

Article

Phylogenomics of One of the World's Most Intriguing Groups of CAM Plants, the Opuntioids (Opuntioideae: Cactaceae): Adaptation to Tropical Dry Forests Helped Drive Prominent Morphological Features in the Clade

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Abstract: Opuntioideae, composed of roughly 370 species, occur in almost every biome in the Americas, from seasonally dry tropical forests (SDTF) to high-elevation Andean grasslands, American deserts and temperate zones. The interrelationships among the three major clades of Opuntioideae (Cylindropuntieae, Opuntieae and Tephrocacteae) are not well resolved, and thus, the ancestral habitat, biogeographic history and evolution of morphological characters, such as large photosynthetic leaves and flattened stems, are poorly understood. To test their geographic origin and evolution of key morphological characters, we built the largest phylogenomic dataset for Cactaceae to date using 103 plastid genes of 107 taxa of Opuntioideae. The subfamily Opuntioideae likely evolved in South America in a combination of seasonally dry tropical forest (SDTF)/desert habitats. Opuntieae most likely evolved in South America in SDTF and, from there, moved into desert regions, Chaco and temperate/subtropical zones, while Tephrocacteae and Cylindropuntieae evolved in South America in desert regions and moved into SDTF, Chaco and temperate/subtropical zones. Analyses of morphological evolution suggest that, although large leaves are plesiomorphic in Opuntioideae, long-lived, photosynthetically active leaves in Cylindropuntieae and Tephrocacteae are homoplasious and do not represent retained plesiomorphy, as is often assumed. Flattened stems are synapomorphic for Opuntieae, possibly representing adaptation to competition for light resources in SDTF, their most likely ancestral area.

Keywords: *Austrocylindropuntia*; biogeography; *Cylindropuntia*; CAM photosynthesis; *Opuntia*; phylogenomics; seasonally dry tropical forests



Citation: Majure, L.C.; Achá, S.; Baker, M.A.; Puente-Martínez, R.; Köhler, M.; Fehlberg, S. Phylogenomics of One of the World's Most Intriguing Groups of CAM Plants, the Opuntioids (Opuntioideae: Cactaceae): Adaptation to Tropical Dry Forests Helped Drive Prominent Morphological Features in the Clade. *Diversity* **2023**, *15*, 570. <https://doi.org/10.3390/d15040570>

Academic Editor: Hong-Hu Meng

Received: 3 March 2023

Revised: 14 April 2023

Accepted: 16 April 2023

Published: 18 April 2023



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1. Introduction

The Cactaceae clade (Caryophyllales, Portulacineae) is an endemic New World group of mostly stem succulents that occur in arid and semi-arid, or at least edaphically arid, areas throughout the Americas [1–3] (although see *Rhipsalis baccifera* (Sol.) Stearn [4–10]). Most recent phylogenetic studies show Cactaceae to be most closely related to Portulacaceae s.s., i.e., *Portulaca* L. [11–16] or Anacampserotaceae [17], or as sister to a Portulacaceae + Anacampserotaceae clade [18–21]. Putative ages for the clade have been proposed based on rates of the molecular evolution of ITS [22], as well as calibrated phylogenies based on fossil constraints outside of Cactaceae [23,24], given that no macrofossils beyond the Pleistocene are known from the clade. All three estimates suggest an Oligocene origin for the crown clade of Cactaceae (ca. 30 Ma, although see [11] for a younger putative age or [25] and [15] for older ages). However, most of the diversity within the group is estimated to

have arisen during the mid-Miocene and later, in concert with reduced CO₂ levels and increased aridification across the Americas [23].

The leafy members of Cactaceae, *Leuenbergeria* and *Pereskia* (i.e., “*Pereskia* s.l.”), have most recently been resolved as subsequent sisters to the rest of the more derived, mostly stem photosynthetic cacti [18,20,26,27]. *Maihuenia* (sometimes placed in *Maihuenioideae* [28]), a C₃ taxon with large, persistent, cylindrical leaves, has been recovered as sister to the major clade Cactoideae [18–20,26,29] or as sister to the Cactoideae + Opuntioideae clade or in other positions [30]. Cactoideae and Opuntioideae are the most diverse groups in the family, with 1000+ and ca. 370 species, respectively [3], and have been recovered as monophyletic in numerous analyses [11,23,26,29–35].

The bulk of phylogenetic work in Cactaceae has been focused on smaller groups within the most diverse subfamily Cactoideae [36–50]. However, renewed interest has also developed for Opuntioideae [51,52], a group that was once placed mostly under a single genus, *Opuntia* (L.) Mill. s.l. [1]. In the past 20 years, numerous phylogenetic studies have focused on Opuntioideae [29,32,35,53,54], including phylogenetic studies of the well-supported tribes Opuntieae DC. (prickly pear cacti and relatives; see [55–58]), Tephrocactae Doweld (Andean *Opuntias* and relatives; see [59,60]) and *Cylindropuntieae* Doweld, the chollas and relatives [29,53,61].

Phylogenetic relationships among the three tribes are contentious, with some analyses reporting Opuntieae as sister to a Tephrocactae + *Cylindropuntieae* clade [29], while other analyses show *Cylindropuntieae* as sister to an Opuntieae + Tephrocactae clade [20]. Plastid data have shown the former, while nuclear data have sometimes shown the latter. However, Kohler et al. [35] also showed that different combinations of plastid genes could resolve either relationship. Thus, this is a pattern deserving further study. Previous results have shown that both Opuntieae and *Cylindropuntieae* likely originated in South America and then subsequently dispersed into North American drylands and elsewhere, where they radiated substantially [29,55]. However, it is still unclear what habitats those two tribes may have evolved in. Likewise, the geographic origin of the mostly Andean Tephrocactae is as yet unexplored.

Cactoideae are considered to be the most derived members of the caulocacti with extremely reduced or microscopic (vegetative plant body) photosynthetic leaves [62,63], while the Opuntioideae apparently exhibit retained ancestral characters, such as relatively large (macroscopic) leaves of the vegetative plant body [29]. However, two members of the Opuntioids, *Quiabentia* from the South American Chaco and Brazilian Caatinga and *Pereskopsis* from Central American and Mexican seasonally dry tropical forests (SDTF), have very large, flattened leaf blades, very similar to those of *Leuenbergeria* and *Pereskia*. Likewise, certain species of *Austrocylindropuntia*, mostly from Andean SDTF or scrub vegetation, produce large, persistent leaves. These leafy taxa have also been reported to exhibit facultative CAM [11,64], a character state reported for other members of Opuntioideae, including *Opuntia* [64,65], as opposed to obligate CAM in most other, more derived Cactaceae [64]. Although often considered to be a retained plesiomorphy [64,66], Majure et al. [29] suggested that the photosynthetically functional leaves of *Quiabentia* and *Pereskopsis* were actually derived secondarily after the drastic reduction in leaf size in most Opuntioids (in concert with increased stem photosynthesis); however, their taxon sampling within Opuntioideae, as well as of outgroup taxa, was greatly limited.

Another key feature in the Opuntioids, specifically the tribe Opuntieae, is the production of flattened stems. This is a morphological feature that is apparently restricted to Opuntieae within the Opuntioids, although other cacti outside of the subfamily have also developed similar flattened stem segments, such as *Epiphyllum*, which grows epiphytically in low-light situations. Interestingly, *Brasilopuntia* and *Consolea*, which oftentimes grow in dense SDTF, produce these flattened stem segments, which are relatively thin and somewhat ephemeral, effectively acting as leaves. It is also curious that species of *Opuntia* occurring in more xeric and high-light conditions often produce copious areoles and spines and/or a pubescent epidermis (Majure, unpubl. data). Thus, there appear to be

morphological characters that are directly affected by and perhaps correlated with specific environments, but these morphological features have not been analyzed in a phylogenetic framework.

Here, we expanded on previous phylogenetic analyses with increased sampling in the subfamily Opuntioideae (107 taxa) to test the relationships among the three major Opuntioideae clades. We also broadly sampled all major clades in Cactoideae (47 taxa) to test the resolving power of our plastome dataset, as well as “leafy” outgroup taxa, such as *Leuenbergeria*, *Maihuea*, *Pereskia*, Aizoaceae, Anacampserotaceae, Didiereaceae, Molluginaceae, Montiaceae and Portulacaceae, to revisit the evolution of large photosynthetic leaves in Opuntioideae, as well as other key morphological characters, such as the curiously flattened stems of Opuntieae. We used our phylogeny to determine the putative ancestral habitat, divergence time estimation and geographic origin of the Opuntioideae clade.

2. Materials and Methods

2.1. Taxon Sampling, DNA Extraction and Sequencing

We sampled broadly across Opuntioideae (107 taxa), incorporating representatives from all major clades of the tribes Cylindropuntieae [29], Opuntieae [55] and Tephrocacteae [59,60]. Our sampling of Cylindropuntieae and Opuntieae was mostly restricted to diploid taxa, based on what we know about ploidy in those clades [67,68]. We also sampled all major clades of the subfamily Cactoideae (47 taxa) based on the topology recovered by Hernández-Hernández et al. [30], as well as both clades of “*Pereskia*” s.l. [26], i.e., *Leuenbergeria* and *Pereskia* s.s. These taxa were either field-collected or sampled directly from herbarium specimens or from the living collections at Desert Botanical Garden (<https://www.dbg.org/research-conservation/living-collections/>; accessed 17 August 2021). Data for *Maihuea*, as well as several other cacti [23], were downloaded from GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>; accessed 17 August 2021), as were raw reads from baited experiments [18], transcriptome sequencing [20] or whole plastome data [69] for Aizoaceae (*Mesembryanthemum*, *Tetragonia*), Anacampserotaceae (*Anacampseros*, *Talinopsis*), Basellaceae (*Anredera*), Didiereaceae (*Didiera*, *Portulacaria*), Molluginaceae (*Mollugo*), Montiaceae (*Calandrinia*, *Cistanthe*) and Portulacaceae (*Portulaca oleracea*) (see Section S1).

Each new taxon sampled here was sequenced using genome skimming based on the methods of Majure et al. [29,49,50]. Briefly, whole genomic DNA was extracted from epidermal, root or tepal tissue, which was homogenized in a mortar and pestle or a tissue homogenizer, such as a bead-beater, and a modified CTAB buffer and direct column cleaning were used in silica columns [29,70]. DNAs were sent to Rapid Genomics for library preparation and sequencing on an Illumina HiSeq X platform using paired-end (150 bp) reads.

All raw reads newly generated here, and any reads downloaded from GenBank (plastome or transcriptome or from baited capture methods), were referenced-mapped to the chloroplast genome of *Cylindropuntia bigelovii* [29] and annotated in Geneious (v. 11.1.5, Biomatters Ltd., Auckland, New Zealand) using our *C. bigelovii* plastome. All genes (coding, rRNA and tRNA; $n = 103$) were subsequently extracted in Geneious from the referenced-mapped raw reads and used for alignment. Alignments were carried out with Mafft [71] and then checked manually. Any genes with $\geq 50\%$ missing data and/or apparent pseudogenes (e.g., *accD*, *ycf1* and *ycf2*; see [29,35]) were removed from the alignment.

2.2. Phylogenetic Analyses and Divergence Time Estimation

Our plastome gene dataset was analyzed using maximum likelihood in Geneious with the RAxML V. 8.0 plugin [72] using the GTR+ Γ model of molecular evolution and undertaking 1000 bootstrap pseudoreplicates. We then used BEAST v2.6.2 [73] to estimate divergence times across Cactaceae. In our BEAST analysis, we searched for optimal partitions in the assembly using the annotated *Cylindropuntia bigelovii* genome [29] and PartitionFinder2 [74]. Furthermore, we tested the unpartitioned assembly for 88 substitu-

tion models using jModeltest 2.1 [75] on CIPRES [76]. We then used treePL v. 1 [77], the RAxML phylogenetic topology and a secondary calibration for the crown age of Cactaceae (28.6 Ma), Cactoideae (24.4) and Opuntieae (25.3) based on ages given in Arakaki et al. [23] to date our phylogeny. We used our treePL chronogram as a starting tree for our analysis in BEAUti and BEAST v2.6.2 [73] on the University of Florida's high-performance computing cluster (HiPerGator). We applied a Gamma site model using the *TPM1uf+I+G* model, as supported by jModeltest with a relaxed log-normal molecular clock with a Yule speciation model. After preliminary analyses, the birthrate and uclMean (uncorrelated log-normally distributed) priors had to be enforced with soft limits ($-\infty, 1 \times 10^{-4}$), because they failed to reach convergence. Additionally, we provided a secondary calibration for the crown age (28.6 Ma) of Cactaceae [23], and we parameterized it with a normal distribution, enforcing monophyly for the family. Lastly, we performed two independent runs of 100 million generations each, sampling every 10,000 generations. The output logs of these analyses were combined with LogCombiner 2.6.2 [78] and inspected in TRACER 1.7.1, discarding the first 10% percent of the generations. The maximum credibility chronogram was produced using TreeAnnotator v2.6.2 [79], using the mean node height values. Finally, we used Figtree v1.4.2. to visualize the resulting chronogram [80].

2.3. Morphological Evolution across Cactaceae

We reconstructed the evolutionary history of several important morphological characters across cacti, including (A) stem shape, (B) leaf size, (C) leaf shape, (D) leaf duration, (E) growth form, (F) growth type and (G) areole number (only in Opuntieae). Stems were coded as either cylindrical or flattened in cross-section. The leaf size of the vegetative stems was coded as either large (macroscopic) or small (microscopic or totally reduced); it should be noted that some Cactoideae produce large leaves on the reproductive parts of the plant [81], which were not considered in our coding here. Leaf shape was coded as flattened (bifacial) or cylindrical (unifacial), and leaf duration was coded as either ephemeral (caducous after the development of the stem) or long-lasting (i.e., retained even after stem development). The growth form was coded as herb, tree, shrub, and liana or lianescent shrub. The growth type was recorded as either monopodial or sympodial, and the areole number for the tribe Opuntieae was coded as having <30 areoles per cladode face or >30 areoles per cladode face based on data compiled by Majure (unpubl. data). We reconstructed the evolution of morphological characters in Mesquite [82] using maximum likelihood (ML) under the Mk1 model of evolution, which allows for an equally probable rate of change from one state to another, or under maximum parsimony (MP) for characters with polymorphic states.

2.4. Ancestral Range Reconstruction

Using our maximum clade credibility chronogram generated in BEAST, we conducted two ancestral range reconstruction analyses in RASP v4.0 [83] and the R package Biogeobears [84–87], including the Biogeographical Stochastic Mapping (BSM) component [88,89] on Hipergator (<https://www.rc.ufl.edu/services/hipergator/>; accessed 4 Mar 2021). First, we designated the ranges based on habitat: (A) seasonally dry tropical forest, (B) desert, (C) temperate/subtropical forest, (D) Chaco and (E) ruderal. Second, we used the ecoregion level 1 units of Griffith et al. [90], in part, based on the distributions of taxa sampled in our phylogeny: (A) Old World, (B) western North American deserts (unit 10), (C) Mexico–Central American tropical dry forests (units 12, 13 and 14), (D) eastern temperate forests and temperate sierras (units 8 and 13), (E) West Indies (unit 16), (F) Chaco (unit 22), (G) Northern, Central and Southern Andes (units, 17, 18 and 19), (H) Eastern Highlands (Brazil—Caatinga) (unit 21) and (I) Pampas (unit 23). We applied the six-model likelihood-ratio test to both analyses: DEC [91,92], DEC+J [86], DIVALIKE [86,93], DIVALIKE+J, BAYAREALIKE [86,94] and BAYAREALIKE+J [86]. We then selected the best model based on the Akaike information criterion (AIC) values [95] and ran a non-stratified ancestral range reconstruction, allowing a maximum of 3 areas per node for the habitat-based reconstruction and 5 for the ecoregion-based reconstruction. We recognize the controversy

surrounding the use of the J parameter in model selection [96]; however, given that most of our taxa occupy single areas, the J parameter is appropriate [97].

3. Results

Our PartitionFinder analysis found support for eight partitions; however, none of these partitions produced significantly different phylogenetic trees (PartitionFinder and corresponding RAxML results not shown) when compared to the concatenated tree. jModeltest recovered the model *TPM1uf+I+G* as the most optimal for the concatenated assembly used in our BEAST analysis.

3.1. Phylogeny and Divergence Time Estimation

Our BEAST (BI) and RAxML (ML) phylogenetic reconstructions recovered a well-supported phylogeny, with most nodes showing 0.95 posterior probability (PP) or 100% bootstrap (bs) support (Figure 1) or higher unless stated otherwise (Supplementary Figure S1). Our divergence time estimation analysis showed very high ESS values (>500) for most parameters, except tree prior (ESS = 38), TreeHeight (ESS = 12), uclDStdev (standard deviation (σ) of the uncorrelated log-normal relaxed clock) (ESS = 19), rate.coefficientOfVariation (ESS = 56), YuleModel (ESS = 38) and birthrate (ESS = 59). In both analyses (ML and BI), Anacampserotaceae + Portulacaceae was resolved as sister to Cactaceae (Figure 1), and *Pereskia* s.s. and *Leuenbergeria* were resolved, although poorly (bs = 59) or moderately (pb = 0.99) supported, as a clade. The Cactaceae crown age was estimated at 28.23 $\pm$ 3.71 Ma (Supplementary Figure S1 and Table S1), followed by a *Leuenbergeria* + *Pereskia* clade (15.19 Ma $\pm$ 7.95 Ma). Cactoideae were strongly supported as sister to Opuntioideae (bs = 100, pb = 1.0), and *Maihuea* was recovered as sister to that clade. *Maihuea* diverged from the rest of Cactaceae at 27.22 $\pm$ 3.87 Ma, while the remaining group Cactoideae + Opuntioideae had a crown age of 25.93 $\pm$ 4.32 Ma. Cactoideae (including *Blossfeldia* + Cactaceae and Core Cactoideae in our topology) diverged around 23.95 $\pm$ 4.07 Ma. Within Cactoideae, the diminutive *Blossfeldia liliputana* was recovered as sister to the rest of the clade. The North American, mostly desert-adapted Cactaceae diverged around 17.52 Ma and were well supported as sister to the *Calymmanthium* + (*Copiapoa* + (Core Cactoideae I + Core Cactoideae II)) clades that consist of North, Central and South American species and that diverged around 20.82 Ma. Within Core Cactoideae I, *Eulychnia* and *Corryocactus* were subsequent sisters to the Pachycereinae + a well-supported clade (bs = 98) formed by *Selenicereus*, *Armatocereus* and *Leptocereus*, which diverged around 16.15 Ma. In Core Cactoideae II, *Parodia* was sister to the Trichocereae + a poorly supported clade (bs = 58) formed by *Rebutia*, *Eriocysce*, *Stetsonia*, *Arrojadoa*, *Pilosocereus* and *Melocactus*. Core Cactoideae II diverged nearly in parallel with Core Cactoideae I, around 16.79 Ma.

The crown age for the well-supported Opuntioideae was recovered at 16.72 Ma (22.18–11.32 Ma), nearly paralleling the divergences of the tribe Cactaceae and Core Cactoideae I and II. The young tribe Opuntieae, which diverged 7.92 $\pm$ 3.94 Ma, was strongly supported as sister to a Tephrocactae + Cyllindropuntieae clade, which diverged from one another around 15.64 Ma ($\pm$ 5.48) Ma. The Caribbean endemic clade *Consolea*, which had a crown age of late Pleistocene origin (0.72 Ma), was resolved as sister to the rest of the Opuntieae, and the hyperdiverse genus *Opuntia* appears to have diverged around 4.38 Ma. *Tacinga* from the Brazilian caatinga biome (tropical dry forest) formed a sister clade to (*Miqueliopuntia* + *Airampoa*) + *Opuntia*. The *Miqueliopuntia* + *Airampoa* clade was very well supported (bs = 99), although its position as sister to *Opuntia* was poorly supported in our ML analysis (bs = 56) and well supported in our BI topology (pb = 1.0). *Opuntia quitensis* was resolved as sister to the rest of the species of *Opuntia*, and the southern South American clade, including *O. quimilo* and *O. arechavaletae*, was sister to the North American clade. The mostly hummingbird-pollinated *Nopalea* clade (sensu [55]) of SDTF was sister to the rest of the North American clades, most of which are desert-adapted. *Maihuea* was sister to the rest of Tephrocactae, and *Pterocactus* was sister to a *Tephrocactus* + (*Austrocylindropuntia* + *Cumulopuntia*) clade. As in Majure et al. [29], the leafy *Quiabentia*

verticillata of the Chaco and *Pereskia* of tropical dry forests of North America were subsequent sisters to a *Micropuntia* + (*Cylindropuntia* + *Grusonia*) clade. The crown ages for *Maihueiopsis* (8.63 Ma) and *Tephrocactus* (8.71 Ma) were older than the crown ages for *Pterocactus* (3.36 Ma) and the *Austrocylindropuntia* + *Cumulopuntia* clade (7.37 Ma) and older than those for subclades of Opuntieae and Cylindropuntieae. *Grusonia* diverged around 3.26 Ma, *Cylindropuntia* diverged 6.39 Ma, and *Micropuntia*, *Pereskia* and *Quiabentia* diverged in the last 3.57–2.77 Ma (see Table 1).

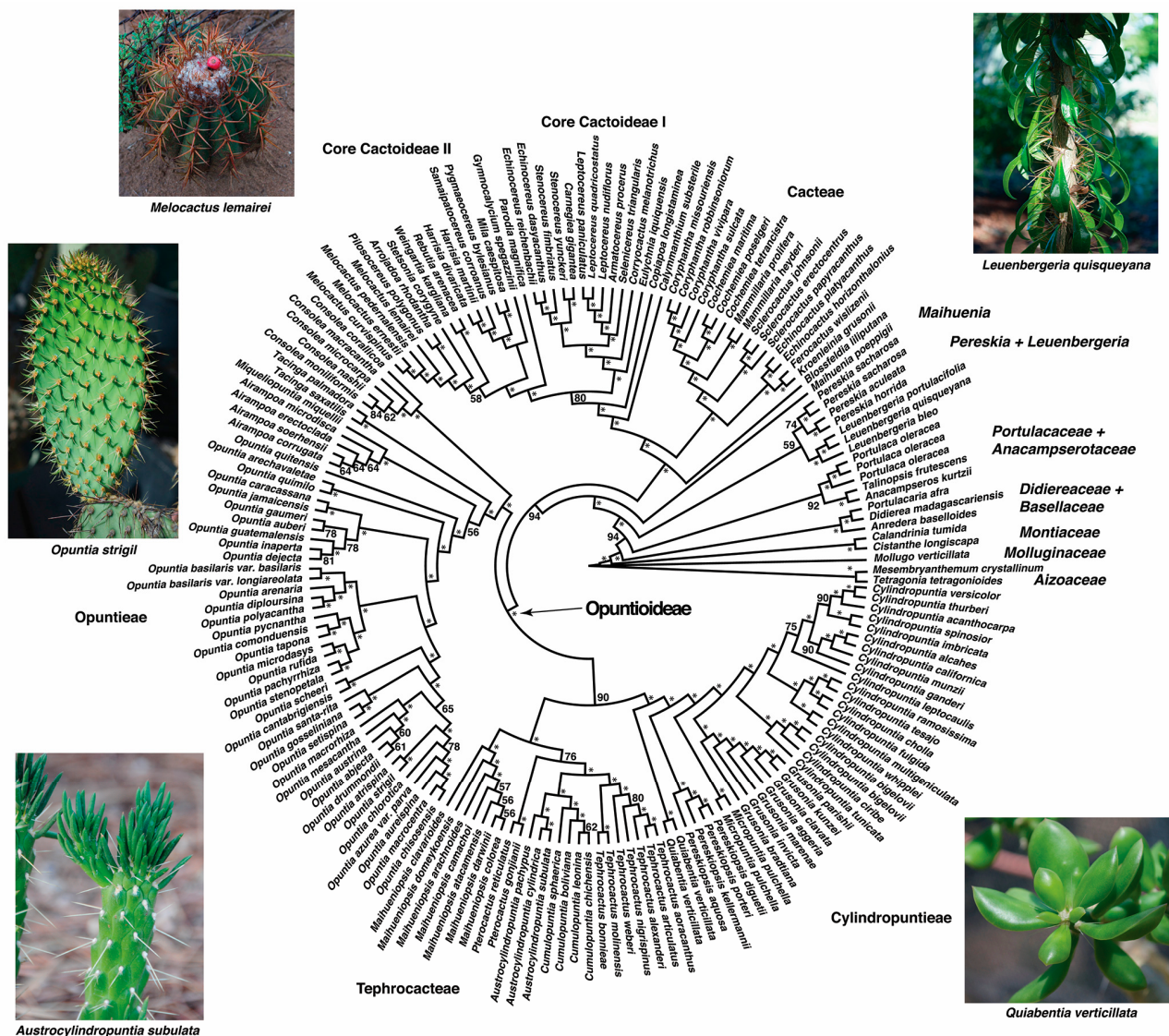


Figure 1. Maximum likelihood topology from RAxML. Cactaceae were recovered as sister to an Anacampserotaceae + Portulacaceae clade. *Pereskia* and *Leuenbergeria* were poorly supported as a clade, and *Maihueiopsis* was supported as sister to a Cactoideae + Opuntioideae clade. Opuntioideae was well supported and consisted of an Opuntieae + (Cylindropuntieae + Tephrocactae) clade. Bootstrap support (bs) is given above branches. * = 100 bs, and support <100 is given. Photos taken by L.C. Majure.

Table 1. Crown ages and confidence intervals from our BEAST analysis.

Clade	Crown Age	CI
<i>Pereskia</i> + <i>Leuenbergeria</i>	15.19	7.24–27.83
<i>Maihuenia</i> + the rest	27.22	23.35–31.23
Cactoideae	25.93	19.88–28.16
Cactaceae	17.53	12.32–23.25
Core Cactoideae I	17.53	12.32–23.25
Core Cactoideae II	20.83	16.39–25.72
Opuntioideae	16.73	11.32–22.18
Opuntieae	7.92	3.98–12.11
<i>Tacinga</i>	1.75	0.16–4.51
<i>Miqueliopuntia</i> + <i>Tunilla</i>	3.53	1.51–5.88
<i>Consolea</i>	0.73	0.14–1.64
<i>Opuntia</i>	4.39	2.52–6.43
Tephrocactae	13.34	8.74–18.86
<i>Tephrocactus</i>	8.72	4.65–13.08
<i>Pterocactus</i>	3.37	0.45–6.95
<i>Austrocylindropuntia</i> + <i>Cumulopuntia</i>	7.37	3.73–11.54
<i>Maihueniopsis</i>	8.65	3.83–13.78
Cylindropuntieae	12.2	7.86–16.55
<i>Quiabentia</i>	3.58	0.37–7.66
<i>Pereskopsis</i>	3	0.87–5.82
<i>Micropuntia</i>	2.77	0.38–5.54
<i>Cylindropuntia</i>	6.4	3.86–9.05
<i>Grusonia</i>	3.26	1.41–5.77

3.2. Morphological Evolution

The ancestral growth form in Cactaceae under parsimony was reconstructed as herbs, shrubs or trees with equal probability, although all modern cacti are woody (see [62]). Both Cactoideae and Opuntioideae were reconstructed as ancestral shrubs but with numerous transitions to trees and several transitions to lianas or linaescent shrubs (Supplementary Figure S2A). Cylindrical stems were symplesiomorphic for Cactaceae, and flattened stems were synapomorphic for Opuntieae, although these reversed back to cylindrical stems in *Miqueliopuntia* (Figure 2A). Monopodial stems were plesiomorphic for Cactaceae, although transitions to sympodial stems occurred in some members of Cactoideae, and sympodial stems were predominant in Opuntioideae, although with a few transitions back to monopodial growth in that clade, such as in *Consolea*, *Austrocylindropuntia*, *Perekiopsis*, *Quiabentia* and *Cylindropuntia leptocaulis* (Figure 2B). Large (macroscopic) leaves were symplesiomorphic for Cactaceae, including Opuntioideae, and small (microscopic) leaves were a synapomorphy of Cactoideae (Figure 2C). Flattened leaves were symplesiomorphic in Cactaceae, and reduced, cylindrical leaves were synapomorphic for the *Maihuenia* (Cactoideae + Opuntioideae) clade. However, large, flattened leaves evolved separately in *Quiabentia* and *Pereskopsis* (Figure 2D). Long-lived leaves were symplesiomorphic for Cactaceae, with ephemeral leaves being synapomorphic for Cactoideae + Opuntioideae. However, long-lived leaves were acquired three more times in Opuntioideae, in *Austrocylindropuntia* and *Quiabentia* and in *Pereskopsis* (Figure 2E). Members of the tribe Opuntieae occurring in tropical dry forests, Chaco, Pampa or temperate forests (e.g., *Elatae*, *Nopalea* and *Humifusa* clades and *O. pachyrrhiza*) exhibited fewer areoles per stem face (<30) than those taxa occurring in drier, more open habitats, with the exception of the Antillean tree genus *Consolea* (with >30), which oftentimes has partially monopodial, flattened stems, as well as the small species of open, dry sandy soils of western Texas and northern Mexico, *Opuntia arenaria* with <30 (Supplementary Figure S2B), which has limited available space for areoles.

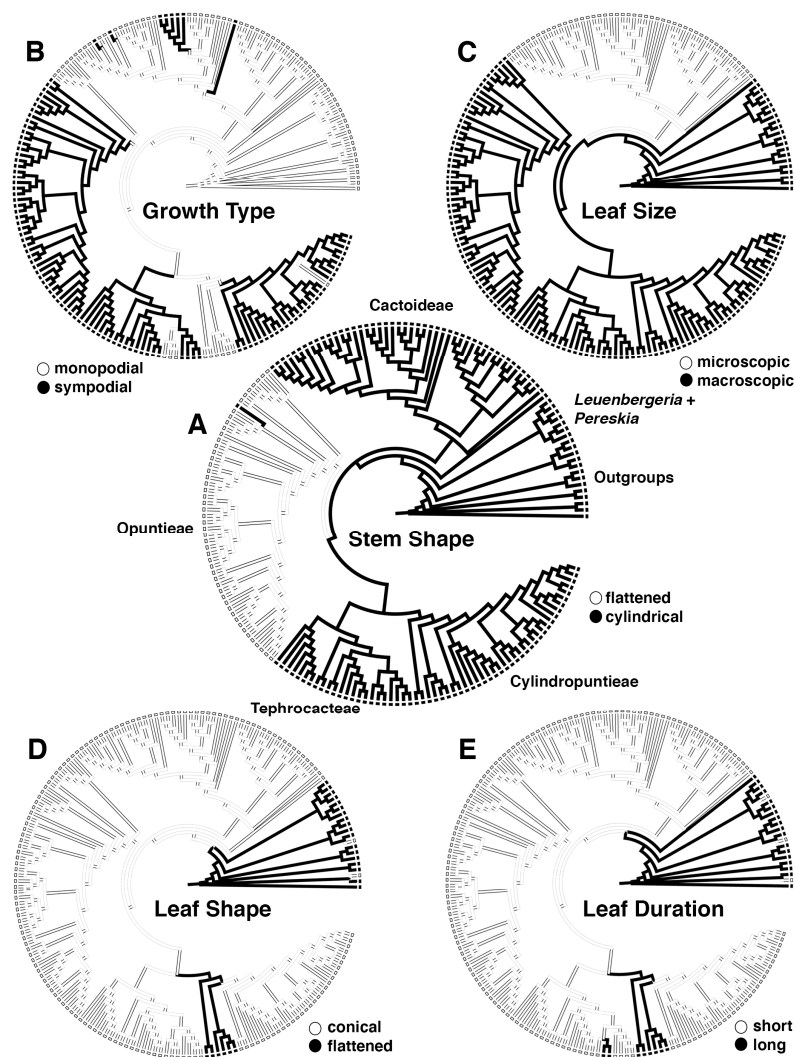


Figure 2. Morphological evolution in Cactaceae. (A) Cylindrical stems (black) were plesiomorphic, and Opuntioideae displayed the synapomorphy (white) of flattened stems. (B) Monopodial stems (white) were plesiomorphic, and sympodial stems (black) evolved several times in Cactoideae, as well as in Opuntioideae, where there were also several changes back to monopodial growth. (C) Large, macroscopic (vegetative) leaves were plesiomorphic (black) in Cactaceae, but near-total leaf reduction was synapomorphic for Cactoideae. (D) Flattened (bifacial) leaves were ancestral in Cactaceae (black) and then re-evolved in Cylindropuntioideae, and (E) long-lived leaves (black) were ancestral and then re-evolved in both Tephrocacteae and Cylindropuntioideae.

3.3. Ancestral Range Reconstruction

3.3.1. Range Defined Based on Habitat

Our RASP analysis showed that the best model was DEC+J (AICc_{wt} = 0.45), followed by DIVALIKE+J (AICc_{wt} = 0.42) (Supplementary Table S1). Based on these results, the habitat with higher speciation corresponded to deserts (106 events), followed by the tropical dry forest/SDTF (33). Most dispersal events happened from the desert to SDTF (10) and from the desert to temperate/subtropical forest (6). Overall, the desert habitat was the highest source of dispersal events (19), and the SDTF habitat was the main recipient of those dispersal events (13). Based on the DEC+J model results, the most recent common ancestor of Cactaceae evolved in a desert habitat (Figure 3A, node 348), with a small probability of an SDTF/desert origin. The Cactoideae + Opuntioideae clade (Figure 3A, node 246) and the Cactoideae (Figure 2A, node 237) evolved in the ancestral desert habitat range. Cactoideae showed dispersal events into SDTF (Figure 3A, nodes 215 and 227), temperate

zones (Figure 3A, nodes 202 and 212) and the Chaco (Figure 3A, node 232), with a potential return to the desert (Figure 3A, node 219). The Cactaceae clade originated in a desert habitat (Figure 3A, node 206), as did the *Calymmanthium* + *Copiapoa* + Core Cactoideae I + Core Cactoideae II clade (Figure 3A, node 235). Core Cactoideae I also originated in a desert habitat (Figure 3A, node 217), and Core Cactoideae II evolved in the Chaco (Figure 3A, node 232). From the Chaco, this clade dispersed into SDTF once (Figure 3A, node 227) and then back to the desert (Figure 3A, node 219).

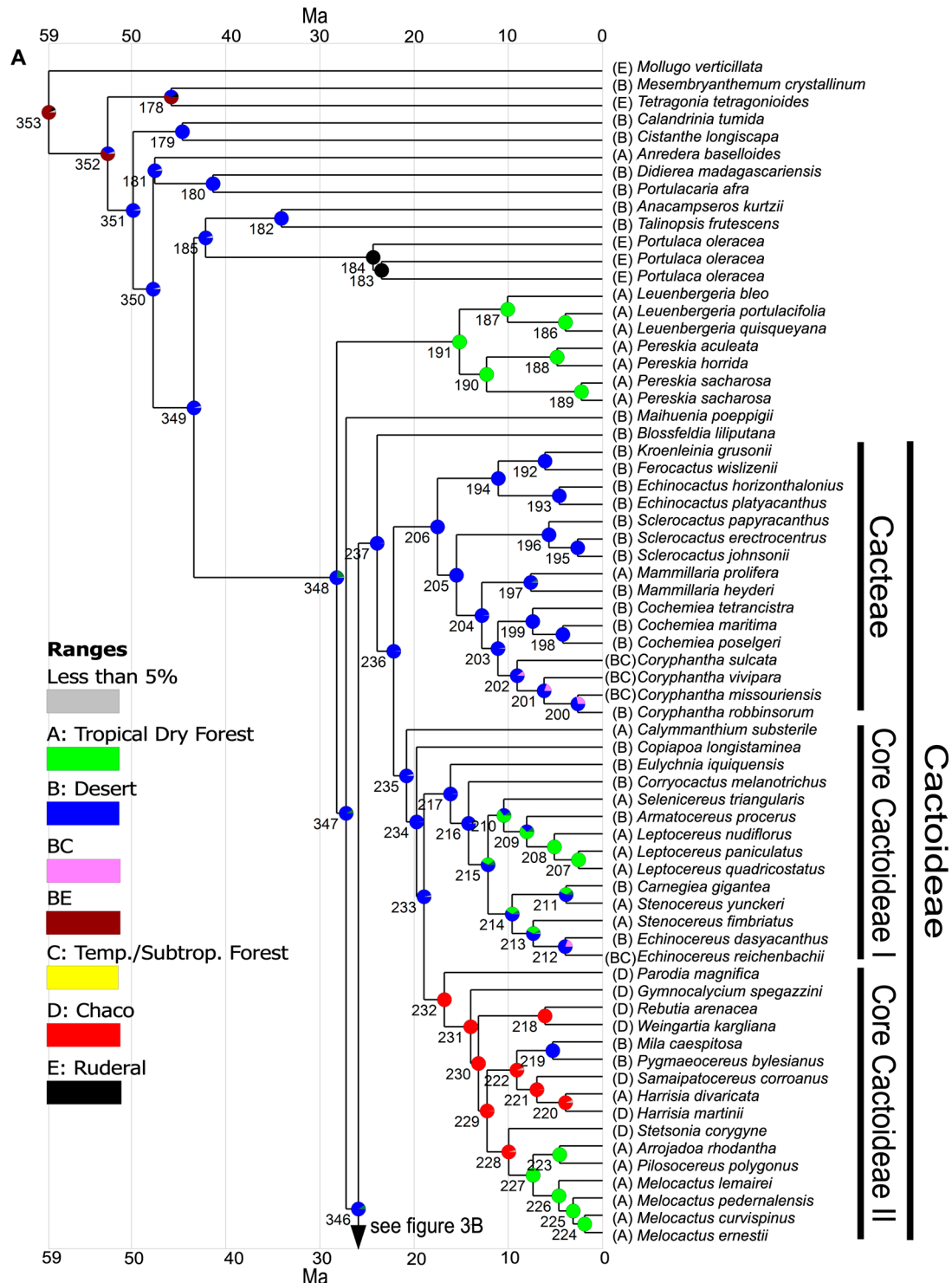


Figure 3. Cont.

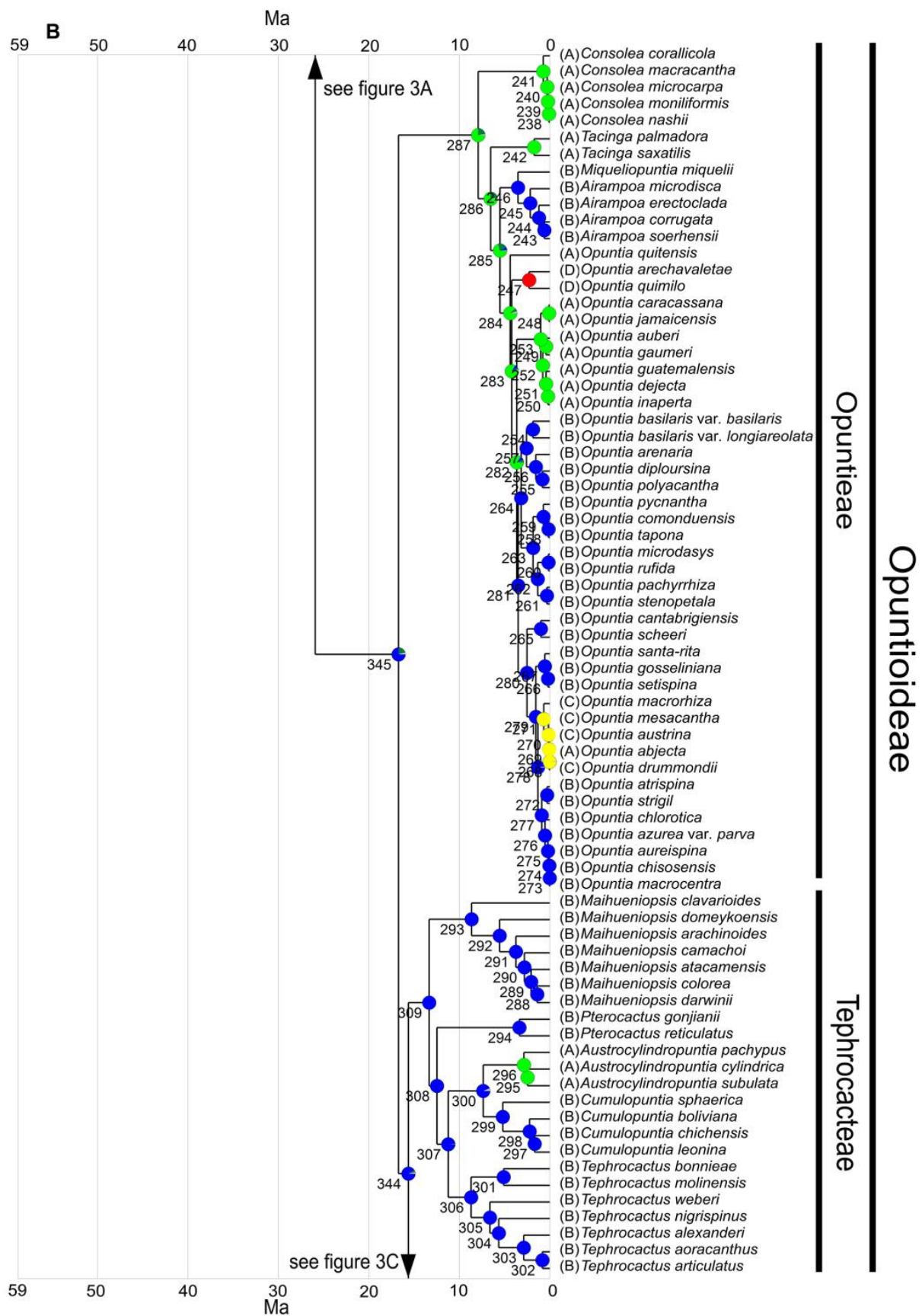


Figure 3. Cont.

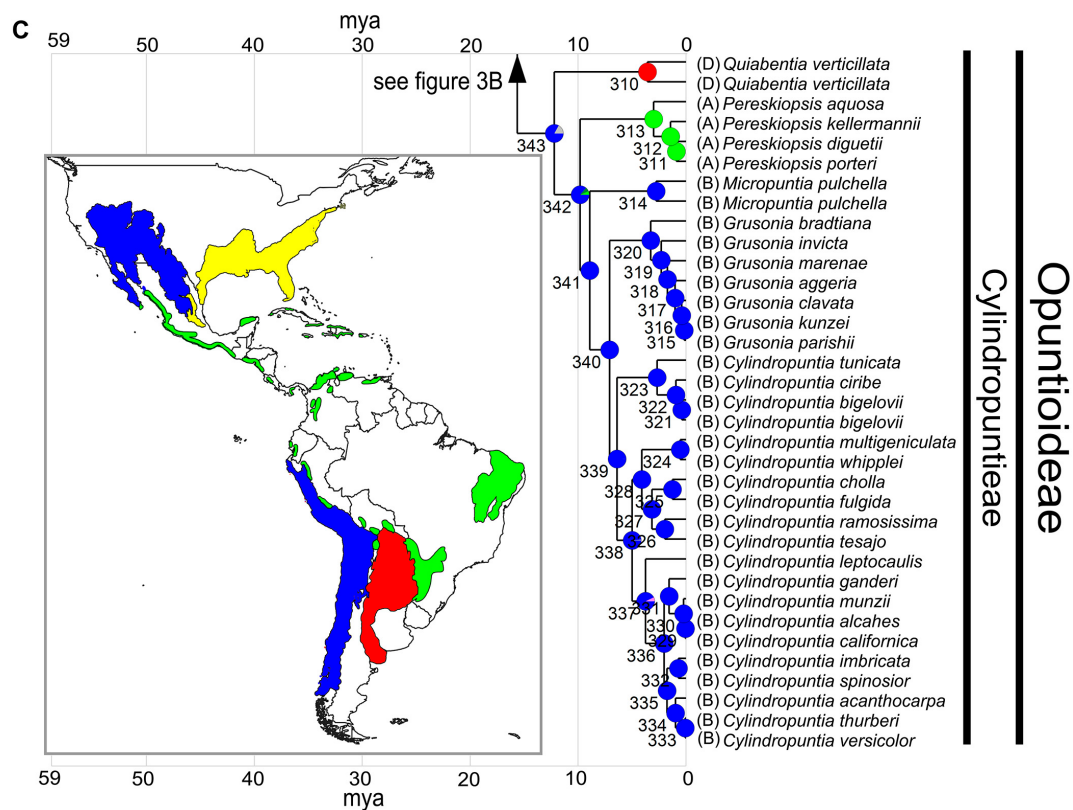


Figure 3. Ancestral habitat reconstruction using our BEAST chronogram. (A) Cactaceae were reconstructed as evolving in a desert environment, while the *Pereskia* clade originated in SDTF, Core Cactoideae I moved into SDTF from the desert, and Core Cactoideae II were reconstructed as evolving in the Chaco before moving into SDTF and back into the desert. (B) Opuntieae were reconstructed as evolving in SDTF and, from there, moving into the desert, Chaco and temperate forest, while Tephrocactaeae were reconstructed as evolving in the desert and then moving into SDTF, and (C) Cyndropuntieae evolved in a desert habitat and then moved into the Chaco and SDTF.

According to the DEC+J model, Opuntioideae originated in a desert habitat (Figure 3B, nodes 345). We found support for the SDTF as the most probable origin of Opuntieae (Figure 3B, node 287), with subsequent dispersal events into Chaco (Figure 3B, node 247) and desert habitats (Figure 3B, node 281) and, finally, two dispersals into temperate/subtropical forest habitats (Figure 3B, node 271). According to our analysis, both Tephrocactaeae and Cyndropuntieae clades evolved in a desert habitat (Figure 3C, node 344, 309 and 343). One dispersal of Tephrocactaeae into SDTF occurred in *Austrocylindropuntia* (Figure 3, node 296). Cyndropuntieae included two early dispersal events: the first from a desert habitat to the Chaco region (Figure 3C, node 310) and the second one from the desert to SDTF (Figure 3C, node 313).

3.3.2. Range Defined Based on Ecoregions

Our Biogeobears analysis found the highest support for the BAYAREALIKE+J model ($AIC_{c_wt} = 1$), followed by the DIVALIKE+J model ($AIC_{c_wt} = 8.20 \times 10^{-7}$) (Supplementary Table S1). The corresponding Biogeographical Stochastic Mapping (BSM) analysis recovered the western North American deserts as the range with the most speciation events (68) followed by the Andean (41) and Caribbean regions (11). Most dispersal events happened from the Andes to three regions: the western North American deserts, the Caribbean and the Chaco, each with three events. Likewise, three dispersal events occurred from the Mexico–Central American SDTF to the western North American deserts. The BSM results also recovered the Andes as the source ecoregion of most dispersal events (14), followed by Mexico–Central American SDTF (7) and the Caribbean region (8). Additionally, the

ecoregion that was the main recipient of dispersal events was the Caribbean region (8), followed by western North American deserts (7) and Mexico–Central American SDTF (7).

Based on the BAYAREALIKE+J results, the most recent common ancestor of Cactaceae had an Andean range (Supplementary Figure S3, node 191). The *Leuenbergeria* + *Pereskia* clade (Supplementary Figure S3, node 192) maintained the ancestral Andean range and subsequently dispersed to the Caribbean and Mexico–Central American SDTF (Supplementary Figure S3, node 193). This clade also included a dispersal to the Chaco region (Supplementary Figure S3, node 197) within *Pereskia*. The Cactoideae + Opuntioideae (Supplementary Figure S3, node 199) and the Cactoideae clade (Supplementary Figure S3, node 200) were reconstructed as originating in the ancestral Andean range. We then observed several range changes within the Cactoideae clade. The Cacteae clade originated in western North American deserts after the dispersal of a MRCA of that clade from the Andean region (Supplementary Figure S3, node 202), while its sister clade Core Cactaceae (*Calymmanthium* + *Copiapoa* + Cactoideae I + Core Cactoideae II) retained the Andean ancestral range (Supplementary Figure S3, nodes 217, 218 and 219). Core Cactoideae I also maintained the Andean ancestral range (Supplementary Figure S3, node 220), with several dispersals into the Caribbean region (Supplementary Figure S3, node 222) and western North American deserts (Supplementary Figure S3, node 227) and a potential dispersal back to the Andean range (Supplementary Figure S3). Core Cactoideae II appears to have evolved in the Chaco region (Supplementary Figure S3, node 231) with a dispersal to the Caribbean region (Supplementary Figure S3, node 241) and Eastern Highlands (Brazil—Caatinga) (Supplementary Figure S3, nodes 242 and 245), with potential movement back to the Andean region (Supplementary Figure S3).

The Opuntioideae clade (Supplementary Figure S3, node 246), as well as the Opuntieae (Supplementary Figure S3, node 247) and Tephrocactaeae + *Cylindropuntia* clades (Supplementary Figure S3, node 297), originated in the ancestral Andean range. We observed several dispersal events within Opuntieae: to the Caribbean region (Supplementary Figure S3, nodes 248 and 263), Eastern Highlands (Brazil—Caatinga) (Supplementary Figure S3, node 253), Chaco and Pampas (Supplementary Figure S3, node 261), western North American deserts (Supplementary Figure S3, node 260), Mexico–Central America SDTF (Supplementary Figure S3, node 265) and Eastern temperate forests (Supplementary Figure S3, node 287). The Tephrocactaeae clade originated in the ancestral Andean range (Supplementary Figure S3, node 298), while *Cylindropuntia* appears to have had a Chaco origin (Supplementary Figure S3 node 320), although this is weakly supported. Finally, the *Pereskopsis* + the rest of *Cylindropuntia* subclade showed a range change to Mexico–Central America SDTF (Supplementary Figure S3, node 323) and then a shift into western North American deserts in the *Micropuntia* + *Cylindropuntia* and *Grusonia* clade.

4. Discussion

4.1. Phylogenetic Relationships in Cactaceae

The sister relationship of (Anacampserotaceae + Portulacaceae) + Cactaceae, as found here, has been recovered in several recent analyses with sufficient taxon sampling [18,20,21]. Although most analyses with sufficient taxon/data sampling have recovered *Leuenbergeria* and *Pereskia* as subsequent sisters to the rest of Cactaceae, our analyses recovered the two clades as sisters, although very poorly supported in ML analyses (bs = 59). It may be likely that the paucity of taxon sampling in our analyses could have affected our resulting topology, although Walker et al. [20] and Wang et al. [21] found high levels of incongruence among gene trees from transcriptome data around the *Pereskia*–*Leuenbergeria* node(s). So, the recalcitrance of this node does not appear to be ameliorated by using plastome data, at least when using just genes. Perhaps entire plastomes would provide better-supported topologies, along with the inclusion of more species from both clades. For example, the Central American *Leuenbergeria lychnidiflora* (not sampled here), which is sister to the rest of *Leuenbergeria* [26], may be key to obtaining a paraphyletic “*Pereskia* s.l.” *Maihuenia* has often been resolved as sister to Cactoideae [20,21,26,29]; however, our plastid gene dataset

consistently resolved *Maihuenia* as sister to a Cactoideae + Opuntioideae clade. It is unclear whether the increased taxon sampling of Cactoideae in our analyses (Figure 1), as compared to previous analyses, may have resulted in this topology. Future work will need to more fully address the placement of *Maihuenia*.

Within Cactoideae, *Blossfeldia* was resolved as sister to the rest of the taxa sampled here, a placement in agreement with other phylogenetic studies [30]. As in Hernández-Hernández et al. [30], we recovered the tribe Cacteae as sister to the Core Cactoideae, which was well supported, and *Calymmanthium* and *Copiapoa* were subsequent sisters to the Core Cactoideae I + Core Cactoideae II clade. In Cacteae, as in Breslin et al. [48], we resolved three separate clades from the Mammilloid clade, *Mammillaria*, *Coryphantha* and *Cochemiea* s.l., but our sampling was not comprehensive enough to evaluate other more recent findings regarding generic delimitation [98]. Within Core Cactoideae I, *Eulychnia* was well supported as sister to the rest of the clade, a placement not previously supported. The structure of Core Cactoideae II was very similar to that described by Hernández-Hernández et al. [30], although with much poorer taxon sampling in our dataset.

Opuntioideae have most often been recovered in phylogenetic analyses as sister to Cactoideae [20,21,23,26,30,32,33], as we found here, although a recent plastome analysis by Yao et al. [15] recovered the Opuntioideae clade as sister to the rest of Cactaceae, a seemingly anomalous placement. The Opuntioideae clade comprises three major clades, the Opuntieae, Tephrocacteae and Cyindropuntieae. Most analyses to date have not resolved relationships among these three clades, or topologies have been poorly supported [30,32,54,59] or based on very poor taxon sampling [20,21]. Majure et al. [29], using plastome data, resolved Opuntieae as sister to a well-supported Tephrocacteae + Cyindropuntieae clade; however, both Opuntieae and Tephrocacteae were very poorly sampled. Our more comprehensive sampling here further lends support for Opuntieae as sister to a Tephrocacteae + Cyindropuntieae clade (bs = 90; Figure 1). Köhler et al. [35] likewise recovered this topology using several datasets derived from plastome data and a series of smaller datasets. Transcriptome datasets, however, have repeatedly recovered a Tephrocacteae + Opuntieae clade as sister to the Cyindropuntieae clade [20,21]. Likewise, a reduced dataset using the top five most highly informative plastid loci across Opuntioideae also recovered that topology [35]. What underlies these topological incongruences is currently unclear; however, it seems possible that selective pressures in loci derived from transcriptome data and homoplasy using rapidly evolving plastid genes that include intergenic spacers, such as in the five-locus dataset of Köhler et al. [35], could influence those differing topologies. The dataset implemented here is also not without its limitations, so to appropriately test this major incongruence among the three Opuntioideae tribes, multiple, independent, single-copy nuclear loci should also be considered, such as in a hyb-seq approach [99].

In contrast to Majure et al. [55], *Opuntia quitensis* was resolved here as sister to the remaining species of *Opuntia*, including the South American (Elatae) clade, consisting of *O. quimilo* and *O. arechavaletae*. Majure et al. [55] recovered the South American clade as sister to the rest and *O. quitensis* as sister to the North American clade, although that placement was poorly supported. Likewise, the *Nopalea* clade within Opuntieae here was resolved as sister to the rest of the North American clades, whereas it was sister to the *Basilares* clade in previous analyses [55]. The *Macrocentra* clade here does not include *O. santa-rita* and its relatives, in contrast to previous analyses, and was recovered as sister to the *Humifusa* clade, although this was not well supported (bs = 64). Likewise, *O. chlorotica* was resolved outside of the *O. santa-rita* clade, in the *Macrocentra* clade, although that species is morphologically very similar to *O. santa-rita*. *Opuntia guatemalensis*, although morphologically slightly different from the other members of the hummingbird-pollinated members of the *Nopalea* clade, was still resolved inside of that clade as in Majure et al. [55], suggesting a transition back to insect pollination from bird pollination in that clade. Incongruencies between our current topology and those of Majure et al. [55] are likely a result of the increased resolution in our current dataset, as well as the use of combined plastid +

nuclear DNA (ITS, *ppc*) in Majure et al. [55]. Species and clade relationships and competing topologies in Opuntieae are being tested further by Köhler et al. (in prep.).

Ritz et al. [59] resolved *Maihueniopsis* (bs = 69) as sister to *Pterocactus*, though this was poorly supported; however, our topology resolved *Pterocactus* as sister to an *Austrocylindropuntia* + *Cumulopuntia* (+ *Tephrocactus*) clade (bs = 76), which was moderately well supported. Although the *Austrocylindropuntia* + *Cumulopuntia* (+ *Tephrocactus*) clade was not well supported (bs = 66) in Ritz et al. [59], we also resolved that clade but with very high support (bs = 100). As in Ritz et al. [59], we resolved *Maihueniopsis clavarioides* and *M. domeykoensis* as subsequent sisters to the rest of the *Maihueniopsis* clade. We also resolved *Tephrocactus bonnieae* and *T. molinensis* in a clade, as well as *T. aoracanthus*, *T. articulatus* and *T. alexanderi* in a clade, as in Ritz et al. [59]. Species limits within *Tephrocactus* were recently tested by Las Peñas et al. [60], and species relationships in our analyses were nearly identical to their strict consensus tree, although their study included more taxa than what was used in our study. Our sampling of *Pterocactus* was extremely limited (to just two species), so we can say little about species relationships. However, Las Peñas and Bernardello [100] recently provided a detailed, species-level phylogeny and cytogenetic analysis of this mostly polyploid genus.

Our topology of Cylindropuntieae here was very similar to that described by Majure et al. [29], although we included fewer taxa in the current analysis, and clades were not as well supported as with nearly all plastome data. As in Majure et al. [29], we recovered the Great Basin endemic *Micropuntia* as sister to the *Grusonia* + *Cylindropuntia* clade, further confirming its placement outside of *Grusonia*.

4.2. Morphological Evolution and Ancestral Habitats

The flattened stems of Opuntieae are clearly synapomorphic for that clade (Figure 2A). In light of SDTF being the most likely putative ancestral area for that clade, flattened stems or large leaves would be necessary to compete with large-leaved species of trees and shrubs in those areas. Thus, the flattened cladodes could act to increase the surface area available for increased photosynthetic rates in those lower-light environments. Many species of *Consolea* have very thin stem segments, which, although long-lived, seem to be shed at a higher frequency than in other groups of desert-adapted species of *Opuntia*, for instance. Other members of the Opuntieae clade, including the tree members of the *Nopalea* clade and *Brasiliopuntia* (not sampled here) inhabiting SDTF, show some of the same characters. So, the large, flattened cladodes effectively act as broad leaves. The same can be seen in certain members of Cactoideae (not sampled here), such as *Epiphyllum* and *Schlumbergera*, which inhabit low-light environments and produce flattened stem segments, presumably for the same purpose (although, developmentally, those flattened stems are quite different from those in Opuntieae).

Likewise, the number of areoles and/or spine production per cladode face appears to be correlated with habitat, with those species occurring in SDTF, Chaco and temperate/subtropical forest regions having far fewer areoles per cladode face (<30), as compared to desert species that produce higher numbers of areoles per cladode face (>30) and thus have the potential to produce more spines (Supplementary Figure S2B). This pattern has been observed in Cylindropuntieae and Tephrocacteae as well (Majure, unpubl. data), but it has not yet been quantified. Certain members of the subfamily Cactoideae also appear to show this pattern, where more ribs (and thus potentially more areoles and spines) are produced in desert environments. The south-facing stem faces notably produce more and deeper ribs, which increases the stem area and can potentially affect photosynthetically active radiation (PAR) interception, stem temperature and CO₂ uptake [101]. Nobel [102–105], in a series of experiments, showed that spines in cacti aided in regulating plant body temperature by reducing shortwave radiation on the stem and thus producing a boundary layer around the stem, which greatly impeded vastly fluctuating stem temperatures. Likewise, spines act to reflect incident PAR, thereby further decreasing potential stem heat loads. Therefore, the production of higher numbers of areoles and the potential for producing

higher numbers of spines for protection against UV radiation and higher temperatures may be correlated with the desert environment, where more extreme temperatures are experienced. So, although Opuntieae may have evolved flattened stems to cope with low-light environments, they could compensate for the high amount of exposed stem surface by clothing their epidermis in spines. Another potential advantage to producing higher areole numbers could be an increase in the passive absorption of water via glochids [106], which are ubiquitous in the areoles of Opuntioideae.

Certain desert members of Opuntieae produce no spines at all (e.g., *Opuntia basilaris*, *O. microdasys* and *O. rufida*) and, on the contrary, produce a dense pubescence that clothes the epidermis and presumably aids in absorbing shortwave radiation, which again helps to regulate plant body temperature [107]. The exhibition of purple betalain pigments by the stems, as always occurs in some prickly pears, such as *Opuntia santa-rita* and *O. macrocentra*, has been suggested by Gibson and Nobel [107] to act to reflect incident PAR, thereby further protecting the plant body from damaging temperatures. However, the effects of pigmentation on cacti are poorly studied.

As proposed by Majure et al. [29], and supported here with much broader taxon sampling, the large, persistent, flattened leaf blades of *Quiabentia* and *Pereskopsis* have been acquired separately from cylindrical, ephemeral-leaved ancestors (Figure 2D,E). Likewise, *Austrocylindropuntia* has acquired persistent leaves separately, and those leaves, although appearing totally cylindrical, show some modifications similar to a bifacial leaf [108]. These large, persistent leaves appear to be correlated with the adaptation to life in SDTF and/or Chaco habitats from desert-adapted ancestors with greatly reduced and ephemeral leaves. From a gross morphological point of view, these taxa have essentially reacquired *Pereskia*/*Leuenbergeria*-like leaves for adaptation to more or less the same types of broad-leaf, tropical dry forests, where those leaves aid to increase photosynthetic capacity.

Bailey [109] analyzed the anatomy of *Pereskopsis* and *Quiabentia* leaves and determined that they were anatomically distinct from the leaves of other leafy Cactaceae, such as “*Pereskia* s.l.”. *Pereskopsis* and *Quiabentia* have palmate or pseudopalmate venation, as opposed to pinnate venation, as is exhibited by *Pereskia* and *Leuenbergeria*. Likewise, the leaves of *Pereskopsis* and *Quiabentia* are truly succulent, meaning that they produce associated parenchymatous tissue, increasing the thickness of the leaves for significant water storage. Bailey [110] analyzed the leaves of *Austrocylindropuntia subulata* and found a similar, although more condensed, palmate venation to that of *Pereskopsis* and *Quiabentia*. He also mentioned the semi-bifacial structure of the leaves, as is seen in cross-section, with the vascular bundles mostly restricted to the adaxial surface instead of the entire leaf becoming adaxialized, as in the truly cylindrical leaves of other Opuntioideae, such as *Opuntia* s.s.

Hernández-Hernández et al. [24] concluded that the growth forms of all other cacti and the subfamily Cactoideae were not comparable and thus excluded *Maihuea*, Opuntioideae and *Pereskia* s.l. from previous analyses of morphological evolution, contrasting that of the growth forms of Cactoideae. However, there is no great distinction between these groups, as most Cactoideae are either trees or shrubs, regardless of branching patterns, etc. Likewise, taxa such as *Armatocereus*, *Cephalocereus* (in part; [111]), *Leptocereus* and *Rhipsalis* (Cactoideae) have nearly identical growth forms to those of most Opuntioideae, showing determinate, sympodial growth of the stem segments, as seen in our reconstructions here (Figure 2B, Supplementary Figure S2A). Likewise, certain Opuntioideae possess indeterminate, monopodial growth (e.g., *Austrocylindropuntia*, *Brasiliopuntia*, *Consolea*, *Cylindropuntia leptocaulis* (at least initially), *Pereskopsis* and *Tacinga funalis*; Figure 2B). So, the great distinction of growth forms among these groups is mostly artificial, as all cacti share a basic body plan having alternate spiral leaf phyllotaxy (these are sometimes coalesced into ribs, as in many Cactoideae and *Grusonia bradtiana* in Opuntioideae; see also [62]). Therefore, we emphasize that variations on a common theme, such as the growth forms associated with succulence across most Cactaceae [112], likely impacted the morphological evolution of Cactaceae as a whole, not just Cactoideae.

4.3. Biogeography of Opuntioideae

The ancestral areas for the Cactaceae as a whole, as well as Opuntioideae, Tephrocacteae and Cyllindropuntieae, were at least in part reconstructed as being the desert, based, of course, on the modern distribution of the species sampled here and an admittedly broad generalization of desert habitats across the Americas. However, according to what we know regarding the formation of deserts in the Americas, as well as the putative ages of these clades (15–28 my), we must infer that the MRCAs of these groups evolved in edaphically arid habitats that perhaps were not as extensive as modern desert regions [113]. Desert formation to the extent that we see them today would account for the rapid radiation of these groups in the mid-Miocene and into Plio- and Pleistocene epochs [23,114].

Our ancestral area reconstruction based on ecoregions revealed, as in other analyses (e.g., [24]), that the Andes were the likely center of origin of Cactaceae. From Andean South America, the clade dispersed essentially throughout the rest of the Americas (Figure 4). This is also true for Opuntioideae, with a putative origin in the Andes and then movement into other dry areas of the Americas. Tephrocacteae, however, remained in the Andes and significantly diversified there, in contrast to Cyllindropuntieae and Opuntieae, which diversified much more substantially outside of the Andes [29,55]. There have been numerous dispersals into western North America, such as in Opuntieae, Cyllindropuntieae [29,55], Cactaceae [43] and Core Cactaceae II [115], and likewise into the Antilles [50,58,115] from South America (Figure 4). So, there has been a repeated pattern of movement out of South America during the diversification of Cactaceae, in general. This has likely been fostered by the expansive drylands of western North America [113] and the seasonally dry tropical forests of the Antilles and Mesoamerica [116].

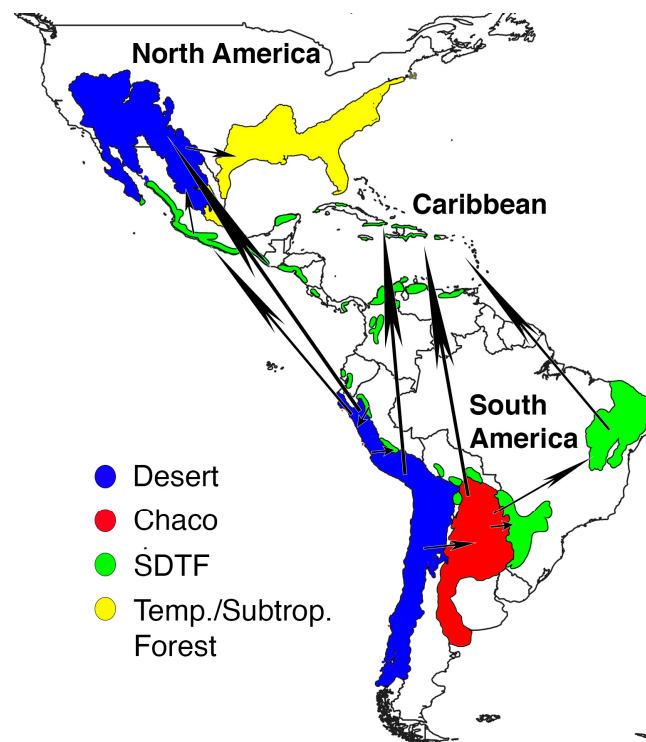


Figure 4. Map of putative dispersal events of Cactaceae across the Americas. Arrows indicate the directionality of dispersal. Cactaceae originated in arid areas of South America, and there were movements between the desert and SDTF on several occasions. Numerous dispersals occurred out of South America into the Caribbean region from the desert, Chaco and SDTF and into western North America, mostly from desert habitats. Movement from the desert to the Chaco and then into SDTF occurred within South America, and movement from SDTF to desert and desert to temp./suptrop. forest occurred in North America.

5. Conclusions

Opuntioideae evolved in the Miocene in the desert regions of South America and, from there, moved into SDTF and temperate regions, including spreading north into western North American deserts (Figure 4), where the bulk of the diversity was generated. Within Opuntioideae, Tephrocactaeae and Cylindropuntieae form a well-supported sister clade to Opuntieae. Opuntioideae exhibit ancestral features in cacti, including large (macroscopic) leaves; however, the production of large, long-lived, photosynthetically active leaves in *Austrocylindropuntia*, *Pereskia* and *Quiabentia* represents homoplasy, having been acquired secondarily, likely as an adaptation to SDTF and Chaco vegetation. The flattened stem segments of the tribe Opuntieae are a synapomorphy for that group and are likely an adaptation for competition in dense SDTF, the putative ancestral habitat for the clade. Areole production appears to increase in hotter, drier conditions within the tribe Opuntieae, which would enable these plants to produce higher numbers of spines to protect stems from UV radiation and associated overheating. Species relationships and major clade relationships are mostly resolved with plastome datasets, which show promise for resolving recalcitrant nodes across the family.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/d15040570/s1>. Figure S1: BEAST analysis showing crown ages for major clades and posterior probability support; Figure S2: Morphological evolution of (A) growth form and (B) areole number in Opuntieae; Figure S3: Ancestral geographic area based on ecoregions using BAYAREALIKE+J model in BioGeobears; Table S1: BioGeoBears model test results for both habitat and ecoregion analyses; Section S1: Accessions used in our plastome analysis.

Author Contributions: Conceptualization, L.C.M.; methodology, L.C.M. and S.A.; formal analysis, L.C.M. and S.A.; investigation, L.C.M.; resources, M.A.B., R.P.-M. and S.F.; data curation, L.C.M. and S.A.; writing—original draft preparation, L.C.M. and S.A.; writing—review and editing, L.C.M., S.A., M.A.B., M.K., R.P.-M. and S.F.; visualization, L.C.M. and S.A.; supervision, L.C.M.; project administration, L.C.M.; funding acquisition, L.C.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded in part by the National Science Foundation, DEB 1735604.

Institutional Review Board Statement: Not applicable.

Data Availability Statement: Our plastid gene dataset, taxon coding and trees generated are available on FigShare (10.6084/m9.figshare.22211935).

Acknowledgments: This work was made possible in part through the Desert Botanical Garden Living collection and financial support from the Desert Botanical Garden. Financial support was also provided in part by the National Science Foundation (DEB 1735604). Permits for material collected for this project were provided by El Ministerio del Medio Ambiente y Recursos Naturales, Dominican Republic, the US National Park Service (Lake Mead National Recreation Area and Big Bend National Park), Arizona, California and Nevada Bureau of Land Management, as well as Big Pine Key Deer Refuge, Florida. The Desert Botanical Garden Herbarium (DES), Arizona State University Herbarium (ASU) and the University of Florida Herbarium (FLAS) serve as repositories for many of the specimens used in our analyses. Lastly, we would like to thank two anonymous reviewers for providing comments on a previous version of this paper.

Conflicts of Interest: The authors have no conflict of interest for the data generated and presented in this study. 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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