

Original Article

Darwin's overlooked radiation: genomic evidence points to the early stages of a radiation in the Galápagos prickly pear cactus (*Opuntia*: Cactaceae)

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ABSTRACT

In the Galápagos Islands, much attention has been devoted to the radiation of iconic species like Darwin's finches, yet the Galápagos Islands offer an overlooked but equally remarkable opportunity for investigating plant radiations. Using a combination of genomic and phenotypic data, we present evidence of the early stages of a radiation in prickly pear cactus (*Opuntia*), a lineage widespread across the archipelago. We show that despite extensive ecophenotypic variation, there is limited genomic differentiation, consistent with the hypothesis that *Opuntia* is in the early stages of the diversification process. Phylogenomic and population genomic analyses show that notwithstanding low genetic differentiation across islands, there is marked geographical structure that is broadly consistent with the palaeogeography of the Galápagos. Because low genetic differentiation stands in stark contrast to the exceptional eco-phenotypic diversity displayed by cacti, it is plausible that the genetic architecture of phenotypic divergence mismatches our genomic sequencing, that phenotypic plasticity precedes genetic divergence and is the source of adaptive evolution, or that introgression influences local adaptation. Models of population relationships including admixture indicate that gene flow is common, probably facilitated by dispersal via animals known to feed on *Opuntia* flowers, fruits, and seeds. Because the prickly pear cacti of the Galápagos are a radiation in the making, they provide an exciting opportunity to investigate the interplay between ecological and genomic mechanisms promoting diversification.

RESUMEN

En las Islas Galápagos, se ha dedicado mucha atención a la radiación de especies icónicas como los pinzones de Darwin, pero las Islas Galápagos ofrecen una oportunidad pasada por alto pero igualmente notable para investigar las radiaciones de las plantas. Utilizando una combinación de datos genómicos y fenotípicos, presentamos evidencia de las primeras etapas de una radiación en el nopal (*Opuntia*), un linaje ampliamente distribuido en todo el archipiélago. Mostramos que a pesar de la extensa variación ecofenotípica, existe una diferenciación genómica limitada, consistente con la hipótesis de que *Opuntia* se encuentra en las primeras etapas del proceso de diversificación. Los análisis filogenómicos y

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genómicos de poblaciones muestran que, a pesar de la baja diferenciación genética entre islas, existe una estructura geográfica marcada que es ampliamente consistente con la paleogeografía de Galápagos. Debido a que la baja diferenciación genética contrasta marcadamente con la excepcional diversidad ecofenotípica que muestran los cactus, es plausible que la arquitectura genética que subyace la divergencia fenotípica no coincida con nuestros datos genómicos, que la plasticidad fenotípica preceda a la divergencia genética y sea la fuente de la evolución adaptativa, o que la introgresión influye en la adaptación local. Los modelos de relaciones poblacionales que incluyen introgresión, indican que el flujo de genes es común, probablemente facilitado por la dispersión a través de animales que se sabe que se alimentan de flores, frutos y semillas de *Opuntia*. Debido a que los nopales de Galápagos son una radiación en formación, brindan una excelente oportunidad para investigar la interacción entre los mecanismos ecológicos y genómicos que promueven la diversificación.

Keywords: biogeography; ecomorphology; Ecuador; phenotypic diversity; phylogeography; plant radiation; population genetics

INTRODUCTION

‘I certainly recognise S. America in ornithology, would a botanist?’

C. R. Darwin (1835, Field Notes, p 30)
soon after arriving to the Galápagos and
noticing the mockingbirds, which he could
associate with birds from mainland South
America. He doubted the same could be said
about the plants.

The origins, persistence, and diversification of species have been studied extensively in oceanic islands where ecological communities are often less complex and environmental conditions provide variation for evolution to flourish (Darwin 1859, Wallace 1869, MacArthur and Wilson 1967, Cerca et al. 2023). The Galápagos Islands are a prime example of such natural laboratories for studying evolution. Studies on the diversification of Galápagos tortoises (Caccone et al. 2002, Miller et al. 2018), iguanas (Rassmann 1997, MacLeod et al. 2015), land snails (Phillips et al. 2020), and Darwin’s finches (Lack 1945, 1947, Bowman 1963, Grant and Grant 2007, Lamichhaney et al. 2015) have provided fundamental biological insight about foundational ideas in evolutionary biology, including speciation, natural selection, and adaptive radiation. Strikingly, and in spite of a relatively well-known flora, studies about the diversification of Galápagos plant lineages have received considerably less attention (Grant and Grant 2024), with the exception of a few classic studies on *Opuntia* prickly pear cacti (Browne et al. 2003, Helsen et al. 2009a, b) and recent work on the salt bush (Arjona et al. 2020) and Darwin’s giant daisies (Fernández-Mazuecos et al. 2020, Cerca et al. 2022). This is surprising given the central role that the plants from the Galápagos, particularly the prickly pear cacti, played in shaping Darwin’s ideas for his theory of evolution (Stöcklin 2009) and in the dietary ecology and diversification of the iconic Darwin’s finches (Lack 1945, Bowman 1963, Grant and Grant 1981, 2014, Olesen et al. 2018). During Darwin’s visit to the Galápagos, he collected about 240 plant species, including cacti, which he sent back to England to be examined by his mentor J.S. Henslow and his close colleague J.D. Hooker. Unlike the birds he collected, Darwin carefully noted down the time of the year and the island where the plants were found. This information proved critical for Hooker who had an intensive correspondence with Darwin and they exchanged their views on the geographical distribution of plants, plant migration possibilities, and island floras. This led Darwin to thank Hooker for his examination of the plant collections, as these observations provided strong support to his revolutionary ideas about the origin and evolution of species (Stöcklin 2009).

The 14 taxa of prickly pear cacti currently recognized in the Galápagos islands display outstanding eco-phenotypic variation, including 4-fold differences in plant height, 100-fold differences in seed size and seed number per fruit, and 5-fold differences in seed hardness (Racine and Downhower 1974, Grant and Grant 1981). In addition, species and populations vary extensively in the size of pads as well as the density and morphology of spines (Stöcklin 2009) (Fig. 1). This pronounced phenotypic variation has led to controversies around the nature and taxonomy of species in Galápagos *Opuntia*. Six species are recognized, with three species further subdivided into multiple subspecies, for a total of 14 taxa (Helsen et al. 2009a). Notably, each recognized taxon is virtually restricted to a single large to mid-size island, with the exception of *Opuntia helleri* which is widespread across multiple islands. Previous work based on the study of variation in eight allozymes (Browne et al. 2003), ten microsatellites (Helsen et al. 2009b), and two sequenced loci (Helsen et al. 2009a) suggested that despite the remarkable phenotypic and ecological variation of *Opuntia* in the Galápagos, there is exceptionally low genetic variation across this radiation. Therefore, whether each of the different recognized taxa represents independently evolving lineages remains unclear.

The prickly pear cacti are a prominent component of the Galápagos flora. They are widespread and occur on all major islands (> 10 km²) and most islets. Although there is considerable uncertainty about the most closely related species on the mainland (Majure et al. 2012), it is likely that *Opuntia* colonized the Galápagos only once from the nearby continent, as have most other native plants of the archipelago (Fajardo et al. 2019). This may have occurred by rafting on floating trees or through dispersal via multiple agents (e.g. sea currents, wind, birds). Studies testing the floatability and viability of *Opuntia* diaspores are lacking and the dispersal agents are unknown. Furthermore, because the reproductive biology of *Opuntia* in the Galápagos remains understudied, it is not known how many individuals, sizes, and plant parts were necessary for the establishment of the initial plant colonists. Nevertheless, it is plausible that vegetative multiplication, common across the genus *Opuntia* (Reyes-Aguero et al. 2006), facilitated the establishment of these plants in the Galápagos. Once in the archipelago, *Opuntia* diversified across islands in multiple environments, ranging from coastal dry open scrublands to higher elevation wet forests with closed canopies (Anderson and Walkington 1971). Yet, the colonization history, intra-island dispersal, and biological mechanisms enabling inter-island dispersal are poorly understood.

One of the most remarkable attributes of the Galápagos prickly pear cactus radiation is the extraordinary phenotypic diversity they display in vegetative and reproductive traits

(Fig. 1). Previous studies have suggested that both environmental and biotic factors may be responsible for such striking phenotypic diversity. In examining the patterns of morphological variation of *Opuntia* on three centrally located islands (Santa Fé, Santa Cruz, and Pinzón), [Racine and Downhower \(1974\)](#) suggested that variation in tree height, trunk diameter growth, and pad production was largely explained by the combined effects

of inter- and intraspecific competition for light and possibly selection due to variable wind intensity. In addition to the extensive variation observed in vegetative growth form, these authors also proposed that the pronounced variation in seed size and seed coat observed among island populations was related to seed dispersal and predation. While mockingbirds, tortoises, and iguanas disperse seeds by consuming either the fruits or the

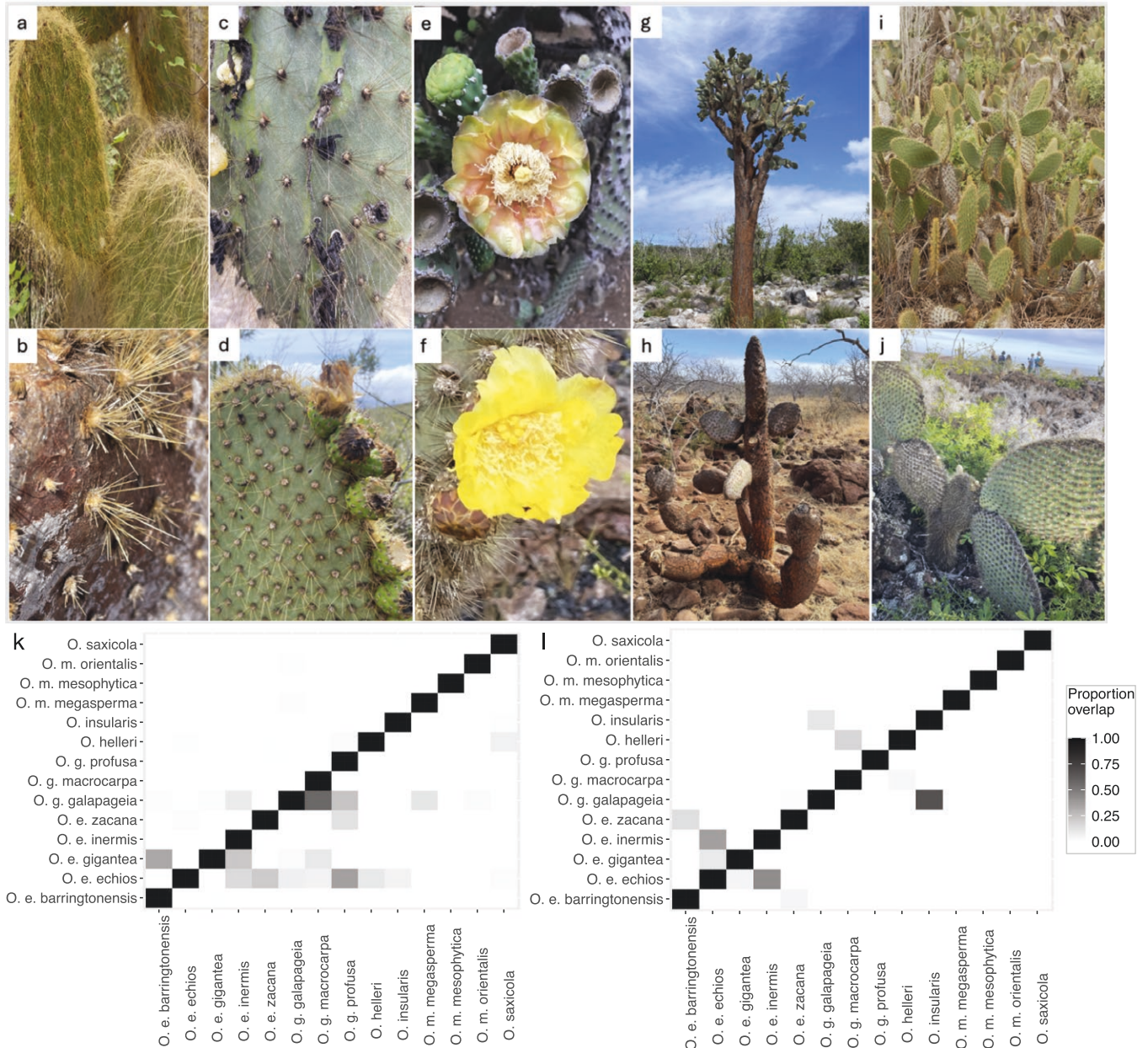


Figure 1. The prickly pear cacti (*Opuntia*) vary extensively in phenotypic and ecological traits across the Galápagos islands. Spines: (A) *Opuntia megasperma* var. *megasperma* from Floreana (photo J.A.C.), (B) *O. echios* var. *zacana* from Seymour (photo G.R.T), (C) *O. galapageia* var. *galapageia* from Santiago (photo G.R.T), (D) *O. saxicola* from Isabela, Volcán Chico (photo J.E.M); Flowers: (E) *O. megasperma* var. *mesophytica* from Santa Cristóbal (photo G.R.T), (F) *O. saxicola* from Isabela, Volcán Chico (photo J.A.C.); Habit: (G) *O. echios* var. *barringtonensis* from Santa Fé (photo J.E.M), (H) *O. echios* var. *zacana* from North Seymour (photo G.R.T), (I) *O. helleri* from Wolf (photo J.A.C.), (J) *O. insularis* from Isabela (photo G.R.T). (K) Pairwise overlap among hypervolumes describing *Opuntia* taxa in the Galápagos in multidimensional vegetative morphospace (8 traits). Most of the overlap occurs between subspecies of the same species. (L) Pairwise overlap among hypervolumes describing *Opuntia* taxa in the Galápagos in multidimensional reproductive morphospace (5 traits). Most of the overlap occurs between subspecies of the same species. Note that this overlap is asymmetric because hypervolumes of different sizes would necessarily differ in their proportion of overlap.

fleshy aril around the seed, the cactus finch (*Geospiza scandens*) is a major seed predator that cracks the seed coat to extract the endosperm (Racine and Downhower 1974). Hence, it is likely that seed predation is an important factor in the diversification of *Opuntia*, and the cactus finch is a prime agent of selection. Subsequent studies are consistent with this hypothesis and show that the interaction between Darwin's finches and prickly pear cacti extends, in fact, to multiple species of ground finches and cacti (Grant and Grant 1981). The intricate relationship between these two lineages is largely modulated by environmental changes such as increased and prolonged periods of drought, which affect plant survival and thus seed availability. Long-term studies of populations of Darwin's finches have shown how such changes have had dramatic effects on the morphology of the beaks of the finches, which are critical for seed manipulation (Grant and Grant 2014). Thus, to some extent, it is plausible there is some degree of coevolution between cactus and *Geospiza* finches in the Galápagos mediated by flowers and seed features, as suggested by classic work on ecological interactions (Lack 1945, 1947, Bowman 1961, 1963, Abbott *et al.* 1977, Smith *et al.* 1978, Grant and Grant 1980). This hypothesis, however, requires rigorous study.

While numerous ecological and genetic studies have examined the radiation of animals interacting with *Opuntia* in the Galápagos (Racine and Downhower 1974, Grant and Grant 2014, Lamichhaney *et al.* 2015), few studies have focused on these cacti to determine the nature of their radiation. Here, we use genomic and phenotypic data to revisit classic work on the prickly pear cacti (*Opuntia*) across the Galápagos archipelago to investigate the evolution of this charismatic lineage of the Galápagos flora (Fig. 1). In particular, we examine whether the *Opuntia* radiation is in the early or late stages of the diversification process. If it is in the late stage, genomic and phenotypic data would show congruent patterns of variation. However, if it is in the early stage, these data would be incongruent. Further, we investigate how genomic variation is distributed across geography to shed light on the spatial context of the history of *Opuntia* in the Galápagos. Given that these plants furnish an important source of food and water to multiple native animals across the archipelago (Anderson and Walkington 1971, Stöcklin 2009) and are critical in the ecology and persistence of some emblematic animal radiations, such as the Galápagos finches in the genus *Geospiza* (Grant and Grant 2014), *Opuntia* can be considered an important element of the Galápagos biota. Understanding the dynamics of the overlooked radiation of prickly pear cacti is therefore essential, not only for revealing the biological impacts of its evolution in other lineages but also for designing conservation strategies to protect the biodiversity of the Galápagos islands as a whole.

MATERIAL AND METHODS

Taxon sampling and DNA extraction

Samples were collected from eight islands across the Galápagos archipelago (Fig. 2; Supporting Information Table S1). The localities selected represented most of the islands across the geographical range of *Opuntia* in the Galápagos. We assigned all specimens to species following the taxonomy of Helsen *et al.*

(2009a) (Table S1). Currently recognized taxa are largely single-island endemics on major islands, and thus island- or taxon-level analyses are almost equivalent. We collected ~1–2 cm² of pad tissue for each specimen, stored it in silica gel, and then transported it to the University of California, Los Angeles, for further analyses. Genomic DNA extraction and purification was performed following a modified version of the CTAB extraction protocol (Doyle and Doyle 1987, Cullings 1992) that incorporates a prewash step to aid in the removal of polyphenols and proteins prior to extraction. Genomic DNA quantification was performed using a Qubit fluorometer v.3.0 (Invitrogen by Thermo Fisher Scientific, Carlsbad, CA, USA) and an Agilent 2200 TapeStation (Agilent Technologies, Santa Clara, CA, USA).

Library preparation and sequencing

To generate sequence data, we prepared quadruple-indexed, triple-enzyme RADseq (3RAD) libraries using the EcoRI, XbaI, and NheI restriction enzymes following the protocol described by Bayona-Vásquez *et al.* (2019). The step-by-step laboratory protocol is also included in the Supporting Information. Prior to sequencing, size selection for fragment lengths of 375–525 bp was performed on a PippinPrep (Sage Science, Beverly, MA, USA). Libraries were pooled and sequenced in one lane of 100PE sequencing on the Illumina HiSeq4000 Sequencing Platform at the Broad Stem Cell Research Center at the University of California, Los Angeles.

RADseq differs from genome sequencing and target-capture methods in terms of information content and methodology (Peterson *et al.* 2012). RADseq focuses on sequencing DNA regions flanking restriction enzyme cut sites, providing a cost-effective means to discover and genotype thousands of markers across the genome without the need for prior genomic information. This approach allows for phylogenetics as well as high-resolution population genetics studies, but it captures only a fraction of the genome, potentially missing some loci of interest. In contrast, whole-genome sequencing offers comprehensive coverage of the entire genome, providing the most detailed information but at a higher cost and with more complex data analysis. Target-capture methods selectively enrich and sequence specific genomic regions of interest, balancing between the breadth of genome sequencing and the focus of RADseq, but requiring prior knowledge of target sequences and potentially missing variation outside these regions. Thus, RADseq is advantageous for its efficiency and cost-effectiveness in large-scale studies, though it is limited in its genomic scope compared to the other methods.

Bioinformatics and single nucleotide polymorphism (SNP) discovery

We demultiplexed, removed adapters, and filtered reads for data quality using the iPyRAD package v.0.9.72 (Eaton and Overcast 2020). Because of the potential hexaploid nature of the Galápagos *Opuntia* (Helsen *et al.* 2009b, Majure *et al.* 2012), we carefully explored and processed the data. Specifically, we assembled the data first in iPyRAD assigning a value of 6 to the maximum number of unique alleles parameter to isolate the six expected haplotypes. In addition, we optimized this assembly by

assigning a value of 6 to the minimum depth filter parameter, a value of 0.94 to the clustering threshold parameter, and a value of 0.75 to the maximum number of shared polymorphic sites in a locus parameter. We repeated the assembly by running Stacks v.2.60 (Rochette 2019) using different M/N combinations as suggested by Paris *et al.* (2017) to optimize rad-assembly parameters. This involved running stacks 10 times using -M (number of mismatches allowed between stacks within individuals) and -N (number of mismatches allowed between stacks between individuals) values between 1 and 10. We ran this protocol four times, specifying different population maps (i.e. groups of populations), namely by islands, by species, by islands excluding outgroups, and by islands excluding outgroups. This led to an optimal combination of -M/-N of 2 for analysis involving the outgroup and -M 1 -N 1 for analysis not including the outgroup. Because preliminary analyses did not differ between the iPyRAD and Stacks assemblies [e.g. principal components analysis (PCA), population structure], we retained the Stacks assembly for subsequent analyses.

Because samples with a high degree of missing data tend to compromise the quality of the final RADseq variant dataset, we followed the protocol established by Cerca *et al.* (2021b) to remove ‘bad apples’. This led to the removal of three outliers with high levels of missing data, resulting in a final dataset of 33 individuals of Galápagos *Opuntia* and two outgroup specimens. For the final dataset, we ran Stacks with and without outgroups, specifying -R 0.8 (minimum percentage of individuals across populations required to process a locus).

Population genetics, phylogenetics, and admixture analyses

For population genetic analyses and to assess patterns of genetic variation among sampling localities, we used multiple approaches. First, we used PCA based on the genetic covariance matrix from genotypes (Patterson *et al.* 2006). Additionally, we used STRUCTURE (Pritchard *et al.* 2000), a model-based clustering algorithm, to assign individuals to genetic populations (i.e. demes) on the basis of their genotypes while simultaneously estimating their proportions of genomic ancestry. To characterize fine-scale population structure, we harnessed the power and resolution afforded by RADseq data to infer recent shared ancestry at multiple successive SNPs (haplotypes) based on nearest-neighbour relationships using the program fineRAD (Lawson *et al.* 2012, Malinsky *et al.* 2018). For the PCA and STRUCTURE analysis, we obtained a VCF file where linkage was reduced by specifying only a single and random SNP per radtag (--write-random-snp), and then cleaned the vcf for coverage and for missing data, obtaining a total of 23 566 SNPs. To run the PCA, we used the libraries vcfr (Knaus and Grünwald 2017), adegenet (Jombart 2008), and ggplot2 (Wickham 2011). For STRUCTURE analysis we used plink (Purcell *et al.* 2007) to convert the VCF into a structure file, and ran STRUCTURE v.2.3.4 (Pritchard *et al.* 2000) specifying K values between 2 and 10 [burn-in of 100 000 and a Markov chain Monte Carlo (MCMC) chain of 100 000]. For plotting the results of this analysis, we used the online server CLUMPAK (Kopelman *et al.* 2015) and its plug-in which uses the Evanno method to determine the optimal K (Evanno *et al.* 2005). We ran fineRADstructure v.0.3.2 (Malinsky *et al.* 2018)

using default settings and annex plotting scripts (<https://www.milan-malinsky.org/fineradstructure>).

To reconstruct the phylogenetic history of *Opuntia* in the Galápagos, we retrieved a collection of FASTA rad loci (--fasta-samples) using Stacks. Since we had a dataset of 35 individuals (70 alleles), we kept loci with more than 59 data points (30 individuals), retaining a total of 10 484 loci (total of 2134 262 bp). We obtained a consensus sequence between the two allele loci using EMBOSS v.6.6.0 consambig as done in Cerca *et al.* (2021a) and concatenated the consensus loci using FASconCAT-G v.1.04 (<https://github.com/PatrickKueck/FASconCAT-G/>). We used the concatenated loci to run IQ-TREE v.2.1.3 (Minh *et al.* 2020a, b) specifying 1000 ultrafast bootstraps. To account for the possibility of tangled phylogenetic relationships, we converted the VCF file into a Nexus file using the script vcf2phyl.py (<https://github.com/edgardomortiz/vcf2phylip>) and used SplitsTree v.5 to estimate a phylogenetic network (Hamming 1950, Bryant and Moulton 2004, Dress and Huson 2004). Lastly, we inferred a ‘population’ tree using a finite-sites mutation model per locus, integrating information over all possible loci/gene trees. For this analysis, we used a coalescent approach implemented in the Bayesian MCMC sampler SNAPP (Bryant *et al.* 2012), which we ran through the BEAST2 package (Bouckaert *et al.* 2014). For SplitsTree and SNAPP, we used a VCF file where filtering included only coverage (--maxDP 200 --max-meanDP 200 --minDP 10 --min-meanDP 10), missing data (--max-missing 0.25), and loci shared (-R 0.8). For computational efficiency, we subsampled the VCF file to 4000 random SNPs for SNAPP.

For admixture analyses, we used the F4 analysis implemented in Dsuite (Malinsky *et al.* 2021). This involved retrieving a VCF file from Stacks (-R 0.8), and filtering it further by coverage (--maxDP 200 --max-meanDP 200 --minDP 10 --min-meanDP 10) and missing data (--max-missing 0.25) using vcftools (Danecek *et al.* 2011). We then ran the algorithm Fbranch of Dsuite specifying the tree obtained in IQ-TREE and plotting it using a collection of ruby scripts from Michael Matschiner (https://github.com/mmatschiner/tutorials/blob/master/analysis_of_introggression_with_snp_data/README.md).

Phenotypic variation

We used the most recent taxonomic monograph of Galápagos *Opuntia* to tabulate the minimum and maximum values reported for 13 phenotypic traits used to describe and delimit each of the 14 recognized taxa (Anderson and Walkington 1971). Vegetative traits included plant height, pad length, pad width, pad thickness, areole width, distance between adjacent areoles, number of spines per areole, and spine length. Reproductive traits included fruit length, fruit width, seed length, seed width, and seed thickness. The combination of minimum and maximum values of these traits delimits a hypervolume of n dimensions (n = the number of phenotypic traits) in the phenotypic space corresponding to each species. We constructed separate hypervolumes for vegetative traits (n = 8) and reproductive traits (n = 5) because some *Opuntia* species seem to differ more in one kind of trait than the other. To determine the distinctiveness of each taxon, we log-transformed all data and estimated the pairwise asymmetric proportion of overlap of all vegetative and reproduction hypervolumes. The proportion of overlap is asymmetric

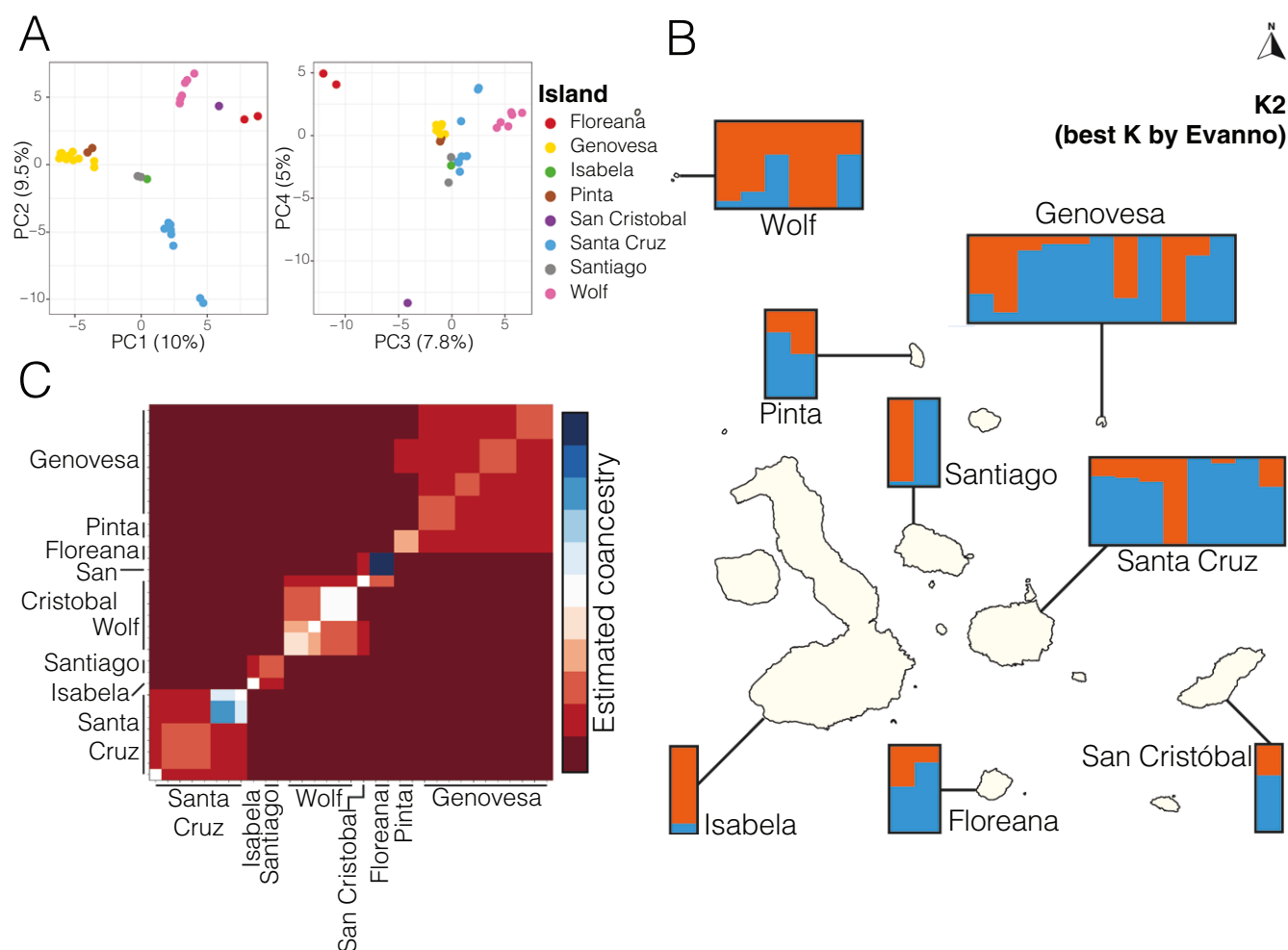


Figure 2. Despite extensive ecophenotypic variation, *Opuntia* shows relatively low genetic differentiation across the Galápagos islands, yet there is a marked geographic structure. (A) Principal component analysis (PCA) to reduce multidimensional genomic space into fewer dimensions. The left panel shows the space defined by PC1 (10% of the variance explained) and PC2 (9.5% of the variance explained), the right panel shows the space defined by PC3 (7.8% of the variance explained) and PC4 (5% of the variance explained). (B) Genomic ancestry shows the optimal partition of the data into two genetic demes shown across islands. (C) Fine-scale population structure based on nearest-neighbor relationships across all individuals sorted by island. Five clusters are readily detected corresponding to single islands or island pairs in close geographic proximity. Further substructure exists within some clusters. Cold colors (blue, top of color legend) correspond to closely related individuals, while warm colors (red, bottom of color legend) correspond to more distantly related individuals.

because the sizes of the hypervolumes corresponding to each species can differ. As a result, some hypervolumes may be contained within others, but not vice versa. We used the library *geometry* (Habel et al. 2023) in R to carry out this analysis.

RESULTS

Population genetic structure of *Opuntia* in the Galápagos archipelago

To examine the population genetic structure of *Opuntia* in the Galápagos islands, we used 3RAD sequencing (Bayona-Vásquez et al. 2019) on 33 individuals representing seven taxa (out of the 14 recognized taxa) across eight islands, and two individuals belonging to an outgroup lineage (*Opuntia dillenii*). Overall, we find that while there is some genetic differentiation among the sampled individuals across the archipelago, such differentiation is not highly pronounced. The first two PC axes account for only 19.5% (PC1 10% of the variance explained; PC2, 9.5%; Fig. 2A)

of the variation in the multivariate genomic space across the Galápagos islands. Using up to four PC axes accounts for 32.5% of the variation (Fig. 2A). Together, these findings indicate that the genomic differentiation of cactus populations across the Galápagos islands, while low, nonetheless reveals some geographical patterns. For instance, PC1 and PC2 broadly separate populations along latitude (Floreana and San Cristóbal in the south separate from Pinta and Genovesa in the north, and Santa Cruz towards the east separate from Wolf in the northwest) while populations at the centre of the archipelago (Santiago and Isabela) cluster together in PC space (Fig. 2A). Genomic ancestry analyses (Pritchard et al. 2000) also reveal low genetic differentiation of populations across the archipelago (Fig. 2B). The optimal partitioning of the data indicates the existence of two demes with all the individuals displaying mixed ancestry, irrespective of island of origin or taxonomic assignment (Supporting Information Fig. S1). This suggests considerable admixture or recent ancestry for all sampled populations.

To characterize fine-scale population structure, we used fineRAD (Lawson *et al.* 2012, Malinsky *et al.* 2018). Clustering results readily reveal the presence of five populations, with marked substructure suggested in some of the populations (Fig. 2C). The five populations correspond to groups of samples from single islands or island pairs in close geographical proximity, namely [Santa Cruz], [Isabela + Santiago], [Wolf], [Floreana + San Cristóbal], and [Pinta + Genovesa], with further substructure within island pairs matching single islands (e.g. Isabela and Santiago, Floreana, and San Cristóbal). Additionally, our results show heterogeneous degrees of differentiation of samples between and within all five populations, with some localities highly differentiated from samples at other localities (e.g. all samples from Floreana and a subgroup of samples within Santa Cruz). Notably, the highly differentiated subpopulations in Santa Cruz correspond to the two subspecies of the species of *Opuntia* present on this island (*Opuntia echios*). Overall, these results suggest shallow genetic divergence across the five populations that are highly restricted geographically and with limited gene flow.

Phylogenetics and admixture of *Opuntia* in the Galápagos archipelago

To reconstruct the radiation of *Opuntia* throughout the Galápagos, we grouped individuals by island and used islands as units of analysis. This approach is consistent with our results on genetic structure (see above), current taxonomy (currently recognized taxa are largely single-island endemics), as well as a reasonable approach to studying the colonization of organisms

across archipelagos. Our phylogenetic reconstructions suggest a recent radiation of prickly pear cacti in the Galápagos, as seen in the tangled phylogenetic network (Fig. 3A), the short internal branches in the species tree (Fig. 3B), and the topological tree discordance using different subsets of loci (Fig. 3C). Both the concatenated and coalescent-based species tree analyses consistently recover two sets of relationships. First, the deepest divergence separates a clade containing San Cristóbal and Floreana, and sometimes Wolf, from the remaining islands (Fig. 3B, C). Second, the individuals from Genovesa and Pinta are always sisters to each other and are nested within a clade including Santiago, Santa Cruz, and Isabela, which show different relationships depending on the loci used to infer phylogenetic relationships. Because incongruent phylogenetic relationships can result from introgression between isolated lineages, we tested for genetic admixture between islands using the F4 statistic (Patterson *et al.* 2012). We detect excess allelic sharing between San Cristóbal and Wolf, between Genovesa and Santa Cruz, and between Wolf and Genovesa and Pinta, indicating admixture between all these islands (Fig. 4).

Phenotypic differentiation of *Opuntia* in the Galápagos archipelago

Our results show that currently recognized taxa (species and subspecies) of *Opuntia* in the Galápagos are strongly differentiated in vegetative and reproductive traits (Fig. 1). In general, taxa do not overlap or overlap minimally in multidimensional morphospace, with marked overlap only occurring between subspecies of the same species (Fig. 1K, L). Only in a few cases,

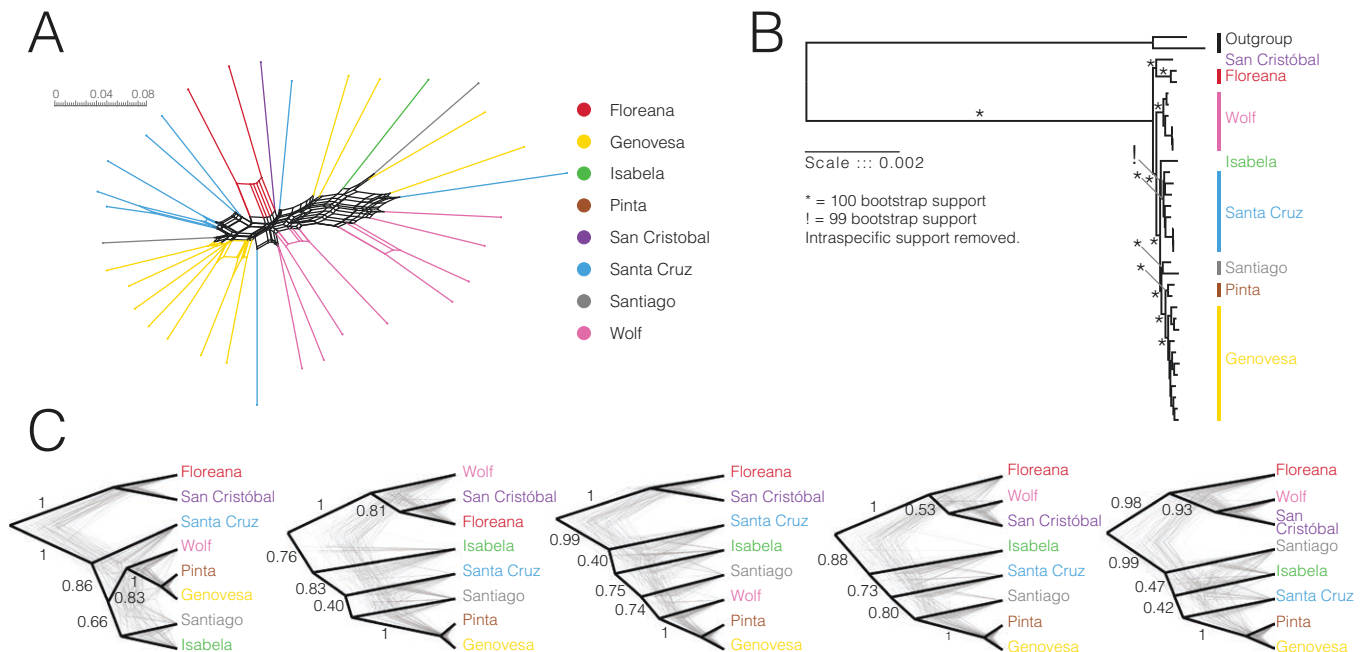


Figure 3. The radiation of *Opuntia* across the Galápagos archipelago is a recent phenomenon. (A) The phylogenetic network shows tangled relationships among all island populations. (B) The phylogenetic tree among all island populations using a concatenated matrix of all loci shows very short internal branches subtending phylogenetic relationships. (C) The phylogenetic trees among all island populations using a coalescent approach (without concatenation) vary according to the loci included in the analysis. Each panel shows trees inferred with 4,000 random sampled loci. The clouds of trees in the background represent trees sampled from the posterior distribution, the black line represents the Maximum Credibility Tree.

(Santiago, Pinzón, Rábida, Santa Cruz, and Floreana), and several other islands emergent then are submerged today. Even in more recent times, during the Last Glacial Maximum (~20 kya), there were many more islands and islets present and there were land bridges connecting three of the present big central islands (Isabela, Fernandina, and Santa Cruz). In sum, such drastic palaeogeographical changes clearly affected the connectivity of populations across islands, thus possibly shaping directly the recent radiation of prickly pear cacti in the archipelago (Helsen *et al.* 2011).

Alternatively, our phylogenetic results could be the outcome of gene flow between geographically isolated localities, perhaps facilitated by pollen and seed dispersers (Grant and Grant 1981) and weak isolating barriers. For instance, we found that San Cristóbal is sister to either Floreana or Wolf, depending on the loci used to infer phylogenetic relationships (Fig. 3B, C). Such a pattern could result from differential gene flow among islands that are separated by more than 300 km as in the case of San Cristóbal and Wolf.

To test formally for genetic admixture between populations in our data set, we used the F4 statistic (Patterson *et al.* 2012). We detected excess allelic sharing between the populations from San Cristóbal and Wolf, Genovesa and Santa Cruz, and Wolf and the grouped populations from Genovesa and Pinta, indicating ancient admixture between all these populations (Fig. 4). Previous studies have suggested that as mainland *Opuntia* species have weak reproductive barriers (Helsen *et al.* 2009a) and because the two sympatric taxa of *Opuntia* present in Santa Cruz island show little to no signal of genetic differentiation based on microsatellite data (Helsen *et al.* 2009b, 2011), gene flow between lineages might be widespread across the Galápagos *Opuntia*. Our results are in general agreement with this idea, yet our evidence for admixture shows that gene flow is more prevalent between certain lineages than others. It remains unclear whether the instances of admixture we report here correspond to cases of hybridization or introgression and it will require more detailed analyses of patterns of reproductive isolation and ecological differentiation between lineages. Likewise, whether genetic admixture fuels or constrains differentiation is not known. Investigating the extent to which gene flow could increase the amount of genetic variation in populations to promote adaptation to local conditions will require identification of the loci underlying the traits of interest and characterization of their evolutionary histories (Todesco *et al.* 2020).

The second notable phylogenetic result concerns the geographical structure of the radiation of Galápagos *Opuntia*. Both the concatenated and coalescent-based species tree analyses recovered consistently the oldest divergence of the radiation to be the separation between San Cristóbal and Floreana, and sometimes Wolf, from the remaining islands (Fig. 3B, C). Because San Cristóbal is the oldest current island (2.4–4.0 Mya) (Geist *et al.* 2014), the deep divergence event separating the *Opuntia* from San Cristóbal is consistent with the history of the islands. However, the age of Floreana (1.5–2.3 Mya) is on par with the ages of several other current islands included in our dataset, such as Santa Cruz (1.1–2.3 Mya) and Wolf (1.6–1.7), as well as with the ages of Pinzón (1.3–1.7 Mya), Rábida (1.3–1.6 Mya), and Santa Fé (2.9–2.9 Mya) not included in our sampling.

Furthermore, Floreana was part of a larger landmass that included Santa Cruz at least 2 Mya (Geist *et al.* 2014). Therefore, although the deep split separating the *Opuntia* from San Cristóbal and the other islands is consistent with the age of this island, it is unclear if the colonization of Floreana involved a stepping-stone dispersal via other older islands such as Española, Santa Fé, or Pinzón, for which we are missing *Opuntia* samples. Including samples from such islands in combination with explicit biogeographical and divergence dating analyses are required to shed light on this possibility.

Sister to the San Cristóbal and Floreana (and sometimes Wolf) clade, the relationships among the remaining islands are broadly consistent with the estimates of island ages and geographical proximity (Fig. 3B, C). Genovesa and Pinta are sister to each other and are nested within a clade which includes Santiago, Santa Cruz, and Isabela. Genovesa and Pinta are some of the youngest islands in our dataset, with ages of 0.3–0.8 and 0.7–0.8 Mya, respectively (Geist *et al.* 2014). Because these islands are also far (~80 km) from the centrally located islands (e.g. Santa Cruz, Isabela, Santiago) and have never been connected to other islands in the archipelago in geological time, our result suggests a recent, long-distance dispersal colonization of *Opuntia* to the islands of Genovesa, Pinta, and possibly Marchena for which we are missing samples in the current study. The ages of Santa Cruz and Santiago islands range between 1.1–2.3 and 0.8–1.4 Mya, respectively. In contrast to other current islands included in this study, these two islands have been part of the same landmass at some point over the last million years (Geist *et al.* 2014). Isabela is one of the youngest islands of the archipelago, with an age estimate of 0.5–0.8 Mya. Further, Isabela is in extremely close geographical proximity to Santa Cruz and Santiago, and it is likely that there was a landbridge connecting Isabela and Santa Cruz during the Last Glacial Maximum (20 kya) (Geist *et al.* 2014). Therefore, our results are consistent with the colonization of the centrally located islands (Santiago, Santa Cruz, and Isabela) from an older island (extinct or extant), with subsequent, repeated movements of plant propagules among all the central islands, probably facilitated by the shared geology and close geographical proximity. Evidence for this scenario comes from the uncertain and incongruent relationships among the populations of these islands (Fig. 3C).

Intriguingly, the relationship between the *Opuntia* from Wolf and from all other islands is the most uncertain. Although such uncertainty is largely explained by instances of gene flow (Fig. 4), Wolf is a small (1.3 km²) and distant island (> 150 km from other islands with the exception of Darwin Island) in the northwest corner of the archipelago, and it is not known how *Opuntia* could have colonized this island. Wolf is approximately as old as Floreana (1.6–1.7 Mya; Geist *et al.* 2014), and hence it might have been available for colonization for a long period of time, yet how *Opuntia* propagules reached this remote location in the first place remains elusive. Future studies will be necessary to test the floatability and viability of *Opuntia* and investigate whether its distribution could be explained by sea dispersal between islands. While the direction of sea currents in the Galapagos archipelago is westward and northwestward (Arjona *et al.* 2020) and this could have facilitated the dispersal of *Opuntia* to Wolf, it is more likely that animals helped disperse these plants. The presence in

Wolf Island of birds known to disperse *Opuntia* seeds elsewhere, such as Darwin's finches (*G. septentrionalis* and *G. magnirostris*: J.A.C. pers. comm.) and mockingbirds, could support this colonization hypothesis. Lava lizards on other islands also act as seed dispersers for *Opuntia* (*O. echios* in Santa Cruz and *O. galapageia* in Pinta) (Hervás-Parejo et al. 2019), but no lava lizards are currently found in Wolf or Darwin islands. A general trend of unidirectional gene flow and historical migration on several species within the archipelago (Caccone et al. 1999, 2002, Arbogast et al. 2006, Benavides et al. 2009, Chaves et al. 2012) resulting from prevailing south to southeast trade winds in the Galápagos (Power 1975, Colinvaux 1984, Geist 1992) could explain the colonization of *Opuntia* propagules or seeds dispersed by animals on these northern islands. Complementing our current sampling with samples from all other current Galápagos islands will certainly help clarify the biogeography and potential dispersal routes of *Opuntia* across the archipelago.

CONCLUSIONS

This study presents genomic evidence of decoupling between phenotypic and genomic differentiation in the Galápagos *Opuntia*. Even though we were only able to obtain one specimen per taxon for several taxa, this does not notably affect our phylogenetic and biogeographical inferences. This is the first study attempting to bridge phylogenetics and population genetic analyses using genomic data in the prickly pear cactus of the Galápagos archipelago to unravel the nature of this radiation and the geographical context of diversification. In the future, denser taxon sampling, further genome sequencing, and explicit phylogenetic dating approaches would improve our findings. While cactus populations from different islands show extreme variation in vegetative and reproductive traits (Fig. 1), the evidence for concomitant genomic variation is weak (Figs 2, 3). This is consistent with the hypothesis that these cacti are at the early stages of a radiation and that gene flow between populations may facilitate local adaptation (Suarez-Gonzalez et al. 2018). To discern whether gene flow might have a contrasting homogenizing effect on local adaptation, further sequencing and experimental work (e.g. common garden experiments, transplant experiments) would be critical in future studies. Alternatively, it is plausible that phenotypic plasticity precedes genetic change and is the source of adaptive divergence (West-Eberhard 1989). Under this scenario, phenotypic plasticity could facilitate the occupation of different habitat types leading to adaptive evolution via advantageous mutations after habitat shifts (Waddington 1953). Biotic interactions with animal taxa might promote gene flow between isolated lineages across the archipelago, and multiple abiotic stressors characterizing volcanic equatorial archipelagos might operate as potential mechanisms of adaptation and act as targets of selection between lineages. Further geographical sampling, deeper genome sequencing, and experimental data are critically needed before these hypotheses can be confronted confidently and the biological mechanisms underpinning this understudied radiation are better understood. Because our findings suggest that this is a radiation in the making, the Galápagos *Opuntia* provides an exciting opportunity to model the influence of ecological interactions on the genomic background of a radiating lineage (Martin and Richards 2019). The extent to which

phenotypic plasticity via genetic assimilation has fuelled adaptation in this scenario is a critical area for continued study. Much remains to be learned about the radiation of prickly pear cacti in the Galápagos.

SUPPLEMENTARY DATA

Supplementary data are available at *Evolutionary Journal of the Linnean Society* online.

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CREDIT STATEMENT

Felipe Zapata (conceptualization, investigation, resources, data curation, data analysis, writing original draft, supervision and project administration, funding acquisition), José Cerca (conceptualization, investigation, resources, data analysis, visualization, writing original draft), Dana McCarney (investigation, data curation), Claudia L. Henriquez (investigation, data curation), Bashir B. Tihamiyu (investigation), John E. McCormack (conceptualization, investigation, resources, data curation), Kelsey R. Reckling (investigation, data curation), Jaime A. Chaves (conceptualization, investigation, resources) and Gonzalo Rivas Torres (conceptualization, investigation, resources, supervision, and project administration). All authors contributed to the discussion of the results and provided their contribution to reviewing and editing the manuscript.

CONFLICTS OF INTEREST

We declare we have no conflicts of interest.

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DATA AND CODE AVAILABILITY

All sequence data have been deposited to the Sequence Read Archive (NCBI): BioProject ID PRJNA1022515 (<https://www.ncbi.nlm.nih.gov/bioproject/1022515>). All processed data files to generate results are available at https://github.com/zapata-lab/ms_opuntia_genomics. All code to run the analyses is available at: https://github.com/zapata-lab/ms_opuntia_genomics

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