight for the historical biogeography of *Opuntia bonaerensis* that must be further explored.

Implications for conservation

Brazil, Uruguay and Argentina are signatories of the Convention on Biological Diversity (CBD), following the Global Strategy for Plant Conservation (GSPC) and are directly dealing with plant knowledge, use and conservation in their territories (Sharrock et al., 2018). Enormous efforts have been undertaken in Brazil to achieve some targets for the development of a functional and widely accessible list of all known plant species of the country (Forzza et al., 2010). However, as the country has long been acknowledged as a world leader in floristic diversity, it is clear that many gaps in our knowledge of the flora still need to be filled (Mittermeier and Mittermeier, 1997; Forzza et al., 2012; BFG, 2015).

Opuntia is a representative genus that exemplifies the increasing of local knowledge regarding its biodiversity. In the first attempt of an authoritative census of the Brazilian flora with scientific credibility to guide conservation planning, just one native species of *Opuntia* was reported as occurring in the country (Forzza et al., 2010, 2012). Later, increasing the efforts to field studies, collections and preparation of materials for herbaria, more than ten species have been documented (Carneiro et al., 2016; Köhler et al., 2018; Zappi and Taylor, 2019; Köhler et al., in prep.).

Here, we provide the first report and confirm the presence of *Opuntia bonaerensis* for the Brazilian and Uruguayan floras. With these new data, we augment the known distribution of the species, previously treated as endemic to Argentina, and expand the conservation efforts for the species. Rio Grande do Sul state has its own Red List of endangered flora, which helps to protect species from the different threats that plants and especially cacti suffer, and is frequently updated (Rio Grande do Sul, 2014). Although *O. bonaerensis* is one of the dozens of *Opuntia* species that have not been yet officially evaluated for its conservation status on the IUCN Red List, its assessment for the local Red List of Rio Grande do Sul is highly recommended for the next revision of the List, considering the limited populations and the ecological significance of the species in the region.

Opuntia bonaerensis is an endemic species of the Pampa biome and the Río de la Plata grassland, one of the largest continuous grassland ecoregions in the Americas covering the vast plains of central-eastern Argentina, Uruguay and part of southern Brazil (Andrade et al., 2018). This is a diverse and historically neglected region for conservation, with less than 3% of its territory under protection, that only now is receiving increasing efforts and strategies for its conservation

(Krapovickas and Giacomo, 1998; Overbeck et al., 2015; Oliveira et al., 2017; Andrade et al., 2018; Vieira et al., 2019). In general, our projections for future scenarios under climate change revealed a north-south distribution shift when comparing to past and current models, with accumulation in the extreme-southern portion of the modern records (Fig. 3). These regions are poorly covered by protected areas, considering only the Natural Reserve of Bahía San Blas and the Ernesto Tornquist Provincial Park (both in Buenos Aires, ARG) as important protected areas that would contain *O. bonaerensis* distributions in worst-case projections.

The records from Mendoza, which are from non-cultivated populations, are in a distinct climatic condition, confirmed by our niche equivalence and similarity tests. It is important to note that there are only two records of difference between the PM and P datasets, which may bias the results of similarity tests. A proper way to do that should be comparing sets with no shared records, which is not possible in this case due to the small number of records in Mendoza. Curiously, ensemble models using the P and PN datasets revealed a slight suitability of presences under the future (RCP 2.6 and RCP 8.5) and current scenarios, respectively, for regions encompassing the Mendoza province (Fig. 3). This, plus the trend of increasing suitability averages for Mendoza records (Fig. S1), suggests that the Mendoza region may act as a future refuge for *O. bonaerensis* under climate change scenarios.

CONCLUSIONS

This study confirms for the first time the presence of *Opuntia bonaerensis* for the Brazilian and Uruguayan flora using molecular phylogenetics and morphology, extending the known distribution of the species and expanding the conservation efforts and strategies for it. Our update on the distribution of *O. bonaerensis* is coincident with the Neotropical Peripampasic Orogenic Arc and suggests new insights for the historical biogeography of the species that must be further explored. The assembled ecological niche models using four different datasets of presence records suggested that the newly revealed Brazilian and Uruguayan populations are putative relicts of a Pleistocene distribution, illuminating for the first time that climatic oscillations during the last 21,000 years may have played an important role in cactus distribution in southern South American Pampean-Chaco regions. Our analyses of climatic suitability trends revealed that the region of Mendoza, previously assumed to be from a non-natural distribution, may act as a future refuge for *O. bonaerensis* under the climate change scenarios explored. Further phylogeographic approaches,

sampling more individuals per population and populational genetic markers should be pursued to reveal a detailed history of the species through its now better-known distribution.

CRediT authorship contribution statement

Matias Köhler: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Validation, Visualization, Writing - original draft, Writing - review & editing. Luíz F. Esser: Formal analysis, Methodology, Validation, Visualization, Writing - original draft. Fabián Font: Data curation, Investigation, Writing - review & editing. Tatiana T. Souza-Chies: Writing - review & editing. Lucas C. Majure: Funding acquisition, Resources, Supervision, Validation, Writing - review & editing.

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi: https://doi.org/10.1016/j.ppees.2020.125543.

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REVEALING A PRICKLY DIVERSIFICATION: THE RAPID AND RECENT RADIATION OF TRIBE OPUNTIEAE (CACTACEAE)

Esse artigo está sendo aprimorado e preparado para submissão ao periódico *Annals of Botany,* ou *Molecular Phylogenetics and Evolution.*

ABSTRACT

Evolutionary radiations are a fundamental topic in the understanding of life diversity. Cacti are one of the most fascinating New World's succulent plant radiations, but some of its major clades still lack detailed investigations about their patterns and processes of diversification. In this study, we explored a chloroplast genome dataset to investigate the major clade relationships within tribe Opuntieae, the celebrated prickly-pear cacti, to assess its morphological evolution, time divergence, historical biogeography, and macroevolutionary dynamics. Our analyses strongly supported Opuntieae as monophyletic with four major clades – 1) *Consolea*, 2) *Brasiliopuntia* + *Tacinga*, 3) *Miqueliopuntia* + *Salmonopuntia* + *Tunilla*, and 4) *Opuntia* – but contentious relationships between some clades were recovered comparing whole plastome and just genes data. Within *Opuntia*, the most diverse clade of Opuntieae, eight major well-supported clades were recovered. Our analyses suggested that Opuntieae represents a shift of increase in diversification rates within Opuntioideae, which means that variation among major clade richness can be explained without invoking heterogeneous diversification dynamics within the tribe. Furthermore, the tribe has undergone a rapid and recent evolutionary radiation during the last ~5 Mya, in which the clade have exhibited long-distance dispersal events that resulted in currently occupying all major arid and semi-arid regions of the Americas. These events have occurred accompanied by phenotypic disparity with the evolution of several homoplasious characters within Opuntieae members, and a prickly history of diversification with no one key feature detected single explaining it across lineages. This study provides the first chloroplast genome phylogenetic framework within a satisfactory taxon sampling of tribe Opuntieae providing a primary big picture of its diversification, but further analyses must investigate unexplored features on the evolutionary history of cacti.

ON THE CONTINUUM OF EVOLUTION: A PUTATIVE NEW HYBRID SPECIATION IN *Opuntia*

Esse manuscrito está sendo preparado para submissão e publicação no periódico *Systematics and Biodiversity*. As novidades nomenclaturais aqui apresentadas não tem intenção de validade.

ABSTRACT

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

Keywords – Biodiversity; Cactaceae; Hybridization; Polyploidy; prickly pear cacti

"THAT'S *Opuntia*, THAT WAS!", AGAIN: A NEW COMBINATION FOR AN OLD AND ENIGMATIC *Opuntia* s.l. (Cactaceae)

Esse artigo está submetido, e em revisão, no periódico *Phytotaxa*.

As novidades nomenclaturais aqui apresentadas não tem intenção de validade.

NEW YET NOT THAT MUCH:
A NEW IDENTITY FOR THE "LION'S TONGUE"

Esse artigo está sendo preparado para submissão no periódico *Muelleria*.
As novidades taxonômicas aqui apresentadas não tem intenção de validade.

CONCLUSÕES E PERSPECTIVAS

Os cactos-palma possuem uma complexa história evolutiva, com um aumento significativo nas taxas de diversificação junto a tribo Opuntieae, revelada com a homoplasia de muitos caracteres morfológicos, e acompanhada por uma rápida e recente radiação ao longo de todas as regiões áridas e semiáridas do continente Americano nos últimos 3 milhões de anos (Köhler et al., *in prep.* – Cap. 7). *Opuntia* é um dos gêneros de Cactaceae mais rico em número de espécies, e a diversidade do grupo é representada por oito principais linhagens evolutivas (clado *Quitensis, Elatae, Nopalea, Basilares, Scheerianae, Setispina, Humifusa* e *Macrocentra*), com perceptível estrutura biogeográfica (Köhler et al, *in prep.* – Cap. 7). A diversificação do grupo é marcadamente situada durante o Pleistoceno e o presente, o que é congruente com hipóteses de que os eventos cíclicos de glaciação/interglaciação podem ter impactado a distribuição de espécies na América do Norte e no sul da América do Sul (Majure et al., 2012a; Köhler et al., 2020b). Além disso, eventos de hibridação, entre espécies próximas e distantes, parece ser um importante mecanismo de evolução no grupo, gerando processos de diversificação e de novidades morfológicas nos cactos-palma (Majure et al., 2012b; Köhler et al., *in prep.* – Cap. 8).

A complexa e espinhosa diversificação dos cactos-palma e relativos (*Opuntia* s.l.) é intrigante, e tem dificultado muito a taxonomia do grupo. Problemas com identificações equivocadas têm sido comuns (Majure et al., 2014; Köhler et al., *in prep.* – Cap. 10), e têm inclusive nebulado a sistemática e o posicionamento filogenético de alguns táxons (Majure & Puente, 2014; Majure, Köher et al., 2020 – Cap. 3; Köhler et al., *in prep.* – Cap. 9). Contudo, recentes avanços têm sido feitos com o uso de dados do genoma plastidial (Köhler et al., 2020a, Köhler et al., *in prep.* – Cap. 7), circunscrevendo de maneira robusta as tribos da subfamília Opuntioideae em três tribos e 17 gêneros: Cylindropuntieae e Tephrocacteae (com cinco gêneros cada), e Opuntieae (com sete gêneros).

Nesse contexto, a integração de diferentes abordagens de estudos torna-se fundamental para melhor entendimento desse grupo de plantas economicamente importantes, e extremamente promissoras em um cenário de mudanças climáticas e aridificação do clima (Nefzaoui, 2009; Park et al., 2018). As estratégias exploradas nessa tese – do campo à genômica – mostraram-se muito relevantes para a produção de conhecimento acerca da evolução e sistemática dos cactos-palma

nas Américas. Por meio de colaborações, da obtenção e uso de variados dados com diferentes técnicas para o estudo da biodiversidade, várias e distintas contribuições foram feitas.

Nossos estudos evidenciaram uma histórica negligência e subestimativa da diversidade dos cactos-palma no sul da América do Sul, e confirmaram a pretérita lacuna de conhecimento sobre o grupo. Ao combinar as diferentes abordagens, revelamos pela primeira vez a ocorrência da palma-platense (*Opuntia rioplatensis*) na flora do Brasil (Köhler et al., 2018), reportando, até o momento, sua restrita ocorrência no território do país ao Parque Estadual do Espinilho, RS. Também reavaliamos a identidade da palma-de-Osten (*O. canterae*), propondo um epitipo e reconhecendo os caracteres morfológicos que a distingue de *O. elata* (Köhler & Majure, 2020).

Combinando os diferentes dados de campo com análises moleculares e de modelagem de nicho ecológico, introduzimos novas perspectivas para o entendimento da distribuição de espécies de cacto-palma no sul da América do Sul (Köhler et al., 2020b). Revelamos que a palma-de-buenos-aires (*Opuntia bonaerensis*), previamente considerada endêmica do centro-sul da Argentina, na verdade, possui uma distribuição mais ampla – ao confirmar a ocorrência da espécie no Uruguai e no sul do Brasil com o uso de dados morfológicos e moleculares. Nossas análises sugerem que a espécie pode ter tido uma distribuição pretérita ampla e contínua ao longo da região pampeana, mas sendo influenciada pelos eventos de glaciação/interglaciação do Pleistoceno.

Coletar cactos não é uma prática usual para botânicos generalistas devido aos espinhos e à suculência das plantas, que demandam uma série de cuidados para processamento do material até a montagem das exsicatas. Isso torna a representatividade de cactos muito baixa – ou até ausente – em determinados herbários e coleções científicas. Assim, os esforços despendidos para amostragem dos cactos-palma ao longo de sua distribuição no sul da América do Sul durante a execução desse projeto – incluindo a revisão de herbários para checagem de materiais históricos e recentes associados aos táxons amostrados – foram essenciais e decisivos para a obtenção de parte dos resultados aqui apresentados, resolvendo problemas taxonômicos e trazendo novidades nomenclaturais (Köhler et al., *in prep.* – Cap. 9 e Cap. 10; Köhler & Majure, 2020), reportando novas ocorrências (Köhler et al., 2018, 2020b.), complementando hipóteses de cenários e panoramas evolutivos (Köhler et al., *in prep.* – Cap. 7 e Cap. 8; Fontenele et al., 2020).

Não são apenas peculiaridades morfológicas, fisiológicas e ecológicas que tornam os cactos tão fascinantes e atrativos. Nossos estudos revelam novas particularidades evolutivas relacionadas com rearranjos e modificações estruturais do genoma plastidial dos cactos-palma (Köhler et al., 2020a), que também podem estar associados com as variadas possíveis adaptações morfo-fisio-ecológicas. Além disso, uma novo cenário eco-evolutivo parece se estabelecer com a detecção e

descrição de novas linhagens de vírus específicas de cactos-palma (Fontenele et al., 2020; Fontenele et al., *in prep.*).

Apesar do considerável progresso no conhecimento aqui apresentado, ainda há várias lacunas a serem preenchidas tangendo a diversidade e a taxonomia dos cactos-palma nas Américas, e que estão em processo de serem elucidadas. Os significativos avanços aqui apresentados impulsionam um novo paradigma para o estudo da evolução e sistemática dos cactos-palma nas Américas. A partir da integração de novas ferramentas, técnicas e métodos, com o desenvolvimento de hipóteses filogenéticas cada vez mais robustas, e amostragens compreensivas ao longo da distribuição dos táxons em suas áreas naturais de ocorrência, importantes passos em direção a uma monografia botânica atualizada do grupo são dados. As ameaças à biodiversidade no Antropoceno, impostas pelo impacto do ser-humano sobre a natureza, fazem com que os esforços para a elaboração de monografias botânicas até então ambiciosas sejam mais do que imperativas e urgentes (Grace et al., 2021). E os cactos precisam disso.

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APÊNDICE I

A NOVEL DIVERGENT GEMINIVIRUS IDENTIFIED IN ASYMPTOMATIC NEW WORLD CACTACEAE PLANTS

por Rafaela S. Fontenele et al. (2020), publicado em *Viruses* 2020, 12(4), 398, https://doi.org/10.3390/v12040398, a qual deve-se referir para citação.

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INTRODUCTION

With the exception of a single species, *Rhipsalis baccifera* (Sols.) Stearn, which is also found in some tropical areas of the Old World, cacti are endemic to the Americas [1]. Cacti have undergone adaptive radiations across a wide variety of edaphically dry environments [1,2], which, together with high degrees of phenotypic diversification within the family, have yielded a broad range of morphological forms [3,4]. Phylogenetic relationships in the family are relatively well-known, and four principle clades have been recovered in analyses (Leuenbergeria, Pereskia, Cactoideae + Maihuenia, and Opuntioideae) [2,5,6]. Cacti are culturally, economically and ecologically important [7]. Since Europeans first arrived in the Americas, cacti have been transported throughout the world [1]: to be grown primarily as ornamentals, but also as a crop for their fruit and stems (known as nopales) and the farming of cochineal insects (Dactylopius spp.), the latter of which are members of the order Hemiptera, used for the production of the carminic acid dye [8].

In 1885, the first evidence of spindle-like structures associated with a virus infection was described from cacti in the genus *Epiphyllum* [9]. Since then, a handful of viruses have been identified in other members of the Cactaceae, all of which belong to the single-stranded RNA virus families *Alphaflexiviridae*, *Betaflexiviridae*, *Puribunyaviridae*, *Tombusviridae* and *Virgaviridae* [10,11,12,13, 14,15,16,17,18]. To our knowledge, no plant-infecting DNA viruses (i.e., viruses belonging to the families *Geminiviridae*, *Nanoviridae,* and *Caulimoviridae*) have ever been found to infect cacti.

High-throughput sequencing (HTS) technologies have led to a dramatic increase in the discovery of novel viruses across ecosystems, and have broadly expanded our knowledge of plant-infecting virus diversity [19,20]. The impacts of these technologies on plant virus discovery are evident within the family *Geminiviridae,* a family of plant viruses for which HTS-based virus discovery projects are uncovering a growing number of divergent lineages. In addition to the nine recognized geminivirus genera — *Becurtovirus*, *Begomovirus*, *Capulavirus*, *Curtovirus*, *Eragrovirus*, *Grablovirus*, *Mastrevirus*, *Topocuvirus* and *Turncurtovirus*; [21,22] — four of which were established based on viruses discovered in large-scale HTS-based virus discovery projects, it is likely that multiple new genera will need to be formed to accommodate 12 other, currently unassigned, divergent geminivirus lineages [23,24,25,26,27,28,29,30,31,32].

Although many of the known geminiviruses cause severe economic losses in a variety of crops (i.e., tomato, maize, cotton, cassava and bean plants) [33,34], many of the newly discovered geminiviruses seem to produce either no symptoms or only very mild symptoms, in the host species from which they were isolated [25,31,35,36,37].

Besides prompting the founding of new geminivirus genera, newly discovered divergent geminivirus lineages are illuminating the deep evolutionary history of this family. The circular single-stranded DNA genomes of the known geminiviruses are encapsidated in twinned icosahedral particles [38] and encode up to seven genes that are bi-directionally transcribed. The only two genes that are detectably conserved across all of these divergent lineages are a replication associated protein gene (*rep*) and a capsid protein gene (*cp*). In addition to these two genes, three others, a replication enhancer protein gene (*ren*), a C4 gene (which encodes a symptom determinant and/or a silencing suppressor), and a transactivation protein gene (*trap*), are possibly conserved across the genera *Begomovirus*, *Curtovirus*, *Eragrovirus*, *Topocuvirus* and *Turncurtovirus*, although in some cases these genes are only putative homologs [21,39,40,41]. Although movement protein genes (*mp*) appear to occur in all known geminivirus genomes [40,41], there is commonly no detectable homology between the movement proteins (MPs) of viruses in the different geminivirus genera.

Geminiviruses are transmitted by a range of insect vectors in the order Hemiptera. In most cases, only one or a few very closely related vector species in a single genus transmit these viruses in each of the different geminivirus genera. Becurtoviruses, curtoviruses, and turncurtoviruses are known to be transmitted by leafhoppers in the genus *Circulifer*, begomoviruses by whiteflies in the genus *Bemisia,* topocuviruses by treehoppers in the genus *Micrutalis*, grabloviruses by treehoppers in the genus *Spissistilus*, and capulaviruses by aphids in the genus *Aphis* [21,33,42,43,44]. In the case

of mastreviruses, however, different virus species are transmitted by insects belonging to different leafhopper species in a number of insect genera including *Cicadulina, Orosius*, *Psammotettix,* and *Nesoclutha* [45].

Although geminivirus research in the past has primarily focused on viruses that are major pathogens of cultivated plants, much recent attention has been given to geminiviruses that circulate within natural ecosystems, especially those at agro-ecological interfaces [46,47,48,49]. The spill-over of viruses between agricultural and natural ecosystems can significantly impact both the preservation of natural ecosystems [50,51] and the emergence of new crop pathogens from these ecosystems [52,53,54].

Here, we describe the characterization of a divergent geminivirus lineage found to infect different cactus species and multiple genera (*Opuntia* spp., *Cylindropuntia* spp. and *Lophocereus schottii*) in the USA and Mexico. The viruses within this lineage have tentatively been grouped with a species named Opuntia virus 1 (OpV1). Infectivity assays involving *Nicotiana benthamiana* and three *Opuntia* spp. confirmed that OpV1 was able to asymptomatically infect *N. benthamiana* and *O. microdasys*.

MATERIALS AND METHODS

Sample Collection and Processing

A total of 527 Cactaceae plant samples (**Supplementary Data 1**) from the Cactoideae and Opuntioideae clades were collected in Argentina (*n* = 14), Bolivia (*n* = 8), Brazil (*n* = 8), Cuba (*n* = 1), Curaçao (*n* = 1), Dominican Republic (*n* = 2), France (*n* = 20), Haiti (*n* = 2), Lebanon (*n* = 1), Morocco (*n* = 1), Mexico (*n* = 31), Paraguay (*n* = 3), Reunion (19), Spain (*n* = 6), Tunisia (*n* = 10), Uruguay (*n* = 5), the United States (*n* = 394) and Venezuela (*n* = 1). Of the cactus samples from the USA, 134 were collected from the cactus collection at the Desert Botanical Garden in Phoenix, Arizona (USA). In addition, 25 non-cactus samples (from the Alliaceae, Amaranthaceae, Apiaceae, Asteraceae, Cucurbitaceae, Lamiaceae, Laureaceae, Malvaceae, Oleaceae and Solanaceae flowering plant families) were also collected from the Desert Botanical Garden in Phoenix, Arizona. Samples were collected using a 3 mm biopsy punch (Robbins Instruments, Chatham, NJ, USA) or scalpel blades. Although none of the sampled cacti were observed to have obvious infection symptoms, 61 plants were infested with cochineal insects (*Dactylopius* sp.). Insects from these 61 plants were

also collected (see **Supplementary Data 1** for details of all the samples analyzed). All samples were stored at −20 °C or dried on silica until processing.

Total DNA was extracted from cactus tissue samples using either the GeneJET Plant Genomic DNA Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA) or DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The cochineal insects (cohorts of 5–10 from a colony) were ground in 200 µl of SM Buffer (0.1 M NaCl, 50 mM Tris/HCl-pH 7.4, 10 mM $MgSO_4$) and subsequently centrifuged for 5 min at 10,000 rpm to pellet cellular material. The supernatant was then used to isolate DNA using the High Pure Viral Nucleic Acid Kit (Roche Diagnostics, Indianapolis, IN, USA). Both plant total DNA and cochineal insect purified viral DNA from each sample were used in a rolling circle amplification (RCA) reaction with the TempliPhi™ kit (GE Healthcare, Chicago, IL, USA), as described by Shepherd et al. [55].

High Throughput Sequencing and Genome Assembly

Aliquots of the RCA product of each sample were pooled (8 to 10 samples per pool) based on sampling location, and sequenced on an Illumina HiSeq 4000 platform (paired-end 2 × 100 bp) at Macrogen Inc. (Seoul, Korea). Raw reads were *de novo* assembled using SPAdes v. 3.12.0 [**56**] and the resulting contigs were analyzed using BLASTx [57] against a GenBank viral RefSeq protein database [**58**]. For contigs with a detectable homology (E-value of $< 10^{-5}$) to known geminiviruses, abutting primers were designed (OpV1_F 5'-GGG CCC CAA TAA GTT CTT TCC AAT GTT TTA GCT TT-3' and OpV1_R 5'-AAA GAG ACT GGC AAA GCA ACT GTA AAT ACG GCA AG-3') to recover potentially full-length virus genomes from plant and insect samples. The primers were used to amplify the geminivirus genomes using KAPA HiFi HotStart DNA polymerase (KAPA Biosystems, USA), following the manufacturer's thermal cycling condition recommendations. Amplicons were resolved in 0.7% agarose gel and those with a size of between 2.5 and 3.5 kb (the expected size-range of geminivirus genomes or genome components) were excised, gel-purified and cloned in the pJET1.2 cloning vector (Thermo Fisher Scientific, Waltham, MA, USA). Cloned amplicons were Sanger sequenced by primer walking at Macrogen Inc. (Seoul, South Korea). Genome assemblies and annotations were performed using Geneious 11.1.5 [59].

Infectivity Assays

One Opuntia-derived geminivirus isolate, OpV1 DBG14_1 (GenBank accession # MN100000) recovered from *O. echios* var. *echios* sampled from the Desert Botanical Garden (Phoenix, AZ, USA) was chosen for the construction of an infectious OpV1 clone. OpV1 F/R

primers were phosphorylated using T4 kinase (New England Biolabs, Ipswich, MA, USA) and subsequently used to amplify the genome from OpV1 DBG14_1. The amplified genome was self-ligated using T4 DNA ligase (Thermo Fisher Scientific, Waltham, MA, USA) to generate a circular genome, which was subsequently amplified by RCA with the TempliPhi™ kit (GE Healthcare, Chicago, IL, USA). The RCA product was then digested with either *Hind*III to generate a linearized full genome copy (FGC; 2945 nt in length), or with both *Hind*III and *Bam*HI to generate a near full-length genome copy (nFGC; 2750 nt in length). The FGC and nFGC were individually cloned into the *Hind*III and/or *Bam*HI restriction enzyme sites of the vector pBlueScript-KS, and Sanger sequenced by primer walking at Macrogen Inc. (Seoul, South Korea). The FGC and nFGC were then cloned in the *Hind*III/*Bam*HI digested pGTV-kan [60] binary vector and used to transform *Escherichia coli* XL1 Blue. To confirm two copies had ligated in tandem, clones were tested by digesting them with *Bam*HI. A clone containing tandemly cloned FGC and nFGC was then used to transform *Rhizobium radiobacter* (synonymous species name for *Agrobacterium tumefaciens*) GV3101. A glycerol stock of this was prepared and stored at −80 °C.

Infection assays were performed on *N. benthamiana*, *O. ficus-indica*, *O. microdasys*, *O. engelmannii*, and *O. santa-rita*. *Rhizobium*-mediated OpV1 infections of *N. benthamiana* were performed in three replicates, with 18 inoculated plants in two replicates and seven in the third, including two negative controls (non-inoculated plants) in each replicate. Five opuntia plants for each species were *Rhizobium*-inoculated, and one plant was used as a negative control. For the *Rhizobium*-inoculations, *R. radiobacter* was grown for 20 h in Luria broth with kanamycin (50 μg/mL) and rifampicin (50 μg/mL). The culture was then centrifuged for 10 min at 4600 rpm to pellet the cells before resuspension in MES buffer (10 mM MES hydrate and 10 mM $MgSO_4$ hepta-hydrate) with acetosyringone 150 μM to an OD of 1.0.

The seven inoculated *Nicotiana benthamiana* plants from the third infection assay were used for Southern blot analysis. We also included two negative control plants (non-inoculated). Total DNA was extracted from the *N. benthamiana plants* as described in "Sample Collection and Processing" and 5 μg total DNA from each plant and a positive control (5 ng of OpV1 PCR amplicon of the genome) were resolved on a 1% agarose gel. The resolved nucleic acid was transferred to a positively charged nylon membrane Hybond-N+ (GE Healthcare, Chicago, IL, USA) and UV-crosslinked. The membrane was hybridized with a digoxygenin-labelled specific probe for the OpV1 full genome. The probe synthesis, hybridization and detection were obtained using the DIG High Prime DNA Labeling and Detection Starter Kit I (Roche, Indianapolis, IN, USA) according to the manufacturer's instructions.

Phylogenetic and Pairwise Identity Analyses

Genome-wide pairwise nucleotide sequence identities between the 79 OpV1 genomes were determined using SDT v1.2 [61]. A genotype demarcation threshold of 95% was selected based on the distribution of pairwise identities and this revealed the existence of 15 genetically distinct OpV1 "genotype groups".

Representative full-length nucleotide sequences from each of these 15 genotype groups, together with the genomes of geminiviruses belonging to the nine classified genera (30 sequences) and those that remained unassigned to a genus (12 sequences), were aligned by MAFFT v.7 [62]. This alignment was used to infer a Neighbor-Joining phylogenetic tree using a Jukes–Cantor substitution model with 1000 bootstrap replicates being used to test branch supports. Branches with < 60% bootstrap support were collapsed using TreeGraph2 [63], and the phylogenetic tree was midpoint-rooted.

The 79 OpV1 genomes were aligned with MAFFT v.7 [62] and the resulting alignment was used to infer a Neighbor-Joining phylogenetic tree using the Jukes–Cantor substitution model with 1000 bootstrap replicates being used to test branch supports. Branches with < 60% bootstrap support were collapsed using TreeGraph2 [63]. The OpV1 genome sequences, with recombination regions removed, were used to infer a Maximum-Likelihood (ML) phylogenetic tree using PHYML 3.0 [64] with the GTR+Γ+I substitution model selected as best fitting by jModelTest [65].

Datasets were also constructed that contained either the inferred Rep or inferred CP amino acid sequences of one, representative of each of the 15 OpV1genotype groups, along with representative sequences of viruses in the nine established geminivirus genera (30 viruses) and sequences from geminiviruses that remain unassigned to any genus (12 viruses). These Rep and CP amino acid datasets were aligned by MAFFT v.7 [62]. The alignments were used to infer ML phylogenetic trees using PHYML 3.0 [64] with the amino acid substitution models rtRev+G+F+I used for the CP dataset and rtRev+Γ+F+I used for the Rep dataset (these models were determined as best fitting by ProtTest; [66]), using the approximate likelihood ratio test (aLRT) of branch support. Branches with < 0.8 aLRT support were collapsed with TreeGraph2 [63] and both ML trees were rooted with sequences of viruses from the family *Genomoviridae*.

Capsid Protein Cluster Analysis

The CP amino acid sequences of all geminiviruses available in GenBank were extracted and clustered using CD-HIT [67] with a 90% identity threshold. A representative from each cluster

was chosen and together with the CP amino acid sequences from representatives of the 15 OpV1 genotypes these were used to generate a sequence similarity network using the Enzyme Function Initiative–Enzyme Similarity Tool (EFI-EST) [68]. The network was created using a similarity score of 60 and E-value threshold of 1×10^{-5}. The network was visualized in Cytoscape v3.7.1 [69] with the organic layout.

Recombination Analysis

The OpV1 genomes (n = 79) were aligned by MAFFT v.7 [62] and recombination analysis was performed by RDP4 v.4.97 [70] with default settings using the detection methods RDP [71], GENECONV [72], BOOTSCAN [73], MAXCHI [74], CHIMERA [75], SISCAN [76] and 3SEQ [77]. Recombination events that were detected by three or more methods with p-values < 0.05 were accepted as credible.

Virus Purification and Transmission Electron Microscopy

A total of 40 g of infected *N. benthamiana* leaves, 21 days post *Rhizobium*-mediated OpV1 infection, was homogenized in 40 mL of extraction buffer (1 × PBS pH 5.2, 10 mg/mL sodium ascorbate, 2 mM PMSF, 1 mM EDTA). The homogenate was filtered through two layers of cheese cloth and two layers of miracloth, and thereafter centrifuged for 30 min at 14,800× *g*. The clarified supernatant was kept at 4 °C overnight and then centrifuged twice for 30 min at 14,800× *g* and the pH was adjusted to 7.0. The supernatant was then centrifuged for 4 h at 32,000 rpm using a Beckman 32 Ti rotor, (Beckman Coulter, Pasadena, CA, USA) onto a 10% sucrose cushion and the pellet resuspended in 1 mL of 1× PBS. A total of 10 µL of a 1:10 dilution of the resuspended pellet was absorbed onto a carbon-coated copper grids for 10 min, washed, and negatively stained with 2% uranyl acetate. The grids were viewed using a Phillips CE 12 transmission electron microscope (Phillips, The Netherlands).

RESULTS AND DISCUSSION

A Novel Cactus-Infecting Geminivirus

In an attempt to determine whether cacti are natural hosts of geminiviruses, we screened a total of 527 cactus samples from 18 countries for the presence of geminiviruses using an HTS approach. Most of the analyzed samples were collected in the USA (n = 394) from botanical

gardens, herbaria and directly from native habitats. Based on geminivirus-like contigs recovered from these samples by HTS, a pair of abutting primers (OpV1 F/R) were designed to recover the full-length geminivirus-like genomes (or at least components of genomes). Amplicons of approximately 3 kb in length were produced using these primers from 31 cactus samples and nine cochineal insect samples.

Of the 31 samples found to contain geminivirus-like sequences, two cactus samples were from Mexico, 29 cactus samples were from the USA (Arizona, $n = 28$; Texas, $n = 1$), and all nine of the insect samples were from the USA. Of the areas in the USA where samples were collected, most ($n = 20$) were from the Desert Botanical Garden. Consequently, 25 additional non-cactus samples were collected from the Desert Botanical Garden to potentially identify alternate hosts. However, none of the non-cactus plant samples were found to contain OpV1-like sequences resembling those found in the cactus samples.

We amplified, cloned, and sequenced geminivirus genome-length DNA fragments (2940 to 2962 nt) from the 31 cactus, and nine insect samples that appeared to contain geminivirus-like DNA. These geminivirus-like genomes were tentatively named Opuntia virus 1 (OpV1), since most of them were retrieved from *Opuntia* spp. (**Table 1**). While some of the cochineal insects from which OpV1 genomes were recovered were collected from plants that also contained OpV1 genomes ($n = 4$) (**Table 1**), in other cases, insects containing OpV1 were collected from plants that did not detectably contain such genomes ($n = 5$) (**Table 1**).

Pairwise identity comparisons of OpV1 sequences to those of other known geminiviruses demonstrated that they all share < 64.9% genome identity with other known geminiviruses, and that all the OpV1 sequences share > 78.4% identity with one another (**Supplementary Data 2 and 3**).

OpV1 sequences all contain at least six recognizable open reading frames (ORFs) that were both capable of encoding proteins with >198 amino acids, and which shared some detectable similarity with known geminivirus-expressed proteins. If these ORFs are indeed genes, then the genome organization of the OpV1 sequences resembles that of viruses in the genus *Begomovirus* with monopartite genomes. On the presumed complementary strand, the OpV1 sequences potentially encode a replication-associated protein (Rep), a replication enhancer protein (Ren), a transactivation protein (TrAP) and a symptom determinant protein (C4) (**Figure 1**). A likely capsid protein (CP) and a possible movement protein (MP) are encoded on the virion strand. Within the OpV1 sequences, in the area corresponding to an intergenic region, there is a conserved nonanucleotide motif, "TAATATTAC", contained within a likely stem–loop structure which, by

analogy with other geminiviruses, is the likely site where virion strand replication is initiated (Figure 1). Within the intergenic region, we identified replication-associated iterative sequences "iterons", the TATA box and conserved late element (CLE)-like sequences (Figure 1). There were two discernible iterons among most OpV1 isolates: a direct repeat adjacent to the *rep* gene TATA box, and an inverse repeat situated 41–42 nt upstream the TATA box. However, in a few OpV1 isolates, two in-tandem iterons are associated with the *rep* TATA box, similar to iterons observed in New World begomoviruses [78]. The specific sequence of the iterons also varied among OpV1 isolates, predominating those with a GGGTCC core sequence, although repeated elements with either GGTGCC, GGAGTC, GGTATY, or GGTGTC core sequences, among others, were also identified in some OpV1 isolates (Figure 1). The functional relevance of those differences is currently unknown. Another OpV1 feature is the position of the TATA box immediately adjacent to the *ori* stem-loop element (Figure 1), a unique arrangement among the geminiviruses.

As with the OpV1 nucleotide sequences, the amino acid sequences of the individual proteins that are likely encoded by these sequences display a considerable amount of diversity. Even the most conserved of these, CP and Rep, respectively, have pairwise amino acid sequence identities that are as low as 74.3% and 77.1% between different isolates.

Based on the distribution of pairwise nucleotide sequence identities shared by the 79 OpV1 sequences, a 95% sequence identity threshold was selected as a cut-off for defining distinct OpV1 genetic groupings. Applying this threshold to sub-classify the OpV1 sequences yielded 15 genotype groupings (Table 1).

It is noteworthy that, out of the 13 instances where more than one OpV1 sequence was isolated from a given plant sample, in seven cases the OpV1 sequences belonged to different genotype groups, i.e., in > 50% of instances where two different sequences were sampled from the same plant, these two sequences shared < 95% pairwise identity (Table 1). In three out of five instances where OpV1 sequences were retrieved from insects that were sampled on a plant from which OpV1 sequences were retrieved, the sequences in the insects were assigned to different genotypes than those to which the sequences in the plants were assigned.

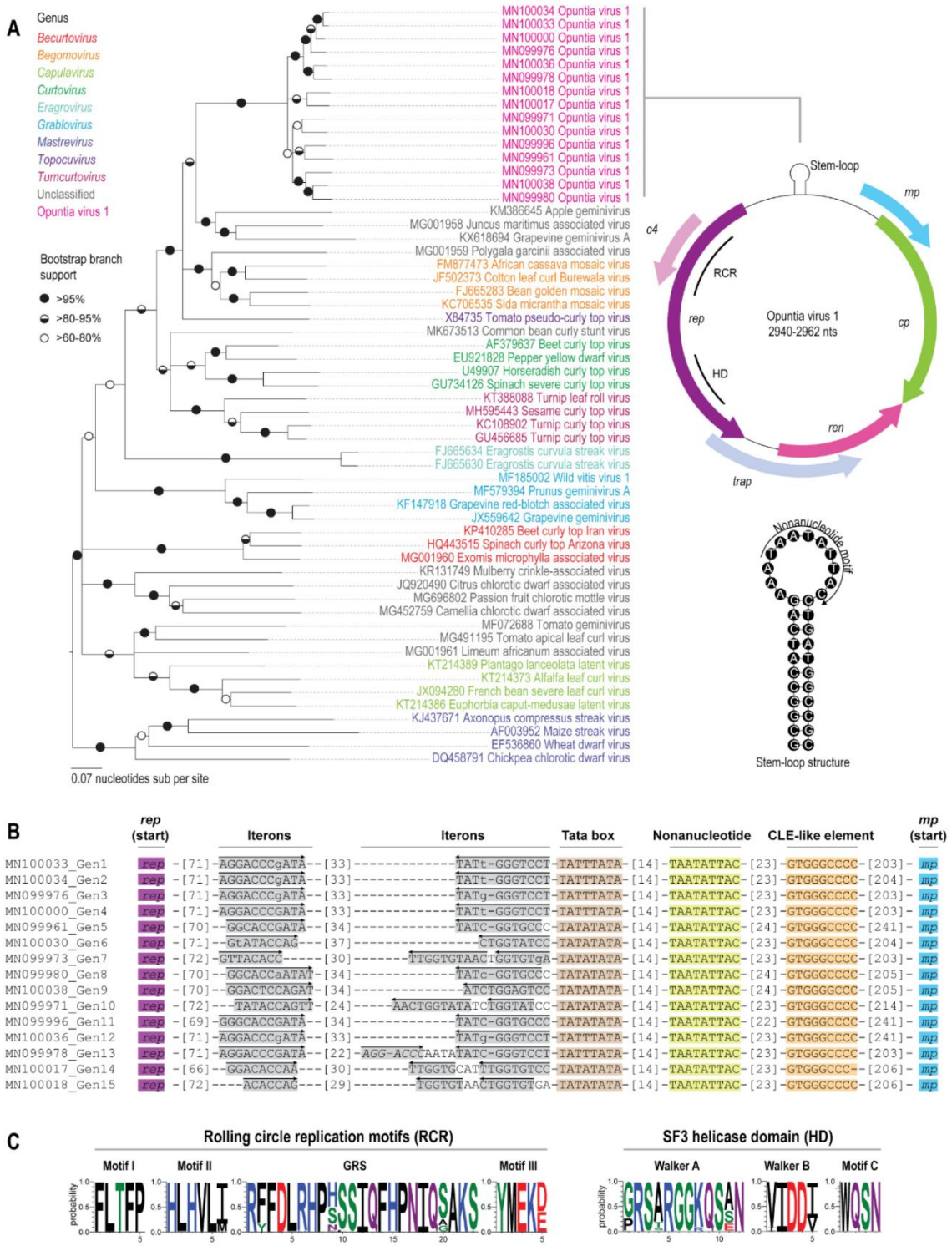

Figura 38 (Figure 1). (**A**). Neighbor-Joining phylogenetic tree of the full-length genome of representatives of the 15 genotypes of OpV1 with those of the family *Geminiviridae*. Branches with < 60% bootstrap support are collapsed, and the tree is midpoint-rooted. The genomic organization of OpV1 and the stem–loop structure containing the nonanucleotide motif are shown to the right of the phylogenetic tree. (**B**). Nucleotide sequence and organization of origin of replication-associated iterative sequences "iterons" in the intergenic region. Arrows indicate the orientation of the iterons with respect to the nonanucleotide sequence. Lower-case letters in an iterated element indicate a nucleotide that does not match in all the iterons from OpV1 viruses of that same genotype. The TATA box, nonanucleotide motif and conserved late element (CLE)-like sequence. (**C**). Graphic representation of the variation in amino acids in the motifs from the SF3 helicase domains and the rolling circle replication motifs present in the Rep sequences of the 79 OpV1s.

Phylogenetic analysis of the full-length genome of OpV1 genotypes with representative geminivirus genome sequences (i.e., including representatives of the nine established geminivirus genera and other geminiviruses that have not yet been assigned to a genus) indicated that the OpV1 sequences could justifiably be assigned to a new geminivirus genus (**Figure 1**). The OpV1 sequences are most closely related to begomoviruses, topocuvirus and the unassigned geminiviruses Polygala garcinii associated virus (MG001959), apple geminivirus, (KM386645), Juncus maritimus associated virus (MG001958), and grapevine geminivirus A (KX618694).

Similarly, phylogenetic analysis of the predicted OpV1 Rep amino acid sequences, together with those of representative geminiviruses, indicated that the OpV1 Rep sequences are most closely related to those of begomoviruses, curtoviruses, topocuviruses, turncurtoviruses and the unclassified geminiviruses common bean curly stunt virus (MK673513)*;* Polygala garcinii associated virus (MG001959); apple geminivirus (KM386645); Juncus maritimus associated virus (MG001958) and grapevine geminivirus A (KX618694) (**Figure 2**). The OpV1 Rep amino acid sequences share < 68.2% identity with those of other geminiviruses.

The predicted OpV1 Rep amino acid sequences all contain predicted rolling circle replication, GRS, SF3 and Walker motifs that are similar to those found in other geminiviruses [**80**]. It is noteworthy that there is variability within these Rep motifs across the different predicted OpV1 Rep amino acid sequences, which further emphasizes the diversity within this group of viruses (**Figure 1**).

Unlike with the Rep amino acid sequences, the predicted OpV1 CP amino acid sequences group phylogenetically within a divergent clade (**Figure 2**). This is likely a consequence of the OpV1 CP amino acid sequences sharing < 28.9% amino acid identity with those of other geminiviruses. Recently, phylogenetic evidence that the CP amino acid sequences of geminiviruses are possibly co-diverging with their specific insect vectors has emerged [**81**]. A sequence similarity network analysis of the CP amino acid sequence of all geminiviruses (with a > 90% identity cut-off) was generated and the association of the known geminivirus CPs with known insect vectors is summarized in **Figure 3**. It is clear that, whenever geminiviruses share an insect vector, their CP amino acid sequences cluster together. As expected, given the divergence of OpV1 CP amino acid sequences relative to those of other geminiviruses, these sequences form their own cluster, implying that they are likely to be transmitted by an insect species that has not previously been associated with geminivirus transmission. Given the association of cochineal insects with the cactus plants from which OpV1 sequences were isolated and the direct isolation of OpV1 sequences from some of these insects, it remains plausible that these insects may be OpV1

transmission vectors. However, controlled insect transmission experiments will be needed to properly test this hypothesis.

The high degree of nucleotide sequence diversity amongst the OpV1 sequences suggests, assuming a similar rate of nucleotide sequence diversification to that seen in other geminiviruses, that OpV1 has likely been circulating in the USA for more than 600 years, i.e., the approximate time it would take mastrevirus and begomovirus species to achieve the degree of diversity observed for the OpV1 sequences [**82**,**83**,**84**,**85**,**86**,**87**]. The lower numbers of OpV1-positive samples found outside the USA certainly represents a sampling bias. Although the number of OpV1-positive cactus plants originating from Mexico were very low (2/31 tested plants), this 6.4% prevalence is not substantially different to the 7.4% OpV1 prevalence in cactus samples from the USA.

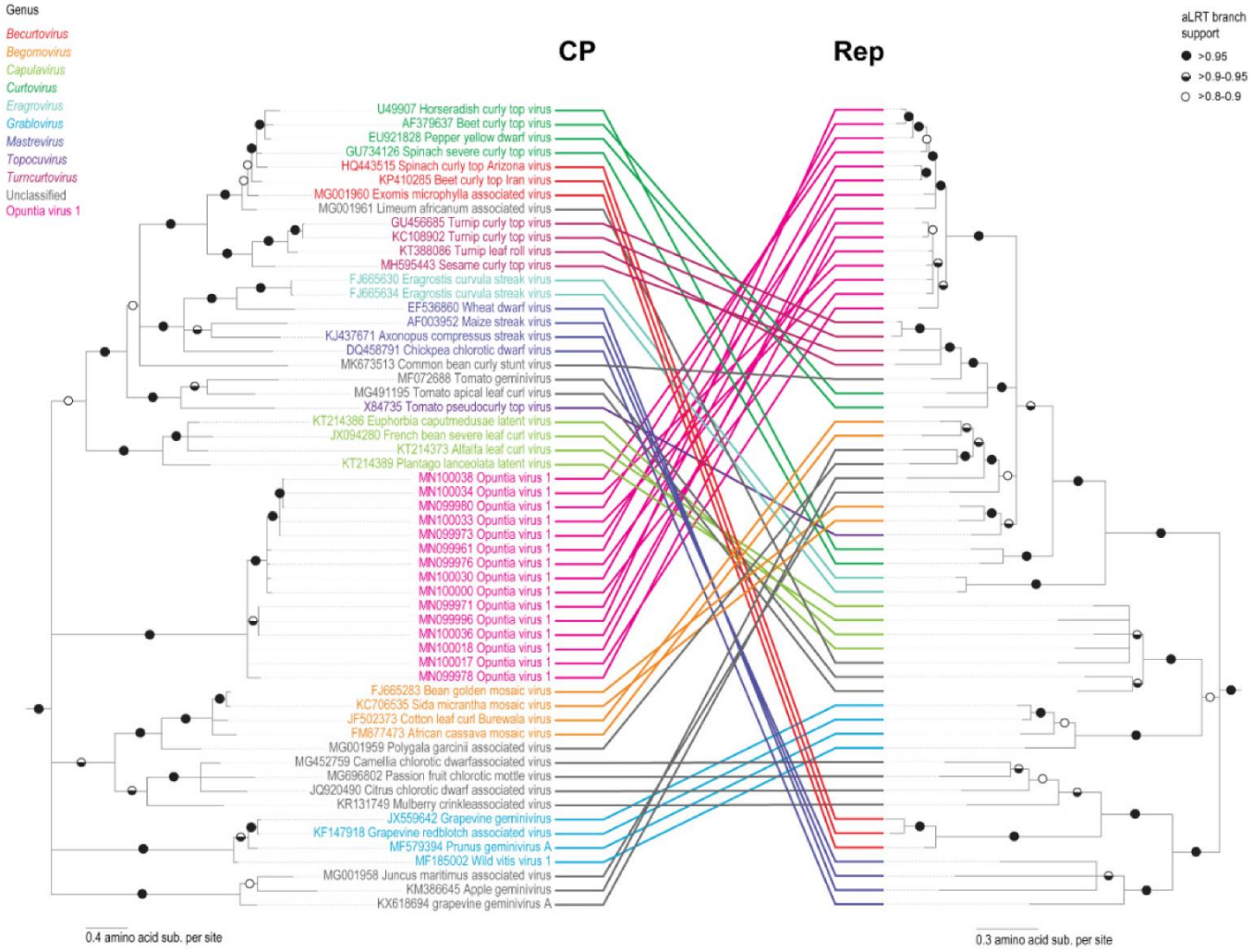

Figura 39 (Figure 2). Maximum Likelihood (ML) phylogenetic trees of the Rep and CP amino acid sequences of the representative 15 genotypes of OpV1 together with other geminiviruses. Branches with aLRT support < 0.8 are collapsed and both trees were rooted with genomovirus [79] sequences.

Testing the Infectivity of the Novel Cactus-Infecting Geminiviruses

To assess the infectivity of OpV1, a *Rhizobium*-infectious clone was created using the isolate DBG_14_1 (MN100000). The infectious clone was generated using ~1.9 unit length DBG_14_1 sequences cloned tandem within the pGTV-kan binary vector [60]. *Rhizobium*-mediated inoculation assays were performed on plants of *N. benthamiana*, *O. ficus-indica*, *O. microdasys*, *O. engelmannii* and *O. santa-rita*. The experiments with *N. benthamiana* were carried out in triplicate, with two replicates consisting of 18 inoculated plants and the third of seven inoculated plants; two negative control (non-inoculated) plants were included in all experiments. The rate of infection in *N. benthamiana* plants varied between replicates. In the initial experiment, five out 18 plants were positive for OpV1 infection and systemic viral infection could be detected at 5 days post inoculation (dpi). In the second experiment, 10 out of 18 plants were positive for OpV1, with systemic viral infection also being detectable at 5 dpi. In the third experiment, samples were only evaluated at 21 dpi and they were all positive for OpV1 by PCR. The Southern blot analysis of the third infectivity assay of *Rhizobium*-mediated OpV1-infected *N. benthamiana* plants corroborated with the PCR results, showing the viral DNA replicative forms in all seven inoculated plants (open circular, linear, covalent closed circular and single stranded) and no viral infection in the negative controls (**Supplementary Figure S1**). We also observed geminate particles in the viral extract of the *Rhizobium*-mediated OpV1 infected *N. benthamiana* leaves (**Supplementary Figure S1**). In the inoculation assays with *O. ficus-indica*, *O. microdasys*, *O. engelmannii* and *O. santa-rita,* five plants (one individual pad) from each species were *Rhizobium*-infiltrated and one plant was kept as a negative control (non-inoculated). Cacti are perennial plants that have slow growth rates, and it is therefore difficult to assess systemic infection. From the inoculated cactus species, only *O. ficus-indica* and *O. microdasys* plants developed new pads over the course of the experiment (~8 months). Wherever new pads were unavailable for sampling, the area of sampling in the originally inoculated pad was selected to be as distant as possible from the spot where the *Rhizobium*-inoculation was carried out. Of the 16 *Opuntia* plants inoculated with OpV1, only one, an individual of *O. microdasys*, was positive for OpV1 by PCR five months post-inoculation.

No symptoms associated with viral infection were observed in either *N. benthamiana* or *O. microdasys*. Infections were further confirmed by recovering viral genomes (which were cloned and sequenced; **Supplementary Data 4**) from the non-inoculated leaves of five *N. benthamiana* OpV1 positive plants and one OpV1 positive *O. microdasys* plant.

Evolutionary Dynamics of the Novel Divergent Geminivirus Group

Given that genetic recombination has been found to occur frequently during the evolution of other geminiviruses and that recombination has been implicated in the genesis of at least four of the currently recognized geminivirus genera [**43**,**46**,**88**,**89**,**90**,**91**,**92**,**93**,**94**,**95**,**96**], we examined the OpV1 sequences for evidence of recombination. In total, we detected 23 well-supported recombination events during the evolution of the 79 OpV1 sequences from their most recent common ancestor. The sizes of genome fragments transferred during these recombination events ranged from approximately 64 to 1171 nt (**Table 2**). Except for the only sequence belonging to genotype 8, all the sequences displayed well supported evidence of at least one recombination event. Some of the OpV1 sequences assigned to genotype 12 display evidence of at least five distinct recombination events. While some of the detected recombination events appear to have occurred quite recently, in that they were only detectable within single OpV1 sequences, others, such as one event that is detectable in all of the genotype 1, 2, 12 and 13 sequences, likely occurred in the more distant past, i.e., prior to the time when the most recent common ancestors of the sequences, sharing evidence of the recombination events, existed.

The largest genome fragment transferred during the detected recombination events was seen in the genotype 6 sequences, and involved the transfer of the ~40% of the genome spanning the intergenic region and the virion strand protein-coding genes.

As has been previously noted for other geminiviruses [**95**,**96**,**97**,**98**], a high proportion of the detected recombination events have breakpoints in the intergenic region at or close to the presumed virion strand origin of replication. Similar to breakpoint patterns seen in other geminiviruses, the Rep/AC4 region of the genome appears to be the genome site outside the intergenic region where recombination breakpoints most frequently occur (**Figure 4**). Conversely, the region of the genome spanning the *ren* and *trap* genes appears to have the lowest frequency of detectable recombination breakpoints.

Identification of Sub/Super- Genomic Molecules

It is noteworthy that during attempts to clone OpV1 sequences, we recovered 12 apparently sub-genome length clones containing OpV1 sequences from nine cactus plants and one cochineal insect (OpV1 sg 9) (**Figure 5**), as well as a sequence containing a full complement of OpV1 DNA together with a 238 nt long sequence insert of unknown origin (i.e., super-genome length) from one cactus plant (OpV1 sg 2). The presence of similar sub-genome length geminivirus-derived

DNA within geminivirus infections, commonly referred to as sub-genomic molecules, have been extensively reported elsewhere [**27**,**99**,**100**,**101**,**102**,**103**,**104**,**105**,**106**]. In addition to deletions, in some cases sub-genomic molecules have also been found to contain sequence insertions, duplications and inversions [**103**,**107**,**108**]. The conservation within sub-genomic molecules of intergenic region sequences—the portion of geminivirus genomes containing the origin of virion-strand replication—indicates that these molecules are, in many cases, potentially either self-replication-competent (if they contain an intact *rep* gene) or are capable of being trans-replicated by non-defective viruses [**109**].

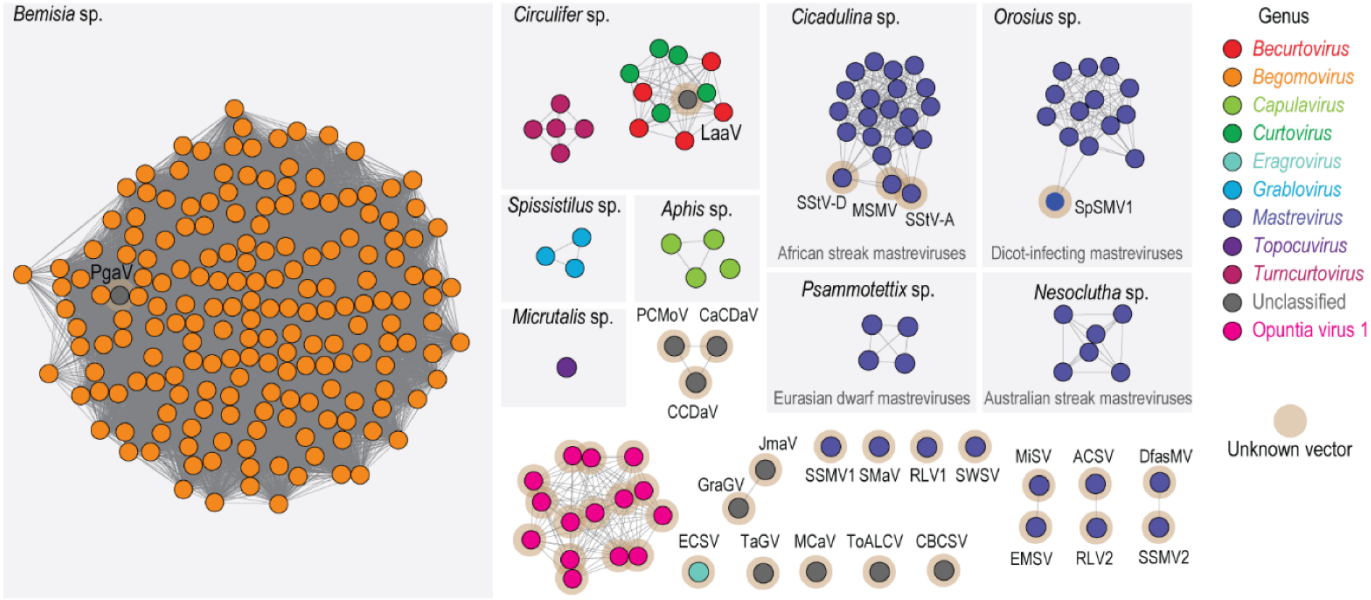

Figura 40 (Figure 4). Sequence similarity network analysis of the CP amino acid sequences of representatives of the 15 genotypes from OpV1, together with those of the geminiviruses present in GenBank (dataset was created with an amino acid identity cut-off of 90%). The clusters are colored based on the genus or group. The genera that have known insect vectors are highlighted in a light grey box with the insect vector name displayed in the top. Clusters or singletons marked with a brown halo have no known insect vector associated with them. ACSV, Axonopus compressus streak virus; CaCDaV, Camellia chlorotic dwarf-associated virus; CCDaV, Camellia citrus chlorotic dwarf-associated virus; DfasMV, dragonfly-associated mastrevirus; CBCSV, common bean curly stunt virus; ECSV, Eragrostis curvula streak virus; EMSV, Eragrostis minor streak virus; GraGV, grapevine geminivirus; JmaV, Juncus maritimus-associated virus; LaaV, Limeum africanum-associated virus; MCaV, mulberry crinkle- associated virus; MiSV, Miscanthus streak virus; MSMV, maize streak Reunion virus; PCMoV, passion fruit chlorotic mottle virus; PgaV, Polygala garcinii-associated virus; RLV1, rice latent virus 1; RLV2, rice latent virus 2; SMaV, switchgrass mosaic-associated virus; SpSMV1, sweetpotato symptomless mastrevirus 1; SSMV1, Sporobolus striate mosaic virus 1; SSMV2, Sporobolus striate mosaic virus 2; SStV-A, sugarcane striate virus A; SStV-D, sugarcane striate virus D; SWSV, sugarcane white streak virus; TaGV, tomato-associated geminivirus; ToALCV, tomato apical leaf curl virus.

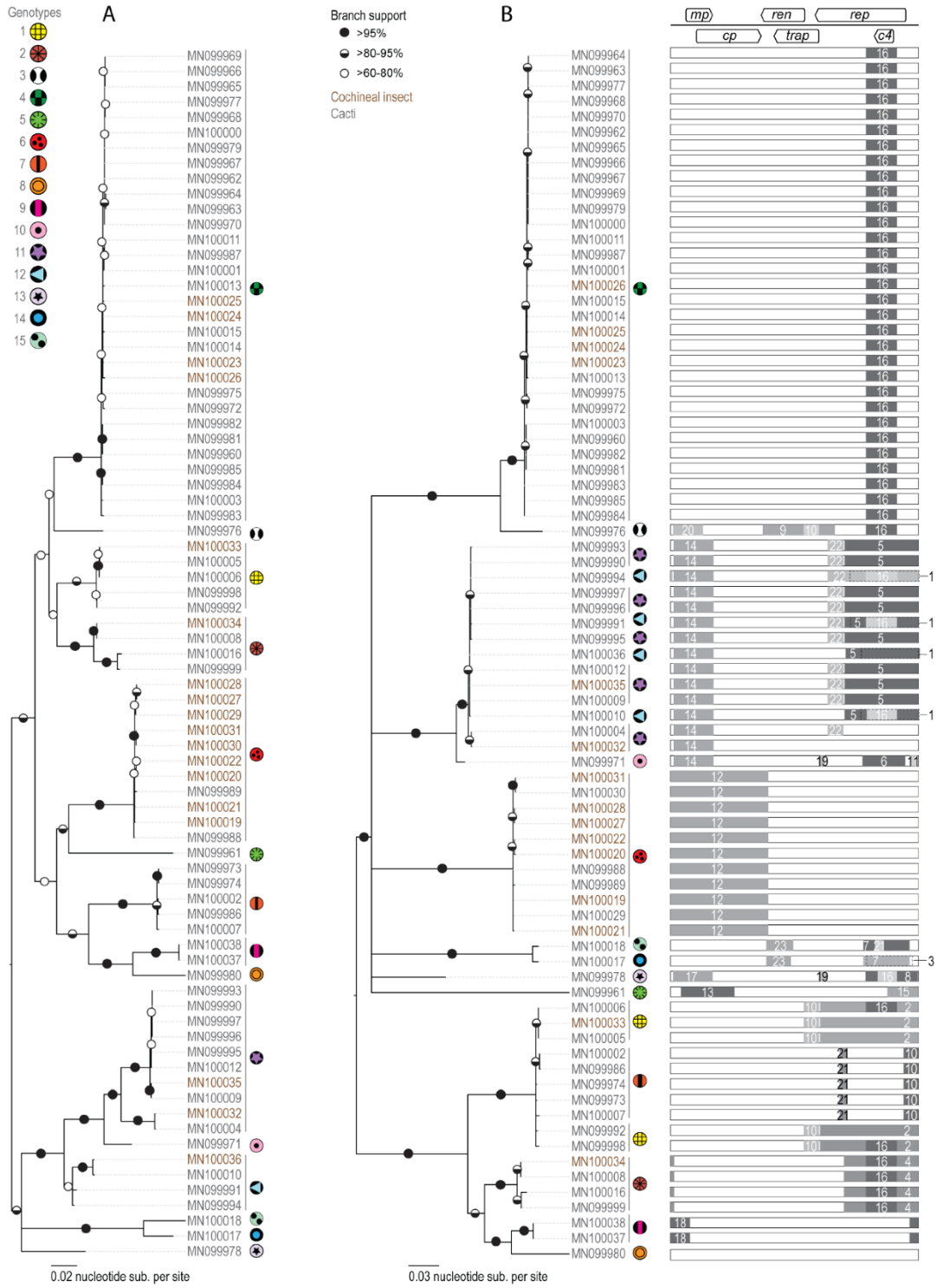

Figura 41 (Figure 4). (A). Neighbor-Joining phylogenetic tree of the 79 OpV1 genomes recovered in this study. Branches with <60% bootstrap support are collapsed. (B). Maximum Likelihood phylogenetic tree of the 79 OpV1 genomes with recombination regions removed. For each genome, a graphic on the right indicates the recombination event with its breakpoint location within the genome. Branches with <60% bootstrap support are collapsed. The 15 genotypes are marked with symbols and genomes that have been recovered from plants (accession numbers in grey) and cochineal insects (accession numbers in brown) are indicated.

From the three cactus samples that we examined (one each of *O. spinosibacca*, *O. rufida* and *O. santa-rita*), we were only able to recover sub-genomic molecules (**Table 1**). None of these sub-genomic molecules (OpV1 sg 10, -11, -13 and -14; **Figure 5**) had a *rep* gene without disruption, which indicates that they would have needed to be trans-replicated by either a non-defective OpV1 variant or some other geminivirus.

OpV1 sg 6 and -14, which were each recovered from different cactus plants, displayed an interesting similarity. Both contain three tandem repeats of the portion of the intergenic region between 22 and 119 nt upstream of the presumed virion strand origin of replication (**Figure 5**). OpV1 sg 6, -8, -11 and -14 all have a similar domain deleted within the Rep coding region (**Figure 5**). The deletions in the Rep-coding region in these molecules are such that the N-terminus of the Rep amino acid sequence has at least two intact rolling circle replication motifs (motif I and II). OpV1 sg 6, -8 and -14 have a second ORF that has an in-frame C-terminus with two helicase motifs (Walker B and motif C). Furthermore, all OpV1 sg molecules except sg 2 and -3 have a deletion spanning the region (743–1566 nt) that encodes the CP, Ren and TrAP proteins (**Figure 5**).

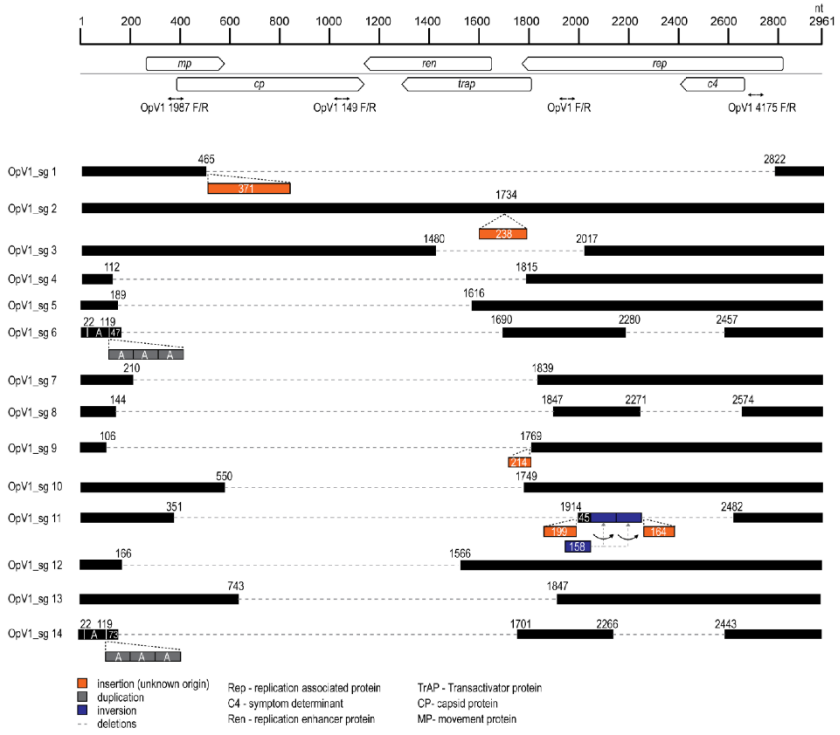

Figura 42 (Figure 5). Structure of OpV1 sub/super-genomic molecules in comparison to an OpV1 parental full-length genome. The areas where deletion occurred are presented by dotted grey lines, insertions, inversions and duplications are represented by orange, blue and grey boxes respectively. The primers pairs used to obtain the sub/super-genomic molecules are shown at their respective binding sites on the parental full-length genome.

Only three of the 14 sub-genomic molecules (OpV1 sg 2, -5, and -12) have an intact Rep coding region and only two have an intact CP coding region (OpV1 sg 2 and -3).

OpV1 sg 2, is larger than the predicted full-length genome of OpV1 with an insert of 238 nt of unknown origin (we have labelled this as super-genomic molecule), and has all coding regions intact, except that encoding the Ren protein (**Figure 5**). Insertions of unknown origin are in OpV1 sg 1 (371 nt), OpV1 sg 9 (214) and OpV1 sg 11 (199/164 nt). In a recent study on beets (*Beta vulgaris*) infected with the geminivirus beet curly top Iran virus (BCTIV), circular molecules labelled as "minicircles" were identified containing large AT-rich host derived sequences, as well as the BCTIV intergenic region containing the origin of replication [**110**]. The minicircles have been proposed to act as a possible mechanism of horizontal gene transfer among host plants. It is important to highlight that the actual diversity of sub/super-genomic DNA molecules that might arise during OpV1 infections is likely higher than those that we have detected here, since the PCR primer binding sites used to amplify these molecules may impact the distributions of the observed deleted regions.

The mechanisms that generate geminivirus sub-genomic molecules are still unclear, although the presence of secondary structures and possible clashes between the replication and transcriptional machinery (due to the bidirectionality of transcription and replication in these viruses) have been suggested as facilitators in this process [**111**]. Some geminivirus sub-genomic molecules have been shown to be packaged into virions and transmitted by their insect vectors [**100**,**112**]; in some cases they can be co-transmitted with their helper/original virus [**103**,**109**] which supports our findings of sub-genomic molecules (OpV1 sg 9) along with full-length OpV1 genomes in the cochineal insect (**Table 1**). The sequence of the sub/super-genomic molecules are provided in **Supplementary Data 5**.

CONCLUDING REMARKS

OpV1, the first reported cactus-infecting DNA virus, is the latest member of the family *Geminiviridae* that will likely require assignment to a novel genus. Despite its high degree of divergence relative to other known geminiviruses—particularly in the CP—OpV1 has numerous similarities with its nearest geminiviruses relatives. OpV1 has a genome organization that is very similar to that of other geminiviruses; it displays patterns of recombination that mirror those of

other geminiviruses, and it forms sub-genomes with patterns of deletion and sequence insertions and rearrangements that are reminiscent of those formed by other geminiviruses.

OpV1 appears to be restricted to the family Cactaceae but has a broad range of host species within the family. In the cactus samples collected to date, no specific host species association to any OpV1 genotype groupings could be inferred. In some cases, we recovered up to five different genotypes from one cactus species and genotype 6 was recovered from 11 different cacti. OpV1 genomes were recovered only from cactus plants in the USA (proportion of plants tested that were positive 7.4%) and Mexico (proportion of plants tested that were positive 6.5%). We were unable to conclusively determine whether cochineal insects are a transmission vector of OpV1, however, it is evident that these insects do acquire the virus upon feeding on infected cactus plants, and therefore it is plausible that they could transmit the virus. Additionally, the host range of cochineal insects is restricted to cacti, further supporting their role as vectors of OpV1, which, to date, has only been identified as infecting cacti. This is also well supported by the CP cluster analyses where the OpV1 CPs form a distinct cluster from those geminiviruses with known insect vectors. Further, we were able to show that cloned OpV1 sequences are capable of initiating asymptomatic systemic infections in *N. benthamiana* and *O. microdasys*. Although we can only confirm that OpV1 is present in the USA and Mexico, it remains plausible that it occurs in other areas of the Americas or parts of the world where cacti are cultivated for agricultural or ornamental purposes.

SUPPLEMENTARY MATERIALS

Supplementary materials can be found at **https://www.mdpi.com/1999-4915/12/4/398/s1**.

AUTHOR CONTRIBUTIONS

Conceptualization, R.S.F. and A.V.; methodology, R.S.F. and A.V.; software, R.S.F., D.P.M and A.V.; validation, R.S.F. and A.V.; formal analysis, R.S.F., J.A.A.-C., G.R.A.-A., D.P.M. and A.V.; investigation, R.S.F., A.M.S., L.C.M., I.N.C., A.B., J.A.A.-C., G.R.A.-A., K.S. (Kara Schmidlin), A.K., K.S. (Kendal Smith), J.S., M.C.L., M.K., M.F.W., W.C.H., R.P.-M., K.V.D., S.K., C.V., D.F., P.R., P.L., S.G.R., S.K., D.P.M., A.V.; resources, R.S.F., A.M.S., L.C.M., I.N.C., A.B, K.S. (Kara Schmidlin), M.C.L., M.K., M.F.W., W.C.H., R.P.-M., K.V.D., S.K., C.V., D.F., P.R., P.L., S.G.R., S.K., D.P.M., A.V.; data curation, R.S.F. and A.V.; writing—original draft preparation,

R.S.F., D.P.M. and A.V.; writing—review and editing, R.S.F., A.M.S., L.C.M., I.N.C., A.B., J.A.A.-C., G.R.A.-A., K.S. (Kara Schmidlin), A.K., K.S. (Kendal Smith), J.S., M.C.L., M.K., M.F.W., W.C.H., R.P.-M., K.V.D., S.K., C.V., D.F., P.R., P.L., S.G.R., S.K., D.P.M., A.V.; visualization, R.S.F. and A.V.; supervision, A.M.S., A.V.; project administration, A.V.; funding acquisition, A.V. All authors have read and agreed to the published version of the manuscript.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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