Population expansion and genetic structure in *Cephalocereus nizandensis* (Cactaceae), a microendemic cactus of rocky outcrops of the Tehuantepec basin, Mexico

Aldo Isaac Juárez-Miranda[1], Amelia Cornejo-Romero[2,‡] & Carlos Fabián Vargas-Mendoza[1,*,‡]

[1]Departamento de Zoología, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, Mexico City, Mexico
[2]Departamento de Botánica, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, Mexico City, Mexico
*Corresponding author: carfvargas@yahoo.com
‡These authors contributed equally to the work

Background and aims – *Cephalocereus nizandensis* is a microendemic columnar cactus that grows isolated in xerophytic enclaves associated with rocky outcrops in the Isthmus of Tehuantepec, in the south of Mexico. Its demographic history and genetic structure were assessed to determine the main events that shaped its current restricted distribution.

Material and methods – Chloroplast intergenic sequences of 40 individuals and inter simple sequence repeats (ISSRs) of 45 individuals from four isolated populations were used to estimate haplotypic and nucleotide diversity, using expected heterozygosity and the Shannon index. AMOVA, population pairwise F_{ST}, and Bayesian clustering analyses were performed to explore the genetic structure. Demographic history was estimated with neutrality tests, mismatch distribution analysis, and Bayesian skyline plots. Phylogenetic relationships and divergence times were determined using a median joining network and a Bayesian molecular clock.

Key results – *C. nizandensis* has a high diversity and moderate genetic differentiation. The lowest elevation locality was found to be the most genetically distinct. The species has undergone a process of population expansion that began 150,000 years ago and has remained without evidence of a population contraction in the transition from the Pleistocene to the Holocene (11,700 years ago).

Conclusions – *C. nizandensis* presents moderate but significant genetic differentiation, which may be due to an early divergence of its populations. Currently observed levels of genetic diversity are the result of historical maintenance of high population sizes and a population expansion approximately in the last 150,000 years, which was sustained independently of the climatic fluctuations of the Early Quaternary, due in part to the stability of the rocky habitat.

Keywords – Cactaceae; historical population expansion; microendemism; Oaxaca, rocky outcrops.

INTRODUCTION

Rocky outcrops are sites where erosion has removed all the soil layers and the bedrock is exposed. They provide various microclimates that generate island habitats, increasing the heterogeneity in the landscape, levels of endemism and β diversity (Twidale 1982; Gibson et al. 2012). Plant species that occur on rocky outcrops are usually adapted to the edaphic conditions of their habitats, while being absent or rare in surrounding habitats (Gibson et al. 2012). In these habitats, the humidity is low, there is almost no water in the soil, and the exposure to the wind is higher compared to neighbouring areas. The concentration of phosphorus and nitrogen is very low as well (Porembski 2007).

Harsh edaphic conditions on outcrops hamper the establishment of seedlings that are not adapted to environmentally extreme conditions. Some adapted traits common in outcrop species are succulent water-storage trunks or caudex, and desiccation-tolerant vegetative tissues (Porembski 2007).

The inorganic and harsh nature of the outcrops provides them with long-lasting landscape characteristics and stable microclimatic conditions; thus, the rocky outcrops can function as ecological refugia for hundreds of thousands of years (Couper & Hoskin 2008; García et al. 2020). This long-term stability of rocky outcrop habitats has allowed the persistence of species and communities in latitudes where their presence cannot be explained by the regional climate. For example, in tropical regions of the Americas (Sarthou et al. 2003; Torres-Ribeiro et al. 2007; Silva et al. 2020), and Africa (Porembski 2007) where rainforests and cloud forests are the most common vegetation, rocky outcrops provide conditions for species adapted to xeric environments (Porembski 2007; Locosselli et al. 2016).

Rocky outcrops host communities of great floristic, ecological, and evolutionary interest. Plant communities of rocky outcrops have an island-like distribution and are thus good study systems to test different hypotheses related to their formation, distribution, and maintenance, particularly with regard to the xerophytic species in these communities (Porembski & Barthlott 2000; Byrne et al. 2019). Consequently, the vegetation of rocky outcrops has been highly studied around the world (Moraes et al. 2005; Bonatelli et al. 2014; de Aguiar-Campos et al. 2020). For example, in eastern Brazil's seasonally dry tropical forests, limestone outcrops have been found to have functioned as litho-refuges during the Quaternary (de Aguiar-Campos et al. 2020). In these ecosystems, population genetics and phylogeographic studies have been conducted in some rupicolous cacti, such as *Praerocerus euchlorus* (F.A.C.Weber ex K.Schum.) N.P.Taylor and the *Pilosocereus auricetus* (Werderm.) Byles & G.D.Rowley complex, which went through population expansion during the Quaternary glacial periods (Moraes et al. 2005; Bonatelli et al. 2014).

Some common characteristics that have been observed in studies of plant genetic variability in rocky outcrop species include moderate genetic diversity within populations, demographically stable populations, and little gene flow between locations (e.g. in *Praerocerus machrissi* Dawson and *P. euchlorus*, Moraes et al. 2005; *Eucalyptus caesia* Benth., Byrne & Hopper 2008; *Acacia woodmaniorum* Maslin & Buscumb, Millar et al. 2017; *Pilosocereus auricetus*, Bonatelli et al. 2014). These demographically stable populations have been maintained for long periods of time suggesting that genetic drift has not been intense in the rocky outcrop populations. In addition, the pattern of isolation by distance between populations is not common, which would suggest an old isolation pattern (Millar et al. 2017). The values of diversity and genetic structure show that the local populations of the species underwent expansion and contraction dynamics, which suggest these outcrops as possible vegetation refuges during the glacial and interglacial periods (Couper & Hoskin 2008; García et al. 2020).

In southern Mexico, on the Isthmus of Tehuantepec, limestone outcrops are found, with associated xerophilous vegetation with a high endemic richness (Pérez-García & Meave 2005; Pérez-García et al. 2009). There are similarities between the rocky outcrops of the Nizanda region in the Isthmus of Tehuantepec and the desert system of North America (for example, the Chihuahua and Sonora deserts; Pérez-García et al. 2010). This similarity makes the Nizanda region an area of biogeographic and evolutionary importance because it is one of the narrowest low-elevation areas that connect the Nearctic and Neotropic biota of North and South America (Nizanda has an average elevation of 140 m a.s.l. and Sierra Madre de Oaxaca, which connects this area, is 3270 m a.s.l.; González-Medrano 1996). In addition, due to its high floristic richness, and its important endemic component it has been proposed that this region may have hosted refuges for Mexican xerophytic flora and limestone outcrops seem good candidate habitats for such refuges (González-Medrano 1996; Pérez-García & Meave 2005; Pérez-García et al. 2010). However, there are no studies on the genetic diversity and the structure or demographic history of any of the species inhabiting these xerophytic enclaves.

In this study, we assessed the population genetics of a dominant and endemic cactus species of these xerophytic enclaves, *Cephalocereus nizandensis* (Bravo & T.MacDoug.) Buxb., Cactaceae. This species grows in limestone outcrops on mountain tops and slopes. It only develops in cavities formed in calcareous rocky outcrops, where organic debris and water accumulate (Bárcenas-Argüello et al. 2010). We hypothesized that the species would display signatures of population expansion processes associated with glacial periods, as observed in similar systems in eastern Brazil (Moraes et al. 2005; Bonatelli et al. 2014), or of long-lasting persistence with isolation, as has been found in other species dwelling on rocky outcrops (e.g. Byrne & Hopper 2008; Millar et al. 2017). In this study we evaluated (1) how much genetic variability this species contains, and how is it distributed, (2) when its populations arose historically, and (3) how its historical demographic dynamic in the middle Pleistocene and in the transition from the Pleistocene to the Holocene.

MATERIAL AND METHODS

Study area

The study area is located in the southern state of Oaxaca, Mexico, in the southeast of the Tehuantepec Isthmus region (fig. 1A, B). The climate is tropical, sub-humid, and highly seasonal. The elevation ranges between 90 and 700 m. The geomorphology is dominated by schist hills abruptly interrupted by exposed limestone outcrops. The geological history of the area shows that this part of Oaxaca was separated in the early Miocene, when the Tehuantepec Fracture caused a significant reduction in highlands (Barrier et al. 1998). The dominant vegetation is tropical dry forest, with considerable savanna areas; but as a result of the edaphic aridification processes facilitated by the limestone outcrops, there are patches of xerophytic shrubs where species such as *Hechtia rosea*, *Comocladia engleriana*, *Pseudosmodingium*

multifolium, *Mammillaria voburnensis*, *M. albilanata*, and *Agave ghiesbreghtii* are common (Pérez-García & Meave 2005). In this region, four isolated patches of *Cephalocereus nizandensis* were chosen for sampling near the towns of Nizanda and La Mata and were named after the closest town (fig. 1C). The coordinates, elevation, and sample sizes are indicated in table 1; each of these localities was considered a population.

Study species

Cephalocereus nizandensis (Bravo & T.MacDoug.) Buxb. (Buxbaum 1965) is a columnar growth cactus, rarely branched, and approximately 3 m in height and 15 cm in diameter. It is characterized by a woolly, differentiated reproductive zone in the apex, called pseudocephalium (Bravo-Hollis & Sánchez-Mejorada 1978; Anderson 2001). This species is micro-endemic to the Nizanda region of Oaxaca, Southern Mexico. It only develops in a few xerophytic enclaves, restricted to a few isolated populations on the peaks and shoulders of an exposed limestone corridor, narrower than 10 km, where the soil is barely a shallow accumulation of organic material inside some cracks (Pérez-García & Meave 2005; Bárcenas-Argüello et al. 2010). These cacti grow both in xerophytic shrubs and tropical dry forest on rocks. However, they are more abundant in xerophytic shrubs, where they are the densest species of the high stratum (Pérez-García & Meave 2005).

Sampling and production of genetic data

Approximately 100 g of photosynthetic tissue per individual was obtained. Only reproductive individuals were sampled, and 1.5 m were left between sampled individuals. Tissue was stored at -80°C until DNA extraction, which was performed using a DNeasy Plant MiniKit (Qiagen, Hilden, Germany). To explore variability in the chloroplast genome, two intergenic regions of chloroplast DNA (cpDNA) were amplified, *rpl32-trnL*[(UAG)] (Shaw et al. 2007) and *trnT-trnL* (Taberlet et al. 1991). The amplification program was as follows: 60 s of initial denaturation at 94°C; 15 s of denaturation at 94°C, 15 s of alignment with temperatures according to supplementary file 1; 15 s extension at 72°C; 30 cycles were used for *rpl32-trnL* region and 40 for *trnT-trnL*. A single sample of both *Cephalocereus apicicephalium* E.Y.Dawson and *Cephalocereus totolapensis* (Bravo & T.MacDoug.) Buxb. were collected and included in the amplification (samples were collected on highway 190 Juchitán-Oaxaca, near the town of Santa María Jalapa del Marqués). Both species are in the same clade as *C. nizandensis* (Bravo-Hollis & Sánchez-Mejorada 1978) and were included as outgroup. The cpDNA PCR products were purified and sequenced by Macrogen, Inc. (Seoul, South Korea). All amplified samples were sequenced in the forward direction, and two individuals per population were sequenced in the reverse direction to verify the sequences.

The sequences were aligned in MUSCLE software (Edgar 2004) and edited in MEGA (Kumar et al. 2016). Sequence ends, 5' and 3', were trimmed enough to avoid the introduction of spurious variability and the variable sites were contrasted against the electropherograms.

Regarding the nuclear genome, a set of nine ISSR primers was tested (in this case, samples of *C. apicicephalium* and *C. totolapensis* were not included). Of these primers, the five that showed variability and reproducibility were used (supplementary file 1). PCRs were carried out in a 25 μL volume for 30 cycles. DNA polymerase Taq Mastermix (RADIANT; Alkali Scientific Inc., Pompano Beach, FL, USA) was used according to the manufacturer's instructions. For ISSR amplification, a touchdown PCR was performed. The starting temperature varied from 54 °C to 56 °C, depending on the primer used (supplementary file 1), and the temperature was reduced by 1 °C in the first nine cycles until it reached 45 °C to 47 °C. ISSR products were separated using horizontal electrophoresis in a 1.5% agarose gel with Tris-Borate-EDTA buffer and visualized using GelRed (Biotium, CA, USA) intercalating agent in a UV light imaging system, Mini-Bis Pro (DNR Bio Imaging Systems, Neve Yamin, Israel), to create a presence/absence database where each band represented a locus. A 100 bp DNA ladder (Omega Bio-TEK M01-02, Atlanta, GA, USA) was used to determine the size of the bands.

Genetic diversity and structure

Genetic variability in cpDNA was estimated as nucleotide diversity (π), number of haplotypes (h), and haplotype diversity (H_d), which were obtained using the DnaSP v.5.0 software (Librado & Rozas 2009). Haplotypes were projected on a map to observe their geographic distribution. To infer the relationships between them, a network of haplotypes was constructed using a median joining algorithm in Network v.4.6.1.3 (Bandelt et al. 1999; Fluxus Technology 2014) including the haplotypes of *C. apicicephalium*, *C. totolapensis*, *C. columna-trajani* (Karw. ex Pfeiff.) K.Schum. (KY624720.1), and *Carnegiea gigantea* Britton & Rose (KY624725.1), the last two species only for the sequence *rpl32-trnL*, which were downloaded from GenBank, to root the network. ISSR variability was explored using the expected heterozygosity (H_e), Shannon diversity index (I), and percentage of polymorphic loci (% P) obtained using POPGENE v.1.32 (Yeh et al. 1997).

The genetic structure of *C. nizandensis* was assessed for both marker types using analysis of molecular variance (AMOVA) and paired F_{ST} in Arlequin v.3.5.5 (Excoffier & Lischer 2010). The AMOVA was initially used to test for differences between all populations, considering these as part of the same hierarchical level, and later, regionalization was tested according to the paired F_{ST} results. In addition, Bayesian clustering was used to assess the genetic clustering of individuals without a priori consideration of geographic location. BAPS v.6.0 (Corander et al. 2003) and STRUCTURE v.2.3 (Pritchard et al. 2000) were used for cpDNA and ISSR markers, respectively. BAPS was used to test for K = 2 to K = 6 cpDNA groups with 10,000 iterations, 10% burn-in period, and 10 replicates. STRUCTURE was used to test for 1 to 10 ISSR groups with 50,000 iterations, 10% burn-in period, and 20 replicates for each K. Default values were used for all other parameters. The most likely number of groups K assigned by BAPS was determined with the posterior probability distribution (Corander et al. 2003) and the most likely K for STRUCTURE analysis

Table 1 – *Cephalocereus nizandensis* populations and diversity indices. Populations latitude (Lat.), longitude (Long.), elevation (Elev.) in meters above sea level (m a.s.l.). Plastid variability (*rpl32-trnL/trnT-trnL* regions): number of samples (N), number of haplotypes (h), haplotypic diversity (H_d), standard deviation (SD), and nucleotide diversity (π). ISSRs variability: number of loci (L), percentage of polymorphic loci (% P), expected heterozygosity (H_e), and Shannon diversity index (I).

Populations	Lat.	Long.	Elev. (m a.s.l.)	cpDNA				ISSR				
				N	h	H_d SD	π SD	N	L	% P	H_e SD	I SD
M1	16.617°	-94.972°	120	8	2	0.25 0.18	0.0007 0.0005	10	55	71.43	0.251 0.165	0.377 0.265
M2	16.609°	-94.961°	89	11	5	0.62 0.16	0.0015 0.0006	12	47	58.44	0.211 0.165	0.314 0.292
N1	16.666°	-95.007°	185	12	6	0.8 0.1	0.0009 0.0002	12	56	68.83	0.235 0.165	0.354 0.276
N2	16.668°	-95.01°	177	9	3	0.42 0.19	0.0011 0.0005	11	49	61.04	0.202 0.165	0.324 0.287
Total				40	14	0.75 0.07	0.0013 0.0003	45	77	98.7	0.296 0.165	0.45 0.165

was estimated using the Evanno et al. (2005) method in STRUCTURE HARVESTER (Earl & von Holdt 2012) and plotted with DISTRUCT v.1.1 (Rosenberg 2004).

Phylogenetic inference and divergence time

Bayesian inference was used to estimate the phylogenetic relationships (Suchard et al. 2018) of the *rpl32-trnL/trnT-trnL* sequences. The ingroup comprised 14 sequences corresponding to the *C. nizandensis* haplotypes, the outgroup was the same as for the Network analysis.

Before doing the phylogenetic reconstruction and with the sequences already aligned, the nucleotide substitution model was defined in the program MEGA v.7.0 (Kumar et al. 2016). In this case, the model with the lowest value of the Bayesian information criterion (BIC) was the HKY (Hasegawa et al. 1985) with a gamma distribution and the shape parameter a = 0.05 (Kumar et al. 2016).

Phylogenetic reconstruction was performed in BEAST v.1.10.4 along with the associated software suite (Drummond et al. 2018) as described below. First, in the BEAUTi v.1.10.4 program (Drummond et al. 2018) the following information was defined: the evolutionary model already mentioned HKY + gamma (a = 0.05) with a substitution rate of 1.1×10^{-9} to 1.6×10^{-9} substitutions per site per year (Wolfe et al. 1987) with a strict molecular clock. The divergence time of the Pachycereinae subtribe (5.28 Mya (95% HPD 7.74–3.47); Hernández-Hernández et al. 2014), to which the genera *Cephalocereus* and *Carnegiea* belong, was used as a calibration point. The length of the Markov chain was defined as 20,000,000 steps sampled every 1,000 steps. The output file of BEAUTi was used to run BEAST program, and the results of the Markov chains generated in BEAST were visually verified in Tracer v.1.7.1 (Rambaut et al. 2018). Additionally, BEAST produces an output file with a set of phylogenetic trees that is summarized in the program TreeAnnotator v.1.10.4 (Rambaut & Drummond 2018), this program finds the best supported tree, with a burn-in of 10%. Using this tree, it was possible to generate information of the posterior probabilities of the nodes, and the HPD (highest posterior density) limits of the node heights. Lastly, the best supported tree was drawn and edited in FigTree v.1.4.4. (Rambaut 2018).

Demographic history based on cpDNA

The demographic history was investigated using four indices. (1) We used F_s neutrality tests (Fu 1997), whose negative values indicate a recent increase in population size under the assumption of neutrality. (2) Using the mismatch distribution analysis (Rogers & Harpending 1992), we calculated the sum of squared differences (SSD) between the observed and the expected mismatch distributions, and (3) Harpending's Raggedness Index (R). These indices take large values for multimodal distributions commonly found in stationary populations, whereas unimodal and smoother distributions are typical of expanding populations (Rogers & Harpending 1992). These analyses were performed using Arlequin v.3.5.5 (Excoffier & Lischer 2010). (4) Historical demographic processes were also inferred by Bayesian Skyline Plot analysis (BSLP), implemented in BEAST (Drummond et al. 2018), which indicates changes in effective population size over time (Drummond et al. 2005). A strict molecular clock was used, with a mutation rate of 1.1×10^{-9} to 1.6×10^{-9} substitutions per site per year (Wolfe et al. 1987) and an MCMC chain with 10,000,000 replicates sampled every 1,000 steps, with a burn-in of 10%.

RESULTS

Genetic diversity and structure

After concatenation and editing, the sequences obtained were 1492 bp in length (523 bp for *trnT-trnL* and 969 bp for *rpl32-trnL*) and 14 haplotypes were found (supplementary file 2). The number of haplotypes per population ranged from two in M1 to six in N1 (fig. 1C; table 1). All haplotypes were private, except for the H02 haplotype, which was only absent

from the M2 population and was found in 18 individuals (48% of the sample). The H04 haplotype was the second most common and was found in 8 individuals (20%), all from population M2, but it was also found in two individuals of *C. apicicephalium* and *C. totolapensis* tested as the outgroup. The H09 haplotype was found in 3 individuals (7.5%) and was private to the N2 population. The rest of the haplotypes were only found in a single individual (fig. 1C).

In the median joining network, most haplotypes are closely related, with one mutational step separating them. Haplotypes from the same or a nearby population were located close to each other in the network, except for the H03 and H06 haplotypes (fig. 1D). Abundance, distribution and connectivity of the H02 haplotype are characteristic of population expansion; this haplotype occupies a central position in the network, with multiple connections with haplotypes of the N1 (H09, H10, and H12) and M2 (H04) populations. Two groups outside the network centre are distinguished: one represents the H01, H05, and H06 haplotypes, found in the M1 and M2 populations, and the other group is formed by the haplotypes H03, H08, H11,

Figure 1 – Location of the *Cephalocereus nizandensis* populations in southern Mexico (A–B), haplotype distribution (C), and haplotype network (D). **A–B**. Maps indicating the location of the study area. **C**. Pie graphic portions are proportional to the abundance of a haplotype within each population; elevation is given in metres above sea level (m a.s.l.). **D**. The graphic represents the relations of the *C. nizandensis* *rpl32-trnT/trnT-trnL* haplotypes. Circles are proportional to the abundance of the haplotypes; lines perpendicular to connections between haplotypes indicate mutational steps; red dots indicate extinct (ext) or not represented haplotypes. Haplotype H04 includes the sequence of species *C. apicicephalium* and *C. totolapensis*. Numbers I–IV refer to the BAPS clusters according to fig. 2. Ccol = *C. columna-trajani*, Cgig = *Carnegiea gigantea*.

H12, H13, and H14, all of which are from the N1 and N2 populations (fig. 1D).

Cephalocereus nizandensis has moderately high levels of genetic diversity. Twelve polymorphic sites were found in the cpDNA sequences, of which six were parsimony informative. The total nucleotide diversity (π) was 0.0013; it varied from 0.0007 in M1 to 0.0015 in M2, and the total haplotype diversity was 0.75 (0.8 in M2 to 0.25 in M1). Seventy-seven ISSR loci were found in total: 76 (98.7%) were polymorphic in at least one population, and 19 loci (14.63%) were private. Both the expected heterozygosity and Shannon diversity index varied little between populations ($H_e = 0.296$; $I = 0.45$; table 1).

The populations are genetically differentiated. Most of the variability lay within populations, although there was significant genetic differentiation among them, both for the chloroplast genome ($F_{ST} = 0.209$; $p < 0.05$) and for the nuclear genome ($F_{ST} = 0.186$, $p < 0.005$; supplementary file 3). Paired F_{ST} indicated that for the cpDNA, populations M1, N1, and N2 showed no differentiation between each other, while the only comparisons significantly different from zero involved population M2: M2 - M1 $F_{ST} = 0.252$, $p < 0.05$; M2 - N1 $F_{ST} = 0.343$, $p < 0.05$; M2 - N2 $F_{ST} = 0.313$, $p < 0.05$ (supplementary file 4). ISSRs revealed significant differences between all pairs of populations (supplementary file 4). BAPS recovered four genetic groups of cpDNA
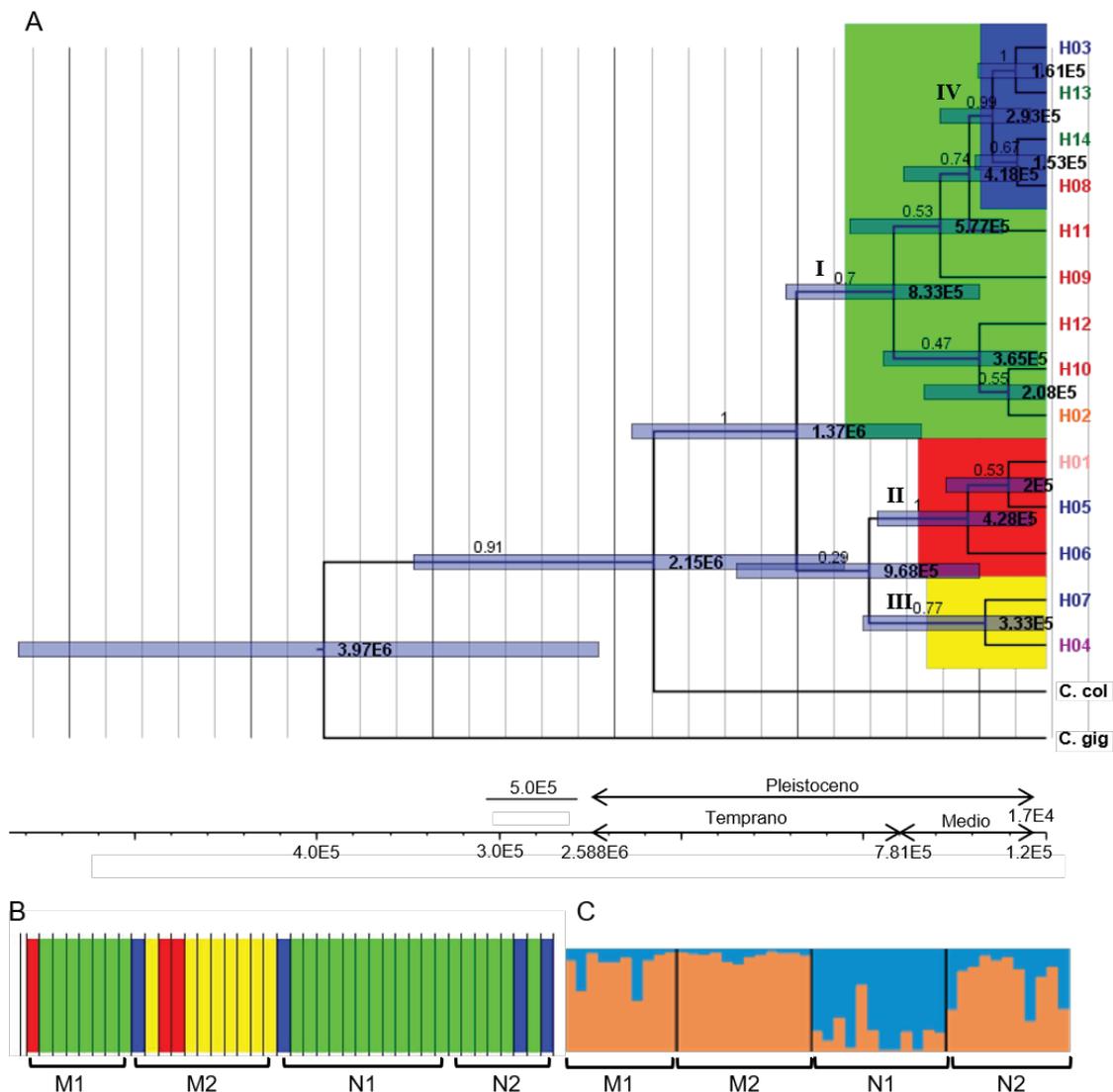

Figure 2 – *Cephalocereus nizandensis* haplotype chronogram (A) and genetic clusters (B and C). **A.** Bayesian inference chronogram. The estimated divergence time for the *rpl32-trnL/trnT-trnL* sequences is illustrated. Posterior probability (on the branches); branch age, (bold text next to nodes), 95% highest posterior density interval (blue bars). Haplotype H04 includes the sequence of species *C. apicicephalium* and *C. totolapensis*. Ccol = *C. columna-trajani*, Cgig = *Carnegiea gigantea*. The boxes correspond to the genetic groups of shown in B in this figure, and the colours of the haplotypes correspond to fig. 1. **B–C.** Genetic groups. *rpl32-trnL/trnT-trnL* clusters obtained with BAPS (**B**) and ISSR clusters obtained with STRUCTURE (**C**), each bar represents an individual, and the colour indicates the probability of an individual belonging to a given group.

Table 2 – *Cephalocereus nizandensis* neutrality tests and mismatch distribution analysis. Fu's neutrality test (F_s) and analysis of mismatch distribution for demographic and spatial expansion: distribution of observed number of differences between pairs of haplotypes (Dist), there are two types of distribution: unimodal (U) or multimodal (M); the sum of squared differences between the observed and the expected mismatch distributions (SSD); the Harpending's Raggedness Index (R).

	F_s	Demographic			Spatial		
		Dist	SSD	R	Dist	SSD	R
M1	0.9714	M	0.309	0.228	M	0.39	0.228
M2	-0.194	M	0.04	0.148	M	0.025	0.148
N1	-2.594	U	0.015	0.14	U	0.015	0.14
N2	1.304	M	0.326	0.644	M	0.099	0.644
Total	-7.224	U	0.214	0.043	U	0.007	0.043

which did not coincide with geographic populations because haplotypes were shared between populations; M2 was somewhat more distinct (fig. 2B). STRUCTURE recovered only two genetic groups with the ISSRs (fig. 2C); N1 is the most distinct population and in the analysis of the pairwise F_{ST} indicated that the most genetically different individuals were found in this population (fig. 2), all the comparisons with this population were the highest (supplementary file 4: N1 - M1 F_{ST} = 0.192, p < 0.05; N1 - M2 F_{ST} = 0.238, p < 0.05; N1 - N2 F_{ST} = 0.176, p < 0.05).

Phylogenetic inference and divergence time

Bayesian analysis (fig. 2A) showed a first divergence in the root of the tree where the genus *Cephalocereus* (with a posterior probability of PP = 0.91) is separated from *Carnegiea gigantea* 3.97 Mya (95% HPD 5.88–2.59). Next, in a clade with a posterior probability of PP = 1.0, *C. nizandensis*, *C. totolapensis*, and *C. apicicephalium* (sharing the H04 haplotype) were grouped and separated from *C. column-trajani* 2.15 Mya (95% HPD 3.47–1.11) in the Early Pleistocene. We can also see in the tree that haplotypes found in this work formed a clade with high statistical support (PP = 1.0). Subsequently, two clades were formed dated to 1.37 Mya (95% HPD 2.27–0.686), the first contains the haplotypes

of group I and IV with a PP = 0.7; most of the haplotypes of the M1, N1, and N2 populations formed clade I. The nesting of the N2 haplotypes (H13 and H14) with respect to the H09 and H11 haplotypes of N1 suggests a possible colonization route in the N1 to N2 direction, probably caused by a population expansion that started approximately 0.577 Mya (95% HPD 1.08–0.234) and culminated with the differentiation of clade IV. The second clade had low statistical support (PP = 0.29) and have haplotypes of groups II and III which belong to the populations M1 and M2 which diverged 0.968 Mya (95% HPD 1.7–0.363).

Historic demography based on cpDNA

Our results suggest that *C. nizandensis* has undergone a process of population expansion, which was mainly registered in the N1 population, while other populations such as M1 showed demographic stability. The neutrality test, Fu's F_s, indicated population expansion for the whole species (F_s = -7.224, p < 0.001) and for the N1 population (F_s = -2.594, p = 0.011; table 2). The mismatch distribution analysis for the entire species' dataset fits the spatial expansion, but there were significant differences from the demographic expansion model. At the population level, N1 was the only population that displayed a signature of spatial expansion (table 2). In the same sense, the Bayesian Skyline Plot (BSLP) showed population expansion from 150,000 years to the present, which increased the effective population size from N_e = 100,000 to N_e = 1,000,000 individuals, without evidence of contraction for the last 250,000 years (fig. 3).

<center>DISCUSSION</center>

Genetic diversity and structure

Cephalocereus nizandensis' genetic variability levels are within values registered for other cacti, but this cactus is distributed in a small area with few populations. The number of cpDNA haplotypes reported for cacti is variable (cpDNA of 11 species of the Cactoideae subtribe; table 3), ranging from 2 to 23. It is important to highlight that the number of haplotypes found in *C. nizandensis* (14) is close to the average, although almost all of these haplotypes have a restricted distribution to only one of the four sampled populations. The haplotype diversity in *C. nizandensis* (H_d = 0.75) is considerably higher than that in other cacti
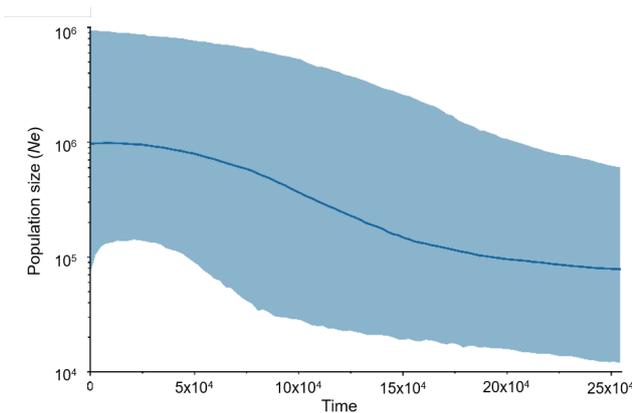

Figure 3 – *Cephalocereus nizandensis* Bayesian skyline plot analysis of historic demography based on cpDNA. Mean effective population size (dark blue line) and 95% confidence interval (light blue area) for the effective population size (N_e) with time in years before present.

Table 3 – Genetic diversity and structure in some cactus species. Studies that assessed population genetics in species of the Cactoideae subtribe: sample size (N), sequence length or number of loci (P_b/NL), differentiation (F_{ST}), number of haplotypes (h), haplotype diversity (H_d), nucleotide diversity (π), expected heterozygosity (H_e), Shannon diversity index (I), and percentage of polymorphism (% P). *Pilisocereus aurisetus* is a complex of eight morphologically similar cactus species.

Species	Markers	N	P_b/NL	F_{ST}	h	H_d	π	References
Pilisocereus aurisetus complex*	*trnT-trnL/trnS-trnG*	157	1464		22		0.0030	Bonatelli et al. (2014)
Cephalocereus columna-trajani	*psbA-trnH/trnT-trnL*	110		0.124	6	0.188	0.0002	Cornejo-Romero et al. (2017)
Cereus hildmannianus	*trnQ-50rps16/psbJ-petA*	119			23	0.55	0.0020	Silva et al. (2018)
Echinopsis terscheckii	*trnH-psbA/psbM-trnD/ trnS-trnG*	48	1247		2	0.507	0.0035	Quipildor et al. (2017)
Average					**13.25**	**0.415**	**0.0022**	

Species	Markers	N	P_b/NL	F_{ST}	H_e	I	% P	
Neobuxbaumia macrocephala	RAPD	84	130	0.106		7.064		Alejos-Velázquez (2002)
Neobuxbaumia mezcalaensis	RAPD	85	128	0.081	0.21	5.853	97.24	Rivera-Montoya (2003)
Stenocereus stellatus	RAPD	155	75	0.106	0.29	0.247	78.97	Cornejo-Romero (2004)
Stenocereus eruca	RAPD	120	75	0.337	0.277		76	Clark-Tapia et al. (2005)
Cephalocereus columna-trajani	RAPD	102	142	0.288	0.31	6.648	60	Vazquez-Montiel (2005)
Cephalocereus totolapensis	RAPD	90	30	0.0008	0.233		67.78	Palleiro-Dutrenit (2008)
Average			**96.67**	**0.15**	**0.26**	**4.95**	**76.00**	
Stenocereus thurberi	ISSR	325	99	0.195	0.207		66.7	Bustamante et al. (2016)
Ferocactus histrix	ISSR	134	27		0.178	0.271	56	Castro-Felix et al. (2014)

(average of 0.415), and its nucleotide diversity ($\pi = 0.0013$) is lower than the average ($\pi = 0.0022$). However, its π values are similar to or higher than those reported in studies that evaluated the same cpDNA regions (Gitzendanner & Soltis 2000; Clark-Tapia & Molina Freaner 2003; Bonatelli et al. 2014; Ornelas & Rodríguez-Gómez 2015; Cornejo-Romero et al. 2017). It should be noted that *C. nizandensis* is as diverse as other *Cephalocereus* species. Nucleotide diversity in *C. nizandensis* was greater than that in *Cephalocereus columna-trajani* ($\pi = 0.00023$; Cornejo-Romero et al. 2017).

The expected heterozygosity in *C. nizandensis* ($H_e = 0.296$) was similar to that found for RAPD and ISSR markers of nine cactus species (average $H_e = 0.26$) and slightly higher than that in *C. totolapensis* ($H_e = 0.233$; Palleiro-Dutrenit 2008); the percentage of polymorphism (98.6%) was higher than the average (76% in RAPD), greater than that in *C. totolapensis* as well (67.8%; Palleiro-Dutrenit 2008) and similar to that reported in *Neobuxbaumia mezcalaensis* (Bravo) Backeb. (97.2%, table 3; Rivera-Montoya 2003; Tapia et al. 2017).

High genetic diversity is common in columnar cacti (Pachyceerinae and Stenoceerinea tribes; Hamrick et al. 2002; Palleiro-Dutrenit 2008; Cornejo-Romero et al. 2014), even in species with restricted or patchy distributions, such as *C. nizandensis* (Ledig et al. 1999, 2001; Moraes et al. 2005; Figueredo et al. 2010; Bonatelli et al. 2014; Khan et al. 2018; Silva et al. 2020). This diversity has been linked to common life history traits in this group, such as long-life cycles and bat- and bird-mediated pollination (Valiente-Banuet et al.

2002) or self-incompatibility (Mandujano et al. 2010). These traits reduce genetic drift and inbreeding effects, favouring outbreeding. This reinforces the notion that within the four *C. nizandensis* populations, genetic drift has had little effect on genetic diversity (Ellstrand & Elam 1993; Hamrick & Godt 1996).

The study shows that *C. nizandensis* presents moderate but significant genetic differentiation, which may be due to an early divergence among its populations. cpDNA pairwise F_{ST} analyses indicated that the most genetically different individuals were found in the M2 population (fig. 2B), which is also found in the lowest elevation location. Environmental differences, such as humidity, temperature or places to germinate, could explain a pattern of local adaptation of plants in different sites (Hamrick et al. 2002; Valiente-Banuet et al. 2002; Nassar et al. 2003; Gutiérrez-Flores et al. 2016), even giving rise to natural selection processes according to the microclimatic conditions of each population (Kim & Donohue 2013). Similarly, the genetic structure of *Stenocereus stellatus* Riccob. has been found to be determined by the evolutionary processes of the landscape, as this determines the recruitment dynamics and gene flow restriction in seeds (Cornejo-Romero 2004; Bustamante et al. 2016). To confirm the existence of local adaptation, it would be necessary to perform specific studies aimed at examining phenotypic differences, such as germination, and/or the genes subjected to natural selection, as well as the measurement of microclimatic variables, that may explain the differentiation (Ohsawa & Ide 2008; Bustamante et al. 2016).

Divergence time and demography history

Cephalocereus genus is divided into two subgenera: *Neodawsonia* (including three species; *C. totolapensis*, *C. apicicephalium,* and *C. nizandensis*) and *Cephalocereus* (including two species *C. senilis* Pfeiff. and *C. columna-trajani*) (Bravo-Hollis & Sánchez-Mejorada 1978), and it is probable that these subgenera diverged approximately 2.5 Mya (95% HPD 3.06–1.24; fig. 2A), during the transition from the Pliocene to the Pleistocene. During this period, the Late Pliocene climate optimum (3.3–2.4 Mya; Tripati et al. 2009) has been related to the most recent radiation of succulent plants (Good-Avila et al. 2006; Arakaki et al. 2011). Recent diversification is a common characteristic in cacti (Arakaki et al. 2011; Hernández-Hernández et al. 2014), as observed in *Pilosocereus* Byles & G.D.Rowley (Perez et al. 2016) and *Cereus* Mill. (Franco et al. 2017; Silva et al. 2020), both genera of South American deciduous tropical forests. The recent divergence time of *C. nizandensis* may explain the retention of ancestral haplotype H04 found in the M2 population of *C. nizandensis*, *C. apicicephalium*, and *C. totolapensis* (figs 1D, 2A)*.* This haplotype could have been lost in the other populations of *C. nizandensis* due to founder events (Ellstrand & Elam 1993).

Other divergences within *C. nizandensis* were estimated to be approximately 0.968 Mya (95% HPD 1.7–0.363) and 0.833 Mya (95% HPD 1.43–0.362; fig. 2A). This period coincides with the most arid stage of the Middle Pleistocene (Russo-Ermolli & Cheddadi 1997). It must be considered that, in recent divergence processes, genetic divergence usually precedes population divergence (Arbogast et al. 2002), which suggests that the Quaternary climate cycles could have influenced the structure of the species (Nistelberger et al. 2015). These estimates precede the climatic events of the last glacial cycle (Last Glacial Maximum LGM, 0.021 Mya), which have been used to explain the population dynamics and genetic structure of plants in arid and semi-arid environments at different latitudes (Nason et al. 2002; Bonatelli et al. 2014; Cornejo-Romero et al. 2017; Quipildor et al. 2017).

On the other hand, Silva et al. (2020) suggested that the Early Quaternary climatic events were more important in the diversification and population divergence of plants from open vegetation, at least in South America. However, the region where *C. nizandensis* is distributed has presented changes in the composition of the vegetation during the Late Quaternary (Gámez et al. 2014), although the inferred demographic expansion for *C. nizandensis* appears to be independent of these climatic fluctuations (fig. 3). An explanation for this may be that despite the altitudinal change in the elements of the tropical dry forest that surround the xerophytic enclaves, population expansion events of *C. nizandensis* were restricted to rocky outcrops. A similar case has been reported in Australia, where several species from the surrounding landscape show distribution changes, while the endemic elements of rocky outcrops have historically stable demographics (Byrne 2008; Broadhurst et al. 2017). The stability of rocky habitats, which reaches hundreds of thousands of years, has been used to explain the demographic stability and accumulation of genetic variability in other species of restricted distribution such as *Stypandra glauca* R.Br. (Tapper et al. 2017) and this is most likely what has happened in *C. nizandensis*.

Conservation considerations

Maintaining genetic diversity is essential for the conservation of species, as this enables the long-term viability of populations and survival in response to environmental changes, disease resistance, and chances of survival in general (Toro & Caballero 2005). Isolated populations are much more prone to extinction; however, a high dispersal capacity or colonization processes from different sources can reduce the probability of extinction (Frankham 1997). The restricted and fragmented distribution of *C. nizandensis* does not seem to be a limiting factor for its conservation, as population densities and genetic connectivity, together with its life history characteristics, have allowed it to sustain a high effective population size that mitigates the effects of genetic drift. Therefore, it maintains moderate to high levels of genetic diversity, and its population has increased in the last thousands of years, as shown in this work.

Despite the fact that populations of *C. nizandensis* naturally show a population expansion, their restricted distribution makes these cacti susceptible to anthropogenic effects due to habitat destruction. Even though the soil where they grow is not functional for agriculture, their populations have suffered from the burning of the nearby forest for the creation of paddocks. Other risks observed were rock extraction and pruned plants (Aldo Isaac Juárez-Miranda pers. obs.). Another danger for this microendemic species is the Transisthmic railway expansion, since there are populations described on both sides of the railway (Bravo-Hollis & Sánchez-Mejorada 1978).

Thus, one possible conservation strategy is to name the highest area where the rocky enclaves are located, a reserve of diversity. This area is not in Mexico's priority protected area, so it is important to include it (Arriaga et al. 2000). It is worth highlighting the tourist attraction and the unique landscape beauty of these "succulent rock gardens" and how this can help preserve these ecosystems similar to the N1 population that shows the highest degree of conservation and least damage, being located in proximity of the tourist trails signposted from the town of Nizanda. Additionally, the M2 population could represent a unique and important conservation unit due to its genetic difference, in terms of some haplotypes. However, given that this species has very few localities, it is recommended to preserve the largest number of populations to achieve effective and efficient conservation strategies (Pezoa 2001).

CONCLUSION

The species that inhabit rocky outcrops are of great interest either because of their restricted distribution or because of their adaptations to these arid environments (Crowther 1982; Porembski 2007). In some cases, rocky outcrops have been shown to be buffers of short- and long-term environmental changes that also act as filters for species unadapted to these areas (Porembski 2007). We can think the outcrop habitats have served as ecological refuges for certain species, as can

be the case of *C. nizandensis* that has expanded its population in the past 150,000 years (Porembski & Barthlott 2000; Pérez-García & Meave 2005; Porembski 2007; Couper & Hoskin 2008). These sites still provide the opportunity to ask unexplored questions, from the point of view of population genetics (Torres-Ribeiro et al. 2007).

In Mexico, the investigation of rocky outcrops is still incipient, and in reality, there is no research on population genetics or phylogeography in species that inhabit outcrops. As far as we know, this is the first work to address this question. Therefore, it would be an important approach that can be used as a model for other species living in these enclaves, such as *Agave nizandensis* Cutak (Agavaceae) which is also associated with rocky substrates in the Nizanda region (Pérez-García 2002; Pérez-García & Meave 2005).

SUPPLEMENTARY FILES

Supplementary file 1 – Primers used for *Cephalocereus nizandensis*.

https://doi.org/10.5091/plecevo.2021.1773.2437

Supplementary file 2 – GenBank accession numbers for *Cephalocereus nizandensis*.

https://doi.org/10.5091/plecevo.2021.1773.2439

Supplementary file 3 – Analysis of molecular variance.

https://doi.org/10.5091/plecevo.2021.1773.2441

Supplementary file 4 – Pair-wise population genetic differentiation based on F_{ST}.

https://doi.org/10.5091/plecevo.2021.1773.2443

Supplementary file 5 – ISSR database for *Cephalocereus nizandensis*.

https://doi.org/10.5091/plecevo.2021.1773.2445

DATASETS

Dataset of cpDNA is available on GenBank as mentioned in supplementary file 2 and ISSR database in supplementary file 5.

ACKNOWLEDGEMENTS

AUTHOR CONTRIBUTIONS

A.I. Juárez-Miranda, A. Cornejo-Romero, and C.F. Vargas-Mendoza conceived, design, and completion fieldwork. A.I. Juárez-Miranda and A. Cornejo-Romero performed laboratory work. A.I. Juárez-Miranda, A. Cornejo-Romero, and C.F. Vargas-Mendoza did the analysis and interpretation of the results. A.I. Juárez-Miranda and C.F. Vargas-Mendoza wrote the manuscript.

REFERENCES

Alejos-Velázquez L.P. 2002. Estudio de la estructura poblacional de *Neobuxbaumia macrocephala* en Tehuacán-Cuicatlán, Puebla, mediante el uso de marcadores moleculares. Bachelor thesis. Facultad de Estudios Superiores Iztacala, UNAM, México. Available from http://132.248.9.195/ppt2002/0309463/Index.html [accessed 29 Mar. 2021].

Anderson E.F. 2001. The cactus family. Timber Press, Portland, USA.

Arakaki M., Christin P.A., Nyffeler R., et al. 2011. Contemporaneous and recent radiations of the world's major succulent plant lineages. *Proceedings of the National Academy of Sciences of the United States of America* 108: 8379–8384. https://doi.org/10.1073/pnas.1100628108

Arbogast B.S., Edwards S.V., Wakeley J., Beerli P. & Slowinski J.B. 2002. Estimating divergence times from molecular data on phylogenetic and population genetic timescales. *Annual Review of Ecology and Systematics* 33: 707–740. https://doi.org/10.1146/annurev.ecolsys.33.010802.150500

Arriaga L., Espinoza J.M., Aguilar C., Martínez E., Gómez L. & Loa E. 2000. Regiones terrestres prioritarias de México. Comisión Nacional para el Conocimiento y uso de la Biodiversidad, México.

Bandelt H.J., Forster P. & Röhl A. 1999. Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution* 16: 37–48. https://doi.org/10.1093/oxfordjournals.molbev.a026036

Bárcenas-Argüello M.L., Gutiérrez-Castorena M.C., Terrazas T. & López-Mata L. 2010. Rock-soil preferences of three *Cephalocereus* (Cactaceae) species of tropical dry forests. *Soil Science Society of America Journal* 74: 1374–1382. https://doi.org/10.2136/sssaj2009.0310

Barrier E., Velesquillo L., Chávez M. & Goulon R. 1998. Neotectonic evolution of the Isthmus of Tehuantepec (southeastern Mexico). *Tectonophysics* 287: 77–96. https://doi.org/10.1016/S0040-1951(98)80062-0

Bonatelli I.A.S., Perez M.F., Peterson A.T., et al. 2014. Interglacial microrefugia and diversification of a cactus species complex: phylogeography and palaeodistributional reconstructions for *Pilosocereus aurisetus* and allies. *Molecular Ecology* 23: 3044–3063. https://doi.org/10.1111/mec.12780

Bravo-Hollis H. & Sánchez-Mejorada R. 1978. Las cactáceas de México, Vol. I. Second edition. UNAM, México.

Broadhurst L., Breed M., Lowe A., et al. 2017. Genetic diversity and structure of the Australian flora. *Diversity and Distribution* 23: 41–52. https://doi.org/10.1111/ddi.12505

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

Byrne M. 2008. Evidence for multiple refugia at different time scales during Pleistocene climatic oscillations in southern Australia inferred from phylogeography. *Quaternary Science Reviews* 27: 2576–2585. https://doi.org/10.1016/j.quascirev.2008.08.032

Byrne M. & Hopper S.D. 2008. Granite outcrops as ancient islands in old landscapes: evidence from the phylogeography and population genetics of *Eucalyptus caesia* (Myrtaceae) in Western Australia. *Biological Journal of the Linnean Society* 93: 177–188. https://doi.org/10.1111/j.1095-8312.2007.00946.x

Byrne M., Krauss S.L., Millar M.A., et al. 2019. Persistence and stochasticity are key determinants of genetic diversity in plants

associated with banded iron formation inselbergs. *Biological Reviews* 94: 753–772. https://doi.org/10.1111/brv.12477

Buxbaum F. 1965. Die Tribus Pachycereae F.Buxb. und ihre Entwicklungswege (Fortsetzung IV). *Kakteen und Andere Sukkulenten* 16(3): 42–45.

Castro-Felix P., Rosas-Espinoza V.C., Díaz-Cardenas B., Pérez-Valencia L.I., Huerta-Martínez F. & Santerre A. 2014. Genetic diversity within a declining natural population of *Ferocactus histrix* (DC) Lindsay. *Plant Species Biology* 29: e21–e30. https://doi.org/10.1111/1442-1984.12028

Clark-Tapia R. & Molina-Freaner F. 2003. The genetic structure of a columnar cactus with a disjunct distribution: *Stenocereus gummosus* in the Sonoran desert. *Heredity* 90: 443–450. https://doi.org/10.1038/sj.hdy.6800252

Clark-Tapia R., Alfonso-Corrado C., Eguiarte L.E. & Molina-Freaner F. 2005. Clonal diversity and distribution in *Stenocereus eruca* (Cactaceae), a narrow endemic cactus of the Sonoran Desert. *American Journal of Botany* 92(2): 272–278. https://doi.org/10.3732/ajb.92.2.272

Corander J., Waldmann P. & Sillanpää M.J. 2003. Bayesian analysis of genetic differentiation between populations. *Genetics* 163: 367–374. Available from https://www.genetics.org/content/163/1/367 [accessed 29 Mar. 2021].

Cornejo-Romero A. 2004. Diversidad genética y estructura clonal de *Stenocereus stellatus* (Cactaceae), en una cronosecuencia edáfica del valle de Tehuacán. MSc thesis, UNAM, Mexico.

Cornejo-Romero A., Medina-Sánchez J., Hernández-Hernández T., et al. 2014. Quaternary origin and genetic divergence of the endemic cactus *Mammillaria pectinifera* in a changing landscape in the Tehuacán Valley, Mexico. *Genetics and Molecular Research* 13: 73–88. https://doi.org/10.4238/2014.January.8.6

Cornejo-Romero A., Vargas-Mendoza C.F., Aguilar-Martínez G.F., et al. 2017. Alternative glacial-interglacial refugia demographic hypotheses tested on *Cephalocereus columna-trajani* (Cactaceae) in the intertropical Mexican drylands. *PLOS ONE* 12(4): e0175905. https://doi.org/10.1371/journal.pone.0175905

Couper P.J. & Hoskin C.J. 2008. Litho-refugia: the importance of rock landscapes for the long-term persistence of Australian rainforest fauna. *Australian Zoologist* 34: 554–560. https://doi.org/10.7882/AZ.2008.032

Crowther J. 1982. Ecological observations in a tropical karst terrain, West Malaysia. I. Variations in topography, soils and vegetation. *Journal of Biogeography* 9(1): 65–78. https://doi.org/10.2307/2844731

de Aguiar-Campos N., Maia V.A., da Silva W.B. de Souza C.R. & dos Santos R.M. 2020. Can fine-scale habitats of limestone outcrops be considered litho-refugia for dry forest tree lineages? *Biodiversity and Conservation* 29: 1009–1026. https://doi.org/10.1007/s10531-019-01923-4

Drummond A.J., Rambaut A., Shapiro B. & Pybus O.G. 2005. Bayesian coalescent inference of past population dynamics from molecular sequences. *Molecular Biology and Evolution* 22: 1185–1192. https://doi.org/10.1093/molbev/msi103

Drummond A.J., Rambaut A. & Suchard M.A. 2018. BEAST. Bayesian evolutionary analysis sampling trees version v1.10.4 Prerelease #bc6cbd9. Available from http://github.com/beast-dev/beast-mcmc [accessed 29 Mar. 2021].

Earl D.A. & von Holdt B.M. 2012. STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output

and implementing the Evanno method. *Conservation Genetics Resources* 4: 359–361. https://doi.org/10.1007/s12686-011-9548-7

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

Ellstrand N.C. & Elam D.R. 1993. Population genetic consequences of small population size: implications for plant conservation. *Annual Review of Ecology and Systematics* 24: 217–242. https://doi.org/10.1146/annurev.es.24.110193.001245

Evanno G., Regnaut S. & Goudet J. 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular Ecology* 14: 2611–2620. https://doi.org/10.1111/j.1365-294X.2005.02553.x

Excoffier L. & Lischer H.E.L. 2010. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources* 10: 564–567. https://doi.org/10.1111/j.1755-0998.2010.02847.x

Figueredo C.J., Nassar J.M., García-Rivas A.E. & González-Carcacía J.A. 2010. Population genetic diversity and structure of *Pilosocereus tillianus* (Cactaceae, Cereeae), a columnar cactus endemic to the Venezuelan Andes. *Journal of Arid Environments* 11: 1392–1398. https://doi.org/10.1016/j.jaridenv.2010.05.020

Fluxus Technology 2014. Free Phylogenetic Network Software. Version 4.6.1.3. Fluxus Technology Ltd. Available from https://www.fluxus-engineering.com/sharenet.htm [accessed 2 Apr. 2021].

Franco F.F., Silva G.A.R., Moraes E.M., et al. 2017. Plio-Pleistocene diversification of *Cereus* (Cactaceae, Cereeae) and closely allied genera. *Botanical Journal of the Linnean Society* 183: 199–210. https://doi.org/10.1093/botlinnean/bow010

Frankham R. 1997. Do island populations have less genetic variation than mainland populations? *Heredity* 78: 311–327. https://doi.org/10.1038/hdy.1997.46

Fu Y.X. 1997. Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. *Genetics* 147: 915–925. Available from https://www.genetics.org/content/147/2/915 [accessed 29 Mar. 2021].

Gámez N., Escalante T., Espinosa D., Eguiarte L.E. & Morrone J.J. 2014. Temporal dynamics of areas of endemism under climate change: a case study of Mexican *Bursera* (Burseraceae). *Journal of Biogeography* 41: 871–881. https://doi.org/10.1111/jbi.12249

Ganopoulos I.K., Xanthopoulou A., Mastrogianni A., Koubouris G. & Madesis P. 2015. Genetic diversity of Barbary fig (*Opuntia ficus-indica*) collection in Greece with ISSR molecular markers. *Plant Gene* 2: 29–33. https://doi.org/10.1016/j.plgene.2015.04.001

García M.B., Domingo D., Pizarro M., Font X., Gómez D. & Ehrlén J. 2020. Rocky habitats as microclimatic refuges for biodiversity. A close-up thermal approach. *Environmental and Experimental Botany* 170: 103886. https://doi.org/10.1016/j.envexpbot.2019.103886

Gibson N., Meissner R., Markey A.S. & Thompson W.A. 2012. Patterns of plant diversity in ironstone ranges in arid south western Australia. *Journal of Arid Environments* 77: 25–13. https://doi.org/10.1016/j.jaridenv.2011.08.021

Gitzendanner M.A. & Soltis P.S. 2000. Patterns of genetic variation in rare and widespread plant congeners. *American journal of botany* 87: 783–792. https://doi.org/10.2307/2656886

González-Medrano F. 1996. Algunos aspectos de la evolución de la vegetación de México. *Botanical Sciences* 58: 129–136. https://doi.org/10.17129/botsci.1493

Good-Avila S.V., Souza V., Gaut B.S. & Eguiarte L.E. 2006. Timing and rate of speciation in *Agave* (Agavaceae). *Proceedings of the National Academy of Sciences of the United States of America* 103: 9124–9129. https://doi.org/10.1073/pnas.0603312103

Gutiérrez-Flores C., García-De León F.J., León-de la luz J.L. & Cota-Sánchez J.H. 2016. Microsatellite genetic diversity and mating systems in the columnar cactus *Pachycereus pringlei* (Cactaceae). *Perspectives in Plant Ecology, Evolution and Systematics* 22: 1–10. https://doi.org/10.1016/j.ppees.2016.06.003

Hamrick J.L. & Godt M.J. 1996. Effects of life history traits on genetic diversity in plant species. *Philosophical Transactions of the Royal Society B: Biological Sciences* 351: 1291–1298. https://doi.org/10.1098/rstb.1996.0112

Hamrick J.L., Nason J.D., Fleming T.H. & Nassar J.M. 2002. Genetic diversity in columnar cacti. In: Fleming T.H. & Valiente-Banuet A. (eds) Columnar cacti and their mutualists: evolution, ecology, and conservation: 93–108. University of Arizona Press, Tucson, USA.

Hasegawa M., Kishino H. & Yano T. 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. *Journal of Molecular Evolution* 22: 160–174. https://doi.org/10.1007/BF02101694

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

Khan G., Ribeiro P.M., Bonatelli I.A.S., Perez M.F., Franco F.F. & Moraes E.M. 2018. Weak population structure and no genetic erosion in *Pilosocereus aureispinus*: a microendemic and threatened cactus species from eastern Brazil. *PLOS ONE* 13(4): e0195475. https://doi.org/10.1371/journal.pone.0195475

Khattab S., El Sherif F., El-Garhy H.A., Ahmed S. & Ibrahim A. 2014. Genetic and phytochemical analysis of the in vitro regenerated *Pilosocereus robinii* by ISSR, SDS-PAGE and HPLC. *Gene* 533: 313–321. https://doi.org/10.1016/j.gene.2013.09.026

Kim E. & Donohue K. 2013. Local adaptation and plasticity of *Erysimum capitatum* to altitude: its implications for responses to climate change. *Journal of Ecology* 101: 796–805. https://doi.org/10.1111/1365-2745.12077

Kumar S., Stecher G. & Tamura K. 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution* 33: 1870–1874. https://doi.org/10.1093/molbev/msw054

Ledig F.T., Conkle M.T., Bermejo-Velázquez B., et al. 1999. Evidence for an extreme bottleneck in a rare Mexican pinyon: genetic diversity, disequilibrium, and the mating system in *Pinus maximartinezii*. *Evolution* 53: 91–99. https://doi.org/10.1111/j.1558-5646.1999.tb05335.x

Ledig F.T., Capó-Arteaga M.A., Hodgskiss P.D., et al. 2001. Genetic diversity and the mating system of a rare Mexican piñon, *Pinus pinceana*, and a comparison with *Pinus maximartinezii* (Pinaceae). *American Journal of Botany* 88: 1977–1987. https://doi.org/10.2307/3558425

Librado P. & Rozas J. 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* 11: 1451–1452. https://doi.org/10.1093/bioinformatics/btp187

Locosselli G.M., Cardim R.H. & Ceccantini G. 2016. Rock outcrops reduce temperature-induced stress for tropical conifer by decoupling regional climate in the semiarid environment. *International Journal of Biometeorology* 60: 639–649. https://doi.org/10.1007/s00484-015-1058-y

Mandujano M. del C., Carrillo-Angeles I., Martínez-Peralta C. & Golubov J. 2010. Reproductive biology of cactaceae. In: Ramawat K. (ed.) Desert plants: 197–230. Springer, Berlin, Germany. https://doi.org/10.1007/978-3-642-02550-1_10

Millar M.A., Byrne M., Coates D.J. & Roberts J.D. 2017. Comparative analysis indicates historical persistence and contrasting contemporary structure in sympatric woody perennials of semi-arid south-west Western Australia. *Biological Journal of the Linnean Society* 120: 771–787. https://doi.org/10.1093/biolinnean/blw005

Moraes E.M., Abreu A.G., Andrade S.C., Sene F.M. & Solferini V.N. 2005. Population genetic structure of two columnar cacti with a patchy distribution in eastern Brazil. *Genetica* 125: 311–323. https://doi.org/10.1007/s10709-005-0716-0

Nason J.D., Hamrick J.L. & Fleming T.H. 2002. Historical vicariance and postglacial colonization effects on the evolution of genetic structure in *Lophocereus*, a Sonoran Desert columnar cactus. *Evolution* 56: 2214–2226. https://doi.org/10.1111/j.0014-3820.2002.tb00146.x

Nassar J.M., Hamrick J.L. & Fleming T.H. 2003. Population genetic structure of Venezuelan chiropterophilous columnar cacti (Cactaceae). *American Journal of Botany* 90: 1628–1637. https://doi.org/10.3732/ajb.90.11.1628

Nistelberger H.M., Byrne M., Coates D. & Roberts J.D. 2015. Phylogeography and population differentiation in terrestrial island populations of *Banksia arborea* (Proteaceae). *Biological Journal of the Linnean Society* 114: 860–872. https://doi.org/10.1111/bij.12464

Ohsawa T. & Ide Y. 2008. Global patterns of genetic variation in plant species along vertical and horizontal gradients on mountains. *Global Ecology and Biogeography* 17: 152–163. https://doi.org/10.1111/j.1466-8238.2007.00357.x

Ornelas J.F. & Rodríguez-Gómez F. 2015. Influence of Pleistocene glacial/interglacial cycles on the genetic structure of the mistletoe cactus *Rhipsalis baccifera* (Cactaceae) in Mesoamerica. *Journal of Heredity* 106: 196–210. https://doi.org/10.1093/jhered/esu113

Palleiro-Dutrenit N. 2008. Estructura poblacional y genética del cactus columnar *Cephalocereus totolapensis* (Bravo et MacDougall) en el Estado de Oaxaca, México. MSc thesis, UNAM, Mexico. Available from http://132.248.9.195/ptd2008/junio/0628791/0628791.pdf [accessed 29 Mar. 2021].

Pérez-García E.A. 2002. Enclaves de vegetación xerofítica en regiones mésicas: caracterización, análisis de su diversidad florística e importancia en el mantenimiento de floras xerofíticas. MSc thesis, UNAM, Mexico. Available from http://132.248.9.195/pdtestdf/0307917/Index.html [accessed 29 Mar. 2021].

Perez M., Bonatelli I., Moraes E. & Carstens M.E. 2016. Model-based analysis supports interglacial refugia over long-dispersal events in the diversification of two South American cactus species. *Heredity* 116: 550–557. https://doi.org/10.1038/hdy.2016.17

Pérez-García E.A. & Meave J.A. 2005. Heterogeneity of xerophytic vegetation of limestone outcrops in a tropical deciduous forest region in southern México. *Plant Ecology* 175: 147–163. https://doi.org/10.1007/s11258-005-4841-8

Pérez-García E.A., Meave J.A., Villaseñor J.L., Gallardo-Cruz J.A., & Lebrija-Trejos E.E. 2010. Vegetation heterogeneity and life-strategy diversity in the flora of the heterogeneous landscape of Nizanda, Oaxaca, Mexico. *Folia Geobotanica* 45: 143–161. https://doi.org/10.1007/s12224-010-9064-7

Pérez-García E.A., Sevilha A.C., Meave J.A. & Scariot A. 2009. Floristic differentiation in limestone outcrops of southern Mexico and central Brazil: a beta diversity approach. *Boletín de la Sociedad Botánica de México* 84: 45–58. https://doi.org/10.17129/botsci.2294

Pezoa A. 2001. Estrategias de conservación de la diversidad biológica. In: Squeo F.A., Arancio G. & Gutiérrez J.R. (eds) Libro rojo de la flora nativa y de los sitios prioritarios para su conservación: región de Coquimbo: 273–280. Ediciones Universidad de La Serena, La Serena, Chile. Available from https://biologia614.webnode.es/_files/200000024-8841f893ba/Conservacion.PDF [accessed 29 Mar. 2021].

Porembski S. 2007. Tropical inselbergs: habitat types, adaptive strategies and diversity patterns. *Brazilian Journal of Botany* 30: 579–586. https://doi.org/10.1590/S0100-84042007000400004

Porembski S. & Barthlott W. 2000. Granitic and gneissic outcrops (inselbergs) as centers of diversity for desiccation-tolerant vascular plants. *Plant Ecology* 151: 19–28. https://doi.org/10.1023/A:1026565817218

Pritchard J.K., Stephens M. & Donnelly P. 2000. Inference of population structure using multilocus genotype data. *Genetics* 155: 945–959.

Quipildor V.B., Kitzberger T., Ortega-Baes P., Quiroga M.P. & Premoli A.C. 2017. Regional climate oscillations and local topography shape genetic polymorphisms and distribution of the giant columnar cactus *Echinopsis terscheckii* in drylands of the tropical Andes. *Journal of Biogeography* 45: 116–126. https://doi.org/10.1111/jbi.13106

Rambaut A. 2018. FigTree. Tree figure drawing toll version 1.4.4. Available from https://github.com/rambaut/figtree [accessed 9 Sep. 2019].

Rambaut A. & Drummond A.J. 2018. TREEANNOTATOR v1.10.4 Prerelease #bc6cbd9. In: BEAST package. Available from http://beast.community/ [accessed 29 Mar. 2021].

Rambaut A., Drummond A.J., Xie D., Baele G. & Suchard M.A. 2018. Posterior summarization in Bayesian phylogenetics using Tracer 1.7. *Systematic Biology* 67: 901–904. https://doi.org/10.1093/sysbio/syy032

Rivera-Montoya I. 2003. Estudio de la variabilidad genética de las cactácea columnar *Neobuxbaumia mezcalaensis*. Bachelor thesis, UNAM, Mexico.

Rogers A.R. & Harpending H. 1992. Population growth makes waves in the distribution of pairwise genetic differences. *Molecular Biology and Evolution* 9: 552–569. https://doi.org/10.1093/oxfordjournals.molbev.a040727

Rosenberg N.A. 2004. DISTRUCT: a program for the graphical display of population structure. *Molecular Ecology Notes* 4: 137–138. https://doi.org/10.1046/j.1471-8286.2003.00566.x

Ruán-Tejeda I., Santerre A., Huerta-Martínez F.M., Iñiguez-Dávalos L.I. & Castro-Félix P. 2014. Genetic diversity and relationships among wild and cultivated *Stenocereus queretaroensis* populations in western Mexico. *Biochemical Systematics and Ecology* 55: 125–130. https://doi.org/10.1016/j.bse.2014.03.005

Russo Ermolli E. & Cheddadi R. 1997. Climatic reconstruction during the middle Pleistocene: a pollen record from Vallo di Diano (southern Italy). *Geobios* 30: 735–744. https://doi.org/10.1016/S0016-6995(97)80176-3

Sarthou C., Villiers J.F. & Ponge J. 2003. Shrub vegetation on tropical granitic inselbergs in French Guiana. *Journal of Vegetation Science* 14(5): 645–652. https://doi.org/10.1111/j.1654-1103.2003.tb02196.x

Shaw J., Lickey E.B., Schilling E.E. & Small R.L. 2007. Comparison of whole chloroplast genome sequences to choose noncoding regions for phylogenetic studies in angiosperms: the tortoise and the hare III. *American Journal of Botany* 94: 275–288. https://doi.org/10.3732/ajb.94.3.275

Silva G.A.R., Antonelli A., Lendel A, Moraes E.M. & Manfrin M.H. 2018. The impact of early Quaternary climate change on the diversification and population dynamics of a South American cactus species. *Journal of Biogeography* 45: 76–88. https://doi.org/10.1111/jbi.13107

Silva G.A.R., Khan G., Ribeiro-Silva S., et al. 2020. Extreme genetic structure in a relict cactus genus from campo rupestre landscapes: implications for conservation. *Biodiversity and Conservation* 29: 1263–1281. https://doi.org/10.1007/s10531-020-01934-6

Suchard M.A., Lemey P., Baele G., Ayres D.L., Drummond A.J. & Rambaut A. 2018. Bayesian phylogenetic and phylodynamic data integration using BEAST 1.10. *Virus Evolution* 4(1): vey016. https://doi.org/10.1093/ve/vey016

Taberlet P., Gielly L., Pautou G. & Bouvet J. 1991. Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Molecular Biology* 17: 1105–1109. https://doi.org/10.1007/BF00037152

Tapia H.J., Bárcenas-Argüello M.L., Terrazas T. & Arias S. 2017. Phylogeny and circumscription of *Cephalocereus* (Cactaceae) based on molecular and morphological evidence. *Systematic Botany* 42: 709–723. https://doi.org/10.1600/036364417X696546

Tapper S.L., Byrne M., Yates C.Y., et al. 2017. Prolonged isolation and persistence of a common endemic on granite outcrops in both mesic and semi-arid environments in south-western Australia. *Journal of Biogeography* 41: 2032–2044. https://doi.org/10.1111/jbi.12343

Toro M.A. & Caballero A. 2005. Characterization and conservation of genetic diversity in subdivided populations. *Philosophical transactions of the Royal society B* 360: 1367–1378. https://doi.org/10.1098/rstb.2005.1680

Torres-Ribeiro K., Opazo-Medina B.M. & Rubio-Scarano F. 2007. Species composition and biogeographic relations of the rock outcrop flora on the high plateau of Itatiaia, SE-Brazil. *Revista Brasileira de Botânica* 30: 623–639

Tripati A.K., Roberts C.D. & Eagle R.A. 2009. Coupling of CO_2 and ice sheet stability over major climate transitions of the last 20 million years. *Science* 326: 1394–1397. https://doi.org/10.1126/science.1178296

Twidale C.R. 1982. Granite landforms. Elsevier Scientific Publishing Company, Amsterdam, The Netherlands.

Valadez-Moctezuma E., Samah S. & Luna-Paez A. 2015. Genetic diversity of *Opuntia* spp. varieties assessed by classical marker tools (RAPD and ISSR). *Plant Systematics and Evolution* 2: 737–747. https://doi.org/10.1007/s00606-014-1112-y

Valiente-Banuet A., Arizmendi M.C., Rojas-Martínez A., Casas C., Silva C. & Dávila P. 2002. Biotic interactions and population dynamics of columnar cacti. In: Fleming T.H. & Valiente-Banuet A (eds) Columnar cacti and their mutualists: evolution, ecology, and conservation: 225–240. University of Arizona Press, Tucson, USA.

Vazquez-Montiel R.F. 2005. Diversidad genética de *Cephalocereus columna-trajani* en cuatro regiones del Valle de Tehuacán-

Cuicatlán con diferentes rangos de temperatura. MSc thesis, UNAM, Mexico.

Wolfe K.H., Li W.H. & Sharp P.M. 1987. Rates of nucleotide substitution vary greatly among plant mitochondrial, chloroplast, and nuclear DNAs. *Proceedings of the National Academy of Sciences of the United Stated of America* 84: 9054–9058. https://doi.org/10.1073/pnas.84.24.9054

Yeh F.C., Yang R.C., Boyle T.B., Ye Z.H. & Mao J.X. 1997. POPGENE, the user-friendly shareware for population genetic analysis. Molecular Biology and Biotechnology Centre, University of Alberta, Canada. Available from https://sites.ualberta.ca/~fyeh/popgene.html [accessed 29 Mar. 2021].

Communicating editor: Myriam Heuertz.