



Pleistocene radiation of coastal species of *Pilosocereus* (Cactaceae) in eastern Brazil



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ABSTRACT

The semiarid region in Northeast Brazil (*Caatinga*) suffered several high moisture periods during the Pleistocene, while neighbouring regions experienced drying events. Effects of this climatic history on the evolution of *Caatinga* xeric flora are poorly understood. Demography and evolutionary relationships between *Pilosocereus arrabidaei* and *Pilosocereus catingicola* (Cactaceae) were investigated using two non-coding intergenic spacers of cpDNA (1424 bp) employing distinct statistical methods, such as Bayesian Inference analysis, haplotype network, AMOVA, neutrality tests and Bayesian Skyline Plot. Our data suggests that species formerly arranged as the informal *Pilosocereus arrabidaei* group do not form a monophyletic clade. *P. arrabidaei* and *P. catingicola* are not reciprocally monophyletic and present very low genetic diversity. The Tajima's D and Fu's Fs statistics provided no significant results. Results suggest a very recent origin for *P. arrabidaei* and *P. catingicola*. The beginning of *P. arrabidaei* and *P. catingicola* diversification dates back to the Pleistocene. Genetic diversity of *P. catingicola* subsp. *salvadorensis* is geographically structured between major rivers of the region, suggesting a history of isolation in interfluvies during Pleistocene climatic cycles.

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1. Introduction

The astonishing biodiversity found in the Neotropical region includes vast arid and semiarid habitats (Antonelli et al., 2015; Antonelli and Sanmartín, 2011; Hughes et al., 2013). The cacti are among the most iconic of many drought-adapted plant groups that evolved and diversified under the xeric conditions of these ecosystems. According to Hernández-Hernández et al. (2014), the basal lineages of Cactaceae probably originated in the Andean region of Chile and Argentina. Yet, this family has a broad distribution

throughout the New World, where its high diversity, with c. 1450 species (Hunt et al., 2006), is distributed in several diversity centres (Barthlott et al., 2015). Eastern Brazil, with its semiarid *Caatinga* and seasonal *Cerrado* is amongst the three most important areas in terms of cactus diversity, and it concentrates a very large proportion of threatened species of this fascinating family (Goettsch et al., 2015).

In order to endure life in semiarid or arid environments plants need a set of adaptive strategies (e.g. Eggli and Nyffeler, 2009; Mauseth, 2006). Since adaptations to survive drought usually make them less competitive in non-seasonal habitats (Taylor, 2012), they often become isolated and endemic in arid lands. Thus, the environmental changes that occurred during the Quaternary probably affected the distribution and evolution of cacti and other drought enduring plants. The global cooling and drying

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events of the Late Miocene and the lowering of CO₂ levels may have caused expansion of dry habitats (Hughes et al., 2013) and favoured CAM plants (Arakaki et al., 2011), respectively. These events may be responsible for the radiation of xeric species throughout South America (e.g. Hernández-Hernández et al., 2014; Moggi et al., 2015). Further, during the Pleistocene, the recurrent climate changes probably contributed to the allopatric differentiation of populations or species in the Neotropics (Bonatelli et al., 2014; Franco and Manfrin, 2013; Taylor, 2012; Turchetto-Zolet et al., 2013).

Although phylogeographic studies of plants from dry South American ecosystems are relatively few (Turchetto-Zolet et al., 2013), Hughes et al. (2013) postulate that the evolution of plants in Neotropical Seasonally Dry Tropical Forests (SDTF) is marked by high geographical phylogenetic structure, allopatric speciation, niche conservatism and well-supported monophyly of individual species in densely sampled gene trees. For these authors, such patterns suggest a scenario of population fragmentation in arid patches (with dispersal limitation), *in-situ* diversification of species pairs or small clades, and persistence of stable populations of species or lineages over long periods of time.

Bonatelli et al. (2014) studied the phylogeography of *Pilosocereus aurisetus* (Werderm.) Byles & G.D. Rowley and allies in southeast and central Brazil. They were able to find several of the phylogenetic/phylogeographic patterns expected for SDTF taxa according to Hughes et al. (2013). This group of *Pilosocereus* Byles & G.D. Rowley seems to have suffered successive cycles of population fragmentation during wet interglacial periods, followed by demographic explosions during dry glacial periods. According to Bonatelli et al. (2014), the isolation in multiple refuges between successive glaciations would result in the allopatric origin and spread of different haplotypes, explaining the polytomic phylogeny of the genus.

This model of diversification can explain the evolution of several Neotropical plant lineages that exhibit similar phylogenetic patterns, like some Bromeliaceae (Jabaily and Sytsma, 2012) and Lamiaceae (Drew and Sytsma, 2012). However, *Pilosocereus* is widely distributed in tropical America from southeastern USA and Mexico to southeastern Brazil and Paraguay (Zappi, 1994), with representatives in geographic regions that have experienced different climatic histories during the Quaternary. Thus, a more complete phylogeographic analysis of this genus may provide a wider understanding of xeric plant evolution in the Neotropics.

At this point, we turn our attention to the informal group proposed by Zappi (1994) to include *Pilosocereus arrabidae* (Lem.) Byles & G.D. Rowley and *Pilosocereus catingicola* (Gürke) Byles & G.D. Rowley, later extended by Hunt et al. (2006) to encompass their presumably close relatives: *Pilosocereus azulensis* N.P. Taylor & Zappi and *P. splendidus* F. Ritter. Together, these species occur over a very large area in eastern Brazil, including both inland and the Atlantic coast (Taylor and Zappi, 2004). Their habitat consists of a semideciduous scrubland or low forest that experienced dry interglacial and wet glacial periods (Auler et al., 2004).

Our aim was to test hypotheses on the phylogeography of *Pilosocereus arrabidae* and allies, as well as the phylogeny of the genus, to increase our understanding of the evolutionary history of the Neotropical xeric flora. Our hypotheses are that: 1) *Pilosocereus arrabidae* and its allies form a basal group in subgenus *Pilosocereus*, based on Zappi (1994); 2) The coastal lineages of *Pilosocereus* recently radiated to northern *Caatinga* from the highland region of the 'Chapada Diamantina' – a putative centre of dispersal for many species from arid environments (Collevatti et al., 2012, 2014; Franco and Manfrin, 2013; Zappi, 1994) through the dry valley of the São Francisco River; 3) Populations of *Pilosocereus* experienced demographic reduction in Northeast Brazil during glacial events, when this region was wetter (Auler et al., 2004); 4) *P. arrabidae* and

allies survived wet periods in a putative dry refuge in the 'Chapada Diamantina' and spread to the coast in the last interglacial period; 5) Unlike *P. aurisetus* group in southeastern Brazil (Bonatelli et al., 2014), populations of *P. arrabidae* and allies should have experienced long-term gene flow due to their almost continuous geographic distribution.

Based on these hypotheses, we predicted that: 1.1) *Pilosocereus arrabidae* and its allies should be part of the most basal lineages in the phylogeny of the genus; 1.2) Taxa of the *P. arrabidae* group should exhibit reciprocal monophyly in cytoplasmic DNA, as is expected in well-established sister species (Avise, 2000; Hubbell, 2001); 1.3) *Pilosocereus arrabidae* and its allies should have an earlier divergence time when compared to the *P. aurisetus* group; 2.1) Inland populations of the *P. arrabidae* group should exhibit a higher number of haplotypes when compared to coastal populations; 2.2) Coastal populations from extremes of its geographic distribution should exhibit lower numbers of haplotypes; 3.1) Demographic analyses should reveal evidence of demographic expansion of *Pilosocereus arrabidae* and allies (that should have happened since the end of the last glacial period); 4.1) *P. arrabidae* and allies should exhibit a high number of haplotypes concentrated in a single place (i.e. 'Chapada Diamantina'); 5.1) Phylogeography of *P. arrabidae* group should not be geographically structured if the habitat occupied by its species can be considered continuous and its representatives experienced predominantly favourable climatic conditions (during interglacial periods, which were longer than the glacial).

2. Materials and methods

2.1. Biological model

Pilosocereus is one of the most diverse genera in tribe Cereeae, with 42 species recognized (Hunt et al., 2006; Zappi and Taylor, 2011) of which most inhabit eastern Brazil (Zappi, 1994). This speciose genus, which is disjunct between Brazil's eastern region and northwestern South America (reaching Mexico and the Caribbean) is in many ways an ideal model to study the diversification of lineages into more seasonal habitats during the Quaternary, as seen by Bonatelli et al. (2014).

According to Hunt et al. (2006), *P. arrabidae* and allies are characterized by the shrubby or tree-like habit, low number of ribs, undifferentiated flower-bearing areoles, acute flower-bud apex and by the robust and wide bat-pollinated flowers (Hunt et al., 2006; Locatelli et al., 1997; Zappi, 1994). *P. arrabidae* occurs along the east coast of Brazil, in the states of Rio de Janeiro, Espírito Santo and southern Bahia (Fig. 1). It inhabits the beach or patches of open shrubby vegetation widespread along sea-level sandy areas, called *Restingas*, which landwards are surrounded by a moister forest matrix (Taylor and Zappi, 2004). According to Taylor and Zappi (2004), *P. catingicola* has two subspecies: subsp. *catingicola*, which inhabits *Caatingas* of the dry valleys of the Brazilian state of Bahia; and subsp. *salvadorensis* (Werderm.) Zappi, which occurs in northern *Restingas* or in the *Caatingas* of the dry valleys, in the states of Bahia, Sergipe, Alagoas, Pernambuco, Paraíba, Rio Grande do Norte and Ceará (Fig. 1). *Pilosocereus azulensis* is known to occur in *Caatingas* of only two adjacent localities in a mountainous region in northeastern Minas Gerais (Hunt et al., 2006). *P. splendidus* provisionally refers to a plant from a unique locality in Northeast Brazil, whose taxonomic status is not yet fully understood (Hunt et al., 2006). Thus, we focused our phylogeographic analyses on *P. arrabidae* and two subspecies of *P. catingicola* (the three original taxa of the group according to Zappi, 1994).

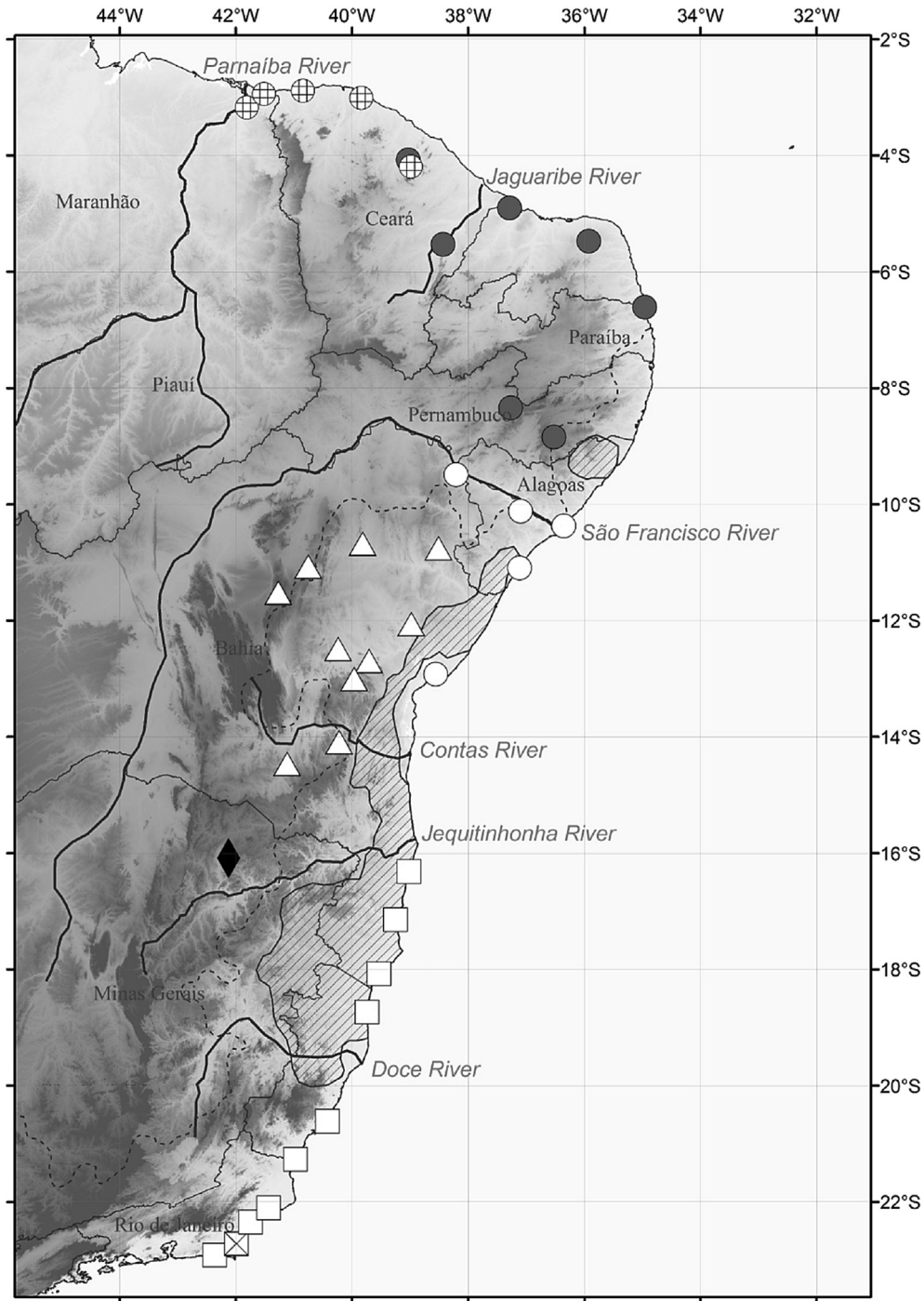


Fig. 1. Geographical distribution of the haplotypes of *Pilosocereus arrabidae* and allies: H3 (white), H4 (diagonal cross), H5 (crosshatched), H6 (gray) and H7 (black). Squares = *P. arrabidae*; Triangles = *P. catingicola* subsp. *catingicola*; Circles = *P. catingicola* subsp. *salvadorensis*; Diamond = *P. pachycladus* subsp. *pachycladus* (*P. cenepequei*). Major eastern Brazilian rivers are shown. Hatched area: historically stable area for Atlantic Forest in the last 21 kyr (according to [Carnaval and Moritz, 2008](#)). Dotted line: Possible expansion of suitable habitat for Atlantic Forest species in the last 21 kyr ([Carnaval and Moritz, 2008](#)). Background: altitude.

2.2. Study area

Studied taxa occur in the Brazilian semiarid region known as *Caatinga*, which harbours a large portion of cactus diversity ([Taylor and Zappi, 2004](#)) and is currently amongst the most neglected regions in South American phylogeographic studies, especially when considering plant species ([Turchetto-Zolet et al., 2013](#)). Semi-deciduous savannas or scrublands, generally classified as SDTF,

are the predominant vegetation types. Geological and palynological data suggest that the northern *Caatinga* experienced several high moisture periods during the late Pleistocene and Holocene (e.g. [Auler et al., 2004](#) and references therein) – that is remarkably different from neighbouring regions, which experienced drying events (e.g. [Ledru et al., 1996](#)) or remained relatively stable ([Colinvaux et al., 2000](#)). There is robust evidence that these moister periods have lasted up to hundreds of thousands of years and may

have been responsible for huge changes in the vegetation and flora of a significant extent of this region (Auler et al., 2004). According to Auler et al. (2004) and Werneck et al. (2011) the unique climatic conditions of the Last Glacial Maximum (LGM) might have allowed taxa from SDTF to mix with rainforest taxa, forming “novel communities with no modern-day analogue”. As terrestrial cacti usually do not inhabit humid habitats, it is expected that these climatic changes in *Caatinga* have played an important role in their phylogeographic pattern (as well as in the phylogeography of other plants adapted to drought-stressed environments).

2.3. Sampling and analyses

The analyses were performed using datasets from *trnT-trnL* and *trnS-trnG* non-coding intergenic spacers of the chloroplast DNA (cpDNA). These markers showed informative intra-specific variation for the genus, at least in the *P. aurisetus* group (Bonatelli et al., 2013, 2014). Small stem tissue explants were obtained from a total of 76 samples: 18 individuals of *Pilosocereus arrabidae* (12 localities), 12 of *P. catingicola* subsp. *catingicola* (12 localities) and 46 of *P. catingicola* subsp. *salvadorensis* (17 localities; Fig. 1; Table 1). One sample of each species included in the *P. arrabidae* group by Hunt et al. (2006) – *P. azulensis* and *P. splendidus* – was also included in phylogenetic analyses. Sampling localities (at least 20 km apart) were recorded with GPS to ensure that the entire known geographic distribution of each taxon in the *P. arrabidae* group was covered. We also used 10 sequences of species belonging to other *Pilosocereus* groups in order to test the monophyletic status of the *P. arrabidae* group (Table 1). These sequences were generated in this study from samples obtained either from field or from living collections. Further, in order to improve the accuracy of divergence time estimates, we combined all sequences generated in this study with sequences from the *P. aurisetus* species group, previously obtained by Bonatelli et al. (2014; TreeBASE: S15695). Such cpDNA dataset includes 157 *trnT-trnL* and *trnS-trnG* sequences from 11 *P. aurisetus* populations, 13 *P. machrisii* populations, 4 *P. jauruensis* populations, 2 *P. vilaboensis* populations, and 1 population from each *P. aureispinus*, *P. parvus* and *P. bohlei* (Bonatelli et al., 2014).

DNA was extracted from tissue samples using the traditional CTAB procedure. The noncoding intergenic spacers *trnT-trnL* and *trnS-trnG* of the cpDNA were amplified with GoTaq[®] Green Master Mix (Promega, Madison, Wisconsin, USA). PCR protocol followed the standard application suggested by the manufacturer, using annealing temperatures described by Bonatelli et al. (2013). The PCR products were purified with EZ-10 Spin Column Purification Kit (Bio Basic Inc, Markham, Ontario, Canada) and sequenced with the ABI 3730 XL DNA Analyzer (Applied Biosystems, Foster City, California, USA).

Samples of both cpDNA regions were sequenced mostly in the forward direction. The reverse sequences were used only when the electropherograms were ambiguous or presented sequencing errors. The tips of the sequences and the homonucleotide repeats (one from *trnT-trnL* and two from *trnS-trnG*) were removed from the analyses due to ambiguity and uncertain homology. Sequences of each marker were then aligned using Muscle multiple aligner (Edgar, 2004). The indels were coded as binary information performing the simple indel coding (Simmons and Ochoterena, 2000) using the software SeqState 1.4.1 (Muller, 2005). A single inversion found in the *trnT-trnL* region detected in some samples of *P. catingicola* subsp. *salvadorensis* was coded as presence/absence information. Haplotype and nucleotide diversity were computed using DNASP 5.1 (Librado and Rozas, 2009).

The two cpDNA regions were subjected to the Incongruence Length Difference Test, performed in TNT (Goloboff et al., 2008) in order to concatenate them in further analyses. The best among 24

substitutions models of nucleotide evolution was chosen for each region using the Bayesian Information Criterion (Schwarz, 1978), implemented in jModelTest v2.1 (Darriba et al., 2012). Phylogenetic analyses were performed using the Bayesian-inference (BI) approach in order to infer phylogenetic relationships among the haplotypes of *P. arrabidae* group and also among representatives of distinct *Pilosocereus* species groups (Table 1), using as outgroups samples from *Micranthocereus auriazureus* (GenBank: JN035614.1, JN035460.1, JN035419.1), *Arrojadoa rhodantha* (GenBank: KC779438.1, KC779313.1) and *P. gounellei* (F.A.C. Weber) Byles & G.D. Rowley (Subgenus *Gounellea* Zappi). The BI analysis was conducted in MrBayes v3.2 (Ronquist et al., 2012), assigning the HKI + I model (Hasegawa et al., 1985) to the nucleotide partition (concatenated data set) and the standard discrete model (Ronquist and Huelsenbeck, 2003) to binary data. The analysis was run for 20⁷ generations, using default parameters and trees sampled every 10² generations using two simultaneous runs, each one with four simultaneous chains. The first 25% of generated trees were deleted as burn-in. The remaining trees were used to summarize a 50% majority-rule consensus tree. FigTree 1.4.0 (available at <http://tree.bio.ed.ac.uk/software/figtree/>) was used to draw the trees. An independent phylogenetic BI analysis was performed only with nucleotide partition following the same above conditions in order to investigate the influence of indels in topology. To investigate whether haplotypes sampled from sympatric populations of *P. arrabidae* or *P. catingicola* and other *Pilosocereus* species were introgressive we used haplotypes from both species found in allopatric populations to make comparisons. The genealogical relationships between the haplotypes were also analyzed by the median-joining network algorithm (Bandelt et al., 1999) implemented in Network v4.6 (Fluxus Technology Ltd., Clare, Suffolk, England). Shared gaps and inversions were double-weighted. The reticulations were resolved through the prioritization of high weight connections.

Analysis of molecular variance (AMOVA) on the *P. arrabidae* and *P. catingicola* dataset was conducted in Arlequin 3.5 (Excoffier et al., 2005), using a pairwise distance matrix and 10,000 permutations. We explored different AMOVA models by grouping samples by taxonomic circumscription, biomes (Atlantic Forest and *Caatinga*) and by large rivers (Fig. 1) that have been cited as ecological barriers for plants in eastern Brazil (Carnaval and Moritz, 2008; Turchetto-Zolet et al., 2013). Arlequin was also used to calculate molecular diversity indices. The correlation between pairwise geographic and genetic distances (measured as the number of substitutions and structural mutations) among sampled localities was analyzed by a Mantel test, implemented by Alleles in Space (Miller, 2005). Two independent runs (with and without logarithmic transformation) were made, using 10,000 permutations.

The time to the most recent common ancestor (T_{MRCA}) was estimated using the Bayesian Markov Monte Carlo method implemented in Beast v1.8 (Drummond et al., 2012). This analysis was run for 10⁷ generations (with samples drawn every 1000 generations) under the “birth-death” model and an uncorrelated lognormal relaxed clock. Two independent runs were performed for each of the extreme cpDNA substitution rates inferred for angiosperms (Wolfe et al., 1987): 0.0011 and 0.0029 subs/site/Myr. The independent runs were combined in LogCombiner v1.8.0 (Drummond et al., 2012) and the results observed in Tracer 1.6 (Rambaut et al., 2014) to check for an effective sample size (ESS) of >200.

The demographic history of *P. arrabidae* and *P. catingicola* was analyzed with D and Fs statistics – Tajima (1989) and Fu (1997), respectively – under a population balance null hypothesis. Demographic history was also investigated with the coalescent-based Bayesian Skyline Plot – BSP, implemented with Beast software for

Table 1
Samples used for phylogeographic analysis (*P. arrabidae* group) and for Bayesian Inference Analysis (species from other groups).

Species/Locality	N	Code	Coordinates (S, W)	Biome	Haplotype
<i>Pilosocereus arrabidae</i> GROUP					
<i>P. arrabidae</i> (Lem.) Byles & G.D. Rowley					
Prado – BA	4	L04	–	Atlantic Forest	H3
Arraial do Cabo – RJ	2	667	22° 55', 42° 14'	Atlantic Forest	H3
Cabo Frio – RJ	1	670	22° 43', 41° 59'	Atlantic Forest	H4
Rio das Ostras – RJ	1	671	22° 28', 41° 54'	Atlantic Forest	H3
Macaé – RJ	2	672	22° 20', 41° 45'	Atlantic Forest	H3
Quissamã – RJ	1	674	22° 06', 41° 26'	Atlantic Forest	H3
Presidente Kennedy – ES	1	675	21° 16', 40° 59'	Atlantic Forest	H3
Marataizes – ES	1	676	21° 01', 40° 48'	Atlantic Forest	H3
Guarapari – ES	2	678	20° 36', 40° 25'	Atlantic Forest	H3
São Mateus – ES	1	679	18° 43', 39° 44'	Atlantic Forest	H3
Mucuri – BA	1	680	18° 04', 39° 32'	Atlantic Forest	H3
Santa Cruz de Cabrália – BA	1	681	16° 18', 39° 01'	Atlantic Forest	H3
<i>P. catingicola</i> (Gürke) Byles & G.D. Rowley subsp. <i>catingicola</i>					
Itaitim – BA	1	584	12° 41', 39° 41'	Caatinga	H3
Nova Itarana – BA	1	586	13° 00', 39° 57'	Caatinga	H3
Manoel Vitorino – BA	1	588	14° 05', 40° 12'	Caatinga	H3
Caraibas – BA	1	592	14° 27', 41° 06'	Caatinga	H3
Itaberaba – BA	1	597	12° 29', 40° 13'	Caatinga	H3
Morro do Chapéu – BA	1	602	11° 31', 41° 15'	Caatinga	H3
Morro do Chapéu – BA	1	604	11° 28', 41° 05'	Caatinga	H3
Jacobina – BA	1	605	11° 04', 40° 45'	Caatinga	H3
Itiúba – BA	1	607	10° 40', 39° 48'	Caatinga	H3
Feira de Santana – BA	1	616	12° 03', 38° 58'	Caatinga	H3
Ribeira do Pombal – BA	1	626	10° 49', 38° 32'	Caatinga	H3
Ribeira do Pombal – BA	1	627	10° 45', 38° 30'	Caatinga	H3
<i>P. catingicola</i> subsp. <i>salvadorensis</i> (Werderm.) Zappi					
Piaçabuçu – AL	1	L02	10° 22', 36° 21'	Atlantic Forest	H3
Aracajú – SE	10	L03	11° 05', 37° 07'	Atlantic Forest	H3
Salvador – BA	1	M08	12° 56', 38° 21'	Atlantic Forest	H3
Camocim – CE	1	11	2° 52', 40° 51'	Caatinga	H5
Buriti dos Lopes – PI	7	437	3° 09', 41° 49'	Caatinga	H5
Luiz Correia – PI	1	439	2° 55', 41° 31'	Caatinga	H5
Itarema – CE	1	443	2° 59', 39° 50'	Caatinga	H5
Caridade – CE	1	497	4° 04', 39° 02'	Caatinga	H6
Caridade – CE	1	498	4° 10', 38° 59'	Caatinga	H5
Alto Santo – CE	1	522	5° 31', 38° 26'	Caatinga	H6
Tibau – RN	1	571	4° 53', 37° 17'	Caatinga	H6
João Câmara – RN	1	577	5° 28', 35° 55'	Caatinga	H6
Mataraca – PB	9	580	6° 36', 34° 57'	Atlantic Forest	H6
Paulo Afonso – BA	1	635	9° 29', 38° 12'	Caatinga	H3
Itabi – SE	2	645	10° 06', 37° 06'	Caatinga	H3
Garanhuns – PE	4	648	8° 50', 36° 36'	Caatinga	H6
Setrânia – PE	3	652	8° 20', 37° 16'	Caatinga	H6
<i>P. azulensis</i> N.P. Taylor & Zappi					
Pedra Azul – MG	1	E02	16° 03', 41° 14'	–	H9
<i>P. splendidus</i> F. Ritter					
Licínio de Almeida – BA	1	E03	14° 32', 42° 31'	–	H15
Species from other groups					
<i>P. chrysostele</i> subsp. <i>cearensis</i> P.J. Braun & Esteves					
Pereiro – CE	1	521	06° 20', 38° 31'	–	H13
<i>P. densiareolatus</i> F. Ritter					
Montes Claros – MG	1	E06	–	–	H10
<i>P. floccosus</i> subsp. <i>quadricostatus</i> (F. Ritter) Zappi					
Pedra Azul – MG	1	M01	16° 03', 41° 14'	–	H9
<i>P. lanuginosus</i> (L.) Byles & G.D. Rowley					
Living collection specimen (from Colombia)	1	M07	–	–	H11
<i>P. multicostatus</i> (F. Ritter)					
Pedra Azul – MG	1	E05	16° 03', 41° 14'	–	H8
<i>P. pachycladus</i> subsp. <i>pachycladus</i> F. Ritter					
Morro do Chapéu – BA	1	603	11° 29', 41° 20'	–	H13
Salinas – MG (described as <i>P. cenepequei</i> Rizzini & A. Mattos-F)	1	M06	16° 01', 42° 03'	–	H7
<i>P. pachycladus</i> subsp. <i>pernambucoensis</i> (F. Ritter) Zappi					
Aiuaba – CE	1	32	6° 37', 40° 07'	–	H12
<i>P. pentaedrophorus</i> subsp. <i>robustus</i> Zappi					
Vitória da Conquista – BA	1	590	14° 49', 40° 50'	–	H14
<i>P. ulei</i> (K. Schum.) Byles & G.D. Rowley					
Arraial do Cabo – RJ	1	669	22° 46', 41° 55'	–	H16

both species. BSP analysis consisted of two independent runs with 10^7 generations (one for each substitution rate). The results were viewed in Tracer to check for an effective sample size (ESS) of >200 .

3. Results

3.1. Phylogenetic relationships and T_{MRC} estimates

Some samples failed to amplify in at least one region even after repeated attempts. These samples (4 of *P. arrabidae*, 1 of *P. catingicola* subsp. *catingicola*, 6 of *P. catingicola* subsp. *salvadorensis*) were removed from the analysis. The same problem occurred with 2 sequences of *P. glaucochrous* (Werderm.) Byles & G.D. Rowley, 2 of *P. pentaedrophorus* (Lab.) Byles & G.D. Rowley subsp. *pentaedrophorus* and 1 of *P. pachycladus* subsp. *pachycladus*. In this case, the available parts of the sequences were successfully used to test for sharing haplotypes between sympatric species. Since there was no significant phylogenetic incongruence between *trnT-trnL* and *trnS-trnG* regions ($P = 0.4743$), all further analyses were performed with these two sequences concatenated. The whole alignment (including gaps) was 1424 bp long.

Excepting *P. bohlei*, all haplotypes from *Pilosocereus* subgenus *Pilosocereus* were clustered in a large unresolved and highly supported clade, which includes internal well-supported branches (Fig. 2). The main results are as follows: 1) the clades N and S described in Bonatelli et al. (2014) were resolved with high statistical support, with the inclusion of *P. splendidus* and *P. densiareolatus* in the former; 2) *P. pachycladus* subsp. *pernambucoensis*, *P. lanuginosus*, *P. pentaedrophorus* subsp. *robustus*, *P. chrysostele* subsp. *cearensis* and *P. pachycladus* subsp. *pachycladus* were clustered in a common branch, with closer relationship between the two latter species; 3) *P. azulensis*, supposedly allied of *P. arrabidae* (Hunt et al., 2006), was included in a highly supported branch with *P. multicostatus* and

P. floccosus, indicating conflict between the current taxonomy and our phylogenetic information; 4) most haplotypes from *P. arrabidae*, *P. catingicola* subsp. *salvadorensis* and *P. catingicola* subsp. *catingicola* were clustered in a well-supported clade, hereafter called clade A, but they did not form a monophyletic clade, as it included one haplotype from *P. pachycladus* (H7; from a population described as *P. cenepequei*); 5) one haplotype of *P. arrabidae* (H4) and one of *P. catingicola* subsp. *salvadorensis* (H5) were unresolved; 6) the phylogenetic relationships of *P. aureispinus*, *P. jauruensis* and *P. ulei* with other species of the polytomic clade remain unclear.

The median-joining network of the haplotypes presented a “starburst” pattern around a median vector in the centre of the network (mv2; Fig. 3). The haplotype which was closest to the centre of the network (diverging by one mutation) is the one shared by *P. aurisetus* and *P. splendidus* (H15). Except for *P. splendidus*, all haplotypes from the *P. arrabidae* group were related to the median vector mv1, which also exhibited a “starburst” pattern. Similarly to what is observed in the BI tree, haplotypes from the *P. arrabidae* group were allocated into distinct branches of the network. The haplotypes of species that co-occur in the locality Pedra Azul – MG (in the southern portion of the *Caatinga*) were closely grouped in the network: the haplotype H9 (shared by *P. azulensis* and *P. floccosus* subsp. *quadricostatus*) was directly linked to *P. multicostatus* (H8).

No haplotype sharing was found between any of the four sympatric pairs of *P. arrabidae* or *P. catingicola* with other species of the subgenus: *P. catingicola* subsp. *catingicola* + *P. pentaedrophorus* subsp. *pentaedrophorus* from Itaitim – BA (population n. 584); *P. catingicola* subsp. *catingicola* + *P. glaucochrous* from Morro do Chapéu – BA (population n. 602); *P. catingicola* subsp. *catingicola* + *P. pachycladus* subsp. *pachycladus* from Jacobina – BA (population n. 605); *P. arrabidae* + *P. ulei* from Armação dos Búzios – RJ (population n. 669).

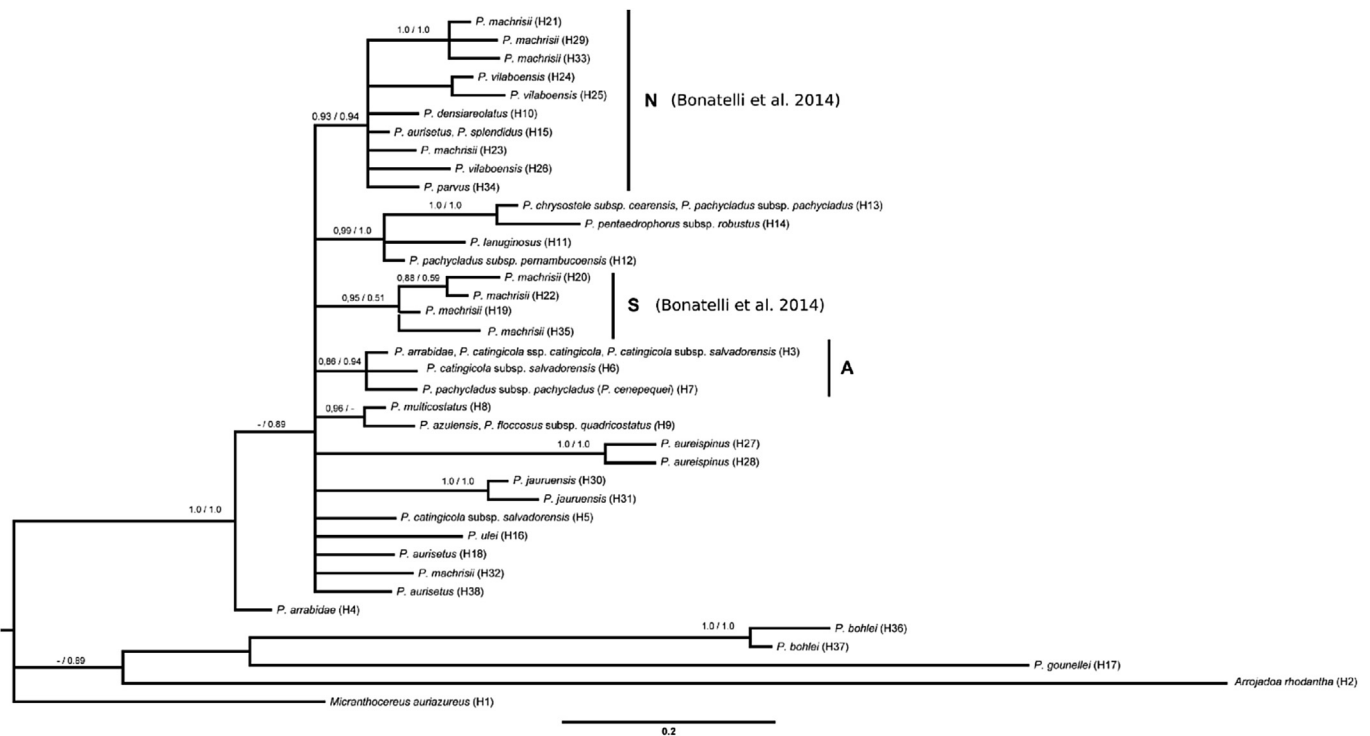


Fig. 2. Bayesian inference consensus tree of *Pilosocereus* Byles & Rowley based on the concatenated sequence of plastid DNA (*trnT-trnL* + *trnS-trnG*). The haplotypes are shown in parenthesis and they are the same as identified in the network and Table 1. Posterior probabilities (above 0.85) obtained in analysis with coded indel and in analysis without this information are sequentially indicated above node branches. Clades N and S recovered from Bonatelli et al. (2014) are shown. The Clade A includes most haplotypes found in *P. arrabidae* group. The scale bar represents the number of substitutions per site.

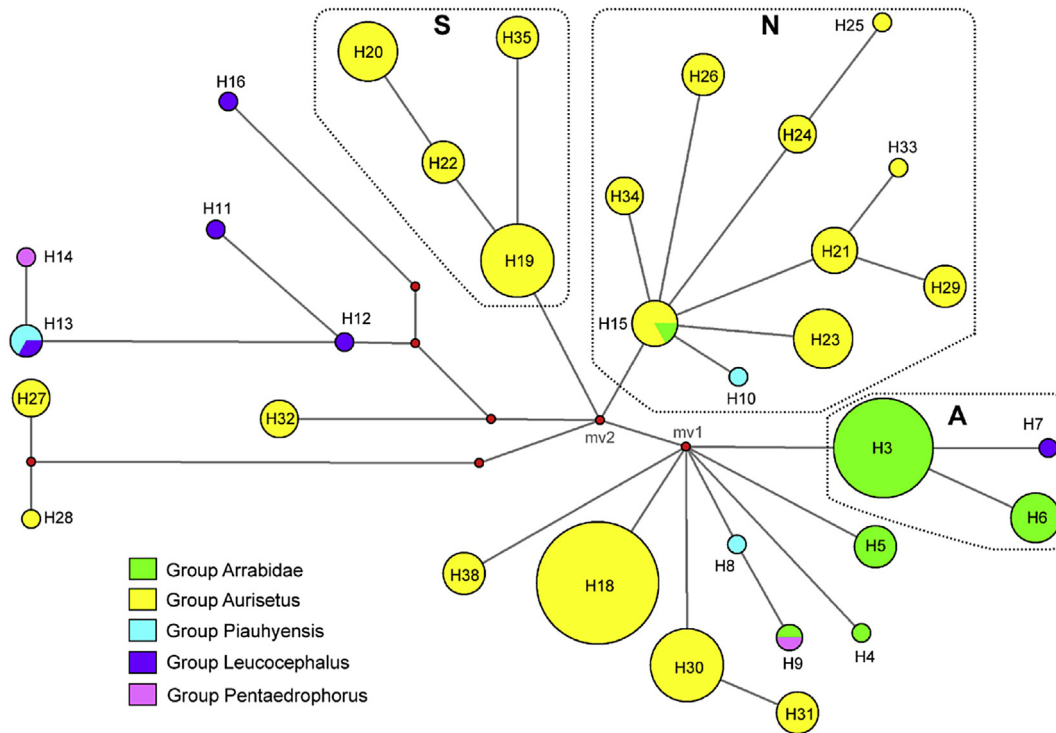


Fig. 3. Haplotype median-joining network of the polytomic clade of subgenus *Pilosocereus*. Each color represents an informal group (sensu Hunt et al., 2006). Correspondence between haplotypes and taxa are presented in Table 1. Clades N and S recovered from Bonatelli et al. (2014) are shown.

Table 2

Minimum and maximum estimates for the T_{MRCA} of *Pilosocereus arrabidae* group under slow and fast substitution rates (Myr).

Clade	Slow rate (HPD 95%)	Fast rate (HPD 95%)
Polytomic clade	2.86 (1.30 – 4.61)	1.79 (0.48 – 1.73)
Clade A	0.36 (0.09 – 0.69)	0.14 (0.04 – 0.27)

Because haplotypes of the *P. arrabidae* group did not form a monophyletic clade in the BI tree, we were not able to estimate the T_{MRCA} for this species group. To overcome this problem we estimated the T_{MRCA} for the large polytomic clade (which included haplotypes from other informal groups of the subgenus *Pilosocereus*) and for the clade A, which can be interpreted as the maximum and minimum diversification times of our focal group, respectively. The T_{MRCA} for the large polytomic clade, estimated using both slow and fast mutation rates, was dated from the middle Pliocene to middle Pleistocene, whereas the T_{MRCA} for the more taxonomically restricted clade A, from middle to late Pleistocene (Table 2).

3.2. Demographic analysis and population structure of *Pilosocereus arrabidae* group

Regardless of the number of samples per population, no within-population variation was found for any species of the *P. arrabidae* group. Only four haplotypes were found for *P. arrabidae* and *P. catingicola* (haplotype diversity = 0.582), which were defined by one inversion, one gap and four nucleotide substitutions (nucleotide diversity = 0.00046). Although most of the taxonomic diversity occurs south of the São Francisco River, most haplotype and nucleotide diversity was found north of this river, where only *P. catingicola* subsp. *salvadorensis* populations are found (northern and southern haplotype diversity = 0.506 vs. 0.045; northern and

southern nucleotide diversity = 0.00075 vs. 0.00010). The geographical distribution of the haplotypes of *P. arrabidae* and *P. catingicola* was in line with the major rivers of eastern Brazil (Fig. 1). The most widespread haplotype (H3) is shared among *P. arrabidae* and both subspecies of *P. catingicola* and it was observed from the São Francisco River southwards to the extreme of *P. arrabidae* distribution. The haplotype H4 is exclusive for *P. arrabidae* and was found in only one locality (Cabo Frio – RJ) in the southernmost part of the species' range. The remaining two haplotypes, H5 and H6, are exclusive for *P. catingicola* subsp. *salvadorensis* and were found northwards from the São Francisco River. However, H6 spreads eastwards and H5 westwards, with a co-occurrence zone near the Jaguaribe River (Fig. 1).

The hierarchical AMOVA considered the grouping based on species and subspecies and showed a rather larger variance component among populations within groups than among groups, weakly supporting taxonomic circumscriptions in the *P. arrabidae* group (Table 3). Furthermore, no variation between population groups was found when populations were sorted by biomes (*Caa-tinga* and Atlantic Forest). In contrast, AMOVA models grouping populations by large rivers revealed larger variance among than within groups and the model with three population groups according to south and north of São Francisco River and west of the Jaguaribe River best explained the amount of variance in the data (90.63% of the genetic variation, $P < 0.0001$). The Mantel test showed weak to moderate correlation between geographic and genetic distances for log transformed pairwise values ($r = 0.3578$, $P = 0.0009$) but no significant correlation for raw values ($r = 0.2367$, $P = 0.1029$).

The Tajima's D and Fu's F_s statistics provided no significant results. The BSP analysis did not provide ESS of >200 for *P. arrabidae* and failed to detect any clear population variation for both subspecies of *P. catingicola*.

Table 3

Analyses of molecular variance on cpDNA variation of *P. arrabidae* and *P. catingicola* samples. F_{ST} , differentiation among populations; F_{SC} , differentiation among populations within groups; F_{CT} , differentiation among groups. * indicates probability values less than 0.01.

Source of variation	df	Variance components	Percentage of variation	Fixation indices
Populations not grouped				
Among population	40	22.57475	100.00	F_{ST} : 1.000*
Within populations	35	0.000000	0.00	
Total	75	22.57475		
Three groups defined by taxonomic circumscriptions				
Among groups	2	6.72803	26.52	F_{CT} : 0.265
Among population within groups	38	18.6400	73.40	F_{SC} : 1.000*
Within populations	35	0.000000	0.00	F_{ST} : 1.000*
Total	75	25.36803		
Two groups according to biomes (Caatinga and Atlantic Forest)				
Among groups	1	-2.06654	-9.57	F_{CT} : -0.095
Among population	39	23.66746	109.57	F_{SC} : 1.000*
Within populations	35	0.000000	0.00	F_{ST} : 1.000*
Total	75	21.60093		
Two groups according to N and S of São Francisco River				
Among groups (North and South)	1	19.55412	61.03	F_{CT} : 0.610*
Among population within groups	39	12.48600	38.97	F_{SC} : 1.000*
Within populations	35	0.000000	0.00	F_{ST} : 1.000*
Total	75	32.04012		
Three groups according to N and S of São Francisco River and E and W of Jaguaribe River				
Among groups (North and South)	2	31.91668	90.63	F_{CT} : 0.906*
Among population within groups	38	3.300780	9.37	F_{SC} : 1.000
Within populations	35	0.000000	0.00	F_{ST} : 1.000*
Total	75	35.21745		
Six groups according to large rivers				
Among groups	5	23.00151	86.19	F_{CT} : 0.861*
Among population within groups	35	3.686380	13.81	F_{SC} : 1.000*
Within populations	35	0.000000	0.00	F_{ST} : 1.000*
Total	75	26.68788		

4. Discussion

Rather than the insufficiency of genetic markers, the unresolved phylogenetic pattern of subgenus *Pilosocereus* seems to be a consequence of a recent event of radiation with maintenance of ancestral polymorphic sites. The plastid genetic markers used for our phylogeny may not have sufficient signal to solve the phylogenetic relationships caused by recent and rapid evolutionary events, such as radiation. Large polytomies with divergence times estimated in a few millions of years have been found for other South American taxa (Drew and Sytsma, 2012; Jabaily and Sytsma, 2012). The arrangement of the haplotypes of *Pilosocereus* subgenus *Pilosocereus* in a polytomic clade as well as the lack of monophyly of the taxa grouped as *P. arrabidae* group refute our first hypothesis. All molecular diversity estimates were lower than those obtained by Bonatelli et al. (2014) for *P. aurisetus* and allies. While Bonatelli et al. (2014) – using the same genetic markers – found 22 haplotypes for 157 individuals of *P. aurisetus* and allies distributed along a 12-degree latitude span, we found only four haplotypes for 76 individuals in a 20-degree span. Moreover, the four haplotypes found for *P. arrabidae* group exhibited only incipient divergence when compared with the *P. aurisetus* group (Bonatelli et al., 2014). On one hand, these results may be explained due to the higher taxonomic diversity being found in *P. aurisetus* group and on the other due to the scattered geographical distribution of species from the *P. aurisetus* group, as they are associated with rock outcrops, having limited population size, thus allowing new haplotypes to rapidly increase in frequency by genetic drift, leading to increasing of haplotype diversity.

Our strategy of calculating minimum and maximum estimates for the T_{MRCA} provided a reliable confidence interval, which was equivalent to that found by Bonatelli et al. (2014) for *P. aurisetus* and allies. Despite the higher number of haplotypes found by Bonatelli et al. (2014) for *P. aurisetus* and allies, according to Hernández-

Hernández et al. (2014) shifts in pollination syndrome and growth habit may change diversification rates in Cactaceae. Thus, because these groups exhibit different pollination syndromes, we were not able to be conclusive about which group is older or more basal: *P. arrabidae* or *P. aurisetus*.

According to Schaal et al. (1998), morphological characters may have a weak relation with genetic variation, especially in the case of recent radiations. This seems to be the case of *Pilosocereus* – since Bonatelli et al. (2014) found taxonomic incongruence for *P. aurisetus* and *P. machrisii* and Menezes et al. (2015) found that morphological patterns of *P. arrabidae* group may be more related to environmental variables than to genetic lineages. Our results reinforce this point of view due to the lack of correspondence between the haplotype network and the informal morphological groups proposed by Zappi (1994) and Hunt et al. (2006). This pattern suggests that the *Pilosocereus* species underwent recent radiation with stochastic lineage sorting.

Considering the haplotype richness of each taxon from the *P. arrabidae* group, the distribution of haplotypes along the east coast of Brazil, as well as the basal placement of H4 (from the southeast) in the phylogenetic tree, we refute our second hypothesis that ancestral lineages of *P. arrabidae* group radiated from the 'Chapada Diamantina'. Instead, we speculate that *P. catingicola* subsp. *catingicola* may have spread from the dry coast towards inland areas through dry valleys. This could be possible especially when considering the sea-level drop during glacial times (Gandini et al., 2014), which must have exposed a huge corridor of sandy sediments of the continental shelf along the coast. This alternative hypothesis is compatible with palaeo-distribution models for Atlantic Forest (Carnaval and Moritz, 2008) and sediment/pollen records (Auler et al., 2004), which denote that the area currently occupied by this taxon (or at least most of it) probably was not suitable for xeric plants until a few thousands of years ago (Fig. 1).

The placement of haplotypes H4 and Clade A in the phylogenetic tree and in the haplotype network suggests that *Pilosocereus* may have spread northwards from the dry coast of Rio de Janeiro State, where the range of many cactus species from inland areas reaches the coast (Taylor and Zappi, 2004). This hypothetical route of colonization of the *Caatinga* by the *P. arrabidaei* group overlaps the current distribution of several xeric legumes that occur along the 'Pleistocene Arc' (Mogni et al., 2015). Such a migration corridor is also compatible with the most probable ancestral area for the BCT Clade reconstructed by Hernández-Hernández et al. (2014).

The lack of intra-populational diversity was not surprising, since this pattern has been observed for other plants from coastal habitats in eastern Brazil (e.g. Lira et al., 2003) and other species of the genus *Pilosocereus* (Bonatelli et al., 2014). Since the demographic tests (BSP Analysis, Tajima's D and Fu's F_s statistics) provided no significant result (possibly due to the low number of haplotypes found for our focal group), we arrived at no conclusion about our third hypothesis, on the influence of Pleistocene climate cycles in the demography of the species from the *P. arrabidaei* group. However, the low genetic diversity found in the *P. arrabidaei* group as well as the star-shaped genealogical pattern of clade A are congruent with a scenario of recent bottlenecks experienced by these species.

The hypotheses of a single refuge in the 'Chapada Diamantina' and high gene flow among populations were definitely refuted for *P. arrabidaei* and allies. The phylogeographic pattern obtained for the group is completely opposite to them, since the number of haplotypes in this region is the lower, and the distribution of haplotypes is geographically structured between the major rivers. We deduce that seed-mediated gene flow has been low enough across these rivers, allowing lineage sorting and random drift and promoting the genetic divergence between populations isolated by rivers.

There is some controversy regarding the effectiveness of rivers as plant species geographic barriers for gene flow in Neotropical species (Turchetto-Zolet et al., 2013), as their pollinators and seed dispersers are often flying animals. In the case of *Pilosocereus* (and other columnar cacti), there are few studies that identify with certainty their effective dispersors in eastern Brazil. Apparently their main dispersing agents are Phyllostomidae bats (Godinez-Alvarez and Valiente-Baunet, 2000; Gomes et al., 2014; Munguia-Rosas et al., 2009) and passeriform birds belonging to various families (Gomes et al., 2008; Munguia-Rosas et al., 2009). Radio-telemetry studies of Phyllostomidae bats show that their home range varies around tens of hectares (Loayza and Loiselle, 2008; Trevelin et al., 2013), sometimes reaching hundreds of hectares (Aguiar et al., 2014). Even though some bats may sporadically fly distances of 47 km in a single night (Meyer et al., 2005), they normally alternate short flights of 500 m with longer ones of up to 3.8 km within their home range (Aguiar et al., 2014). Similar studies considering passeriform birds show that their home range is even smaller, comprising only a few hectares (e.g. Duca et al., 2006; Reichard and Ketterson, 2012). This shows that both Phyllostomidae bats and passeriform birds are potentially able to cross over the rivers mentioned above. Although these gaps might have been wider in the past (during fluctuations of the sea level), nowadays the width of the São Francisco River mouth is around 2 km, and the Doce River is c. 1 km. However, taking into consideration the flight and home ranges of these animals, we consider that long flight events are not very frequent. Moreover, in the case of bats, it is necessary to consider that the seed content in their faeces is relatively low and that these are not frequently deposited in adequate spots for their germination (Godinez-Alvarez and Valiente-Baunet, 2000), thus diminishing the probability of effective long distance dispersal.

In this sense, the fact that the seeds of the plants here studied are dispersed by birds or bats does not contradict our results. The importance of the rivers in the genetic structure of *P. arrabidaei* and *P. catingicola* was reinforced by the AMOVA results, which showed that genetic variation is partitioned mainly among geographic regions delimited by the São Francisco and Jaguaribe rivers. Besides, examples of rivers acting as geographical barriers are not rare for Cactaceae of Eastern Brazil. According to Nigel P. Taylor (personal observation) *Pereskia bahiensis* and *Stephanocereus leucostele* (both of which are widespread in the *Caatinga* of Bahia state) fail to cross the São Francisco River into a very similar habitat in Pernambuco state. In our case, the majority of discontinuities in the geographic distribution of *P. arrabidaei* and *P. catingicola*, as well as in their genetic structure, coincide with some riverine boundaries (the Doce, Jequitinhonha, Contas, São Francisco and Jaguaribe rivers) rather than to biome boundaries. These rivers coincide with occurrence gaps and genetic discontinuities for plant of other families (e.g. Novaes et al., 2010; Ribeiro et al., 2011; Pinheiro et al., 2016). The unique exception is the gap found between the distribution of *P. arrabidaei* and *P. catingicola* in Southern Bahia, between the Contas and Jequitinhonha rivers. This gap also can be attributed to habitat unsuitability – i.e. an extensive historically stable area occupied by humid Atlantic Forest in this region (according to Carnaval and Moritz, 2008, Fig. 1), which may have been sufficient to prevent the establishment and growth of terrestrial cactus seedlings.

5. Conclusions

With almost all predictions refuted, it becomes important to trigger some reflections upon the evolution of *Pilosocereus* and the Neotropical flora as a whole. The results here presented suggest that some Neotropical xeric plants, like *Pilosocereus*, may be currently undergoing a speciation process. Thus, when studying the evolution of Neotropical flora, it is important to consider the recent climatic history of each biogeographic region, as well as the possible influence of rivers in seed-mediated gene flow, especially for xeric plants. Have broad rivers inhibited the movement of fruit-eating seed vectors and thereby caused the partitioning of plant populations leading on to speciation? This might be a plausible explanation. If we take into account the converse situation (of rivers drying up during the Pleistocene climatic fluctuations and thereby permitting the movement of diaspores), it is possible to explain the disjunct distributions of other cactus species, such as *Arrojadoa penicillata*, *Harrisia adscendens* and *Tacinga funalis* across the São Francisco River (Taylor and Zappi, 2004: 67). If the phylogeographic pattern of *P. arrabidaei* and allies is a consequence of the climatic and topographical history of the region, similar patterns should be expected in other groups of xeric plants that occur in the *Caatinga*.

Also, the evolutionary processes responsible for the diversification of the Neotropical xeric flora may operate simultaneously in various ways, depending on the characteristics and climatic history of each biogeographic region, as seen for the *P. arrabidaei* group (Northeast Brazil) and *P. aurisetus* group (Southeast Brazil). Thus, phylogenetic and phylogeographic patterns expected for SDTF taxa (like those presented by Hughes et al., 2013) may have variants, as well as the refuge models during periods of adverse environmental conditions.

Especial attention must also be given to the polarization of morphological traits for plants that live in arid environments (for functional and taxonomical purposes). In the case of *Pilosocereus*, the delimitation of informal taxonomic groups is grounded on the hypothesis that the traits of the tree-like *P. arrabidaei* group are plesiomorphic within the subgenus *Pilosocereus* – which now seems to be incorrect. It seems that the diversification of

Pilosocereus occurred through many parallel evolutionary events in different regions, and that the tree-like habit and the bat pollinated flowers of the *P. arrabidae* group are related to a recent colonization of a closed shrubby vegetation of *Caatinga*.

Although we did not find significant evidence that the Pleistocene wet periods in northeastern Brazil affected the demography of *P. catingicola* (probably due to our low number of haplotypes), the most obvious explanation for the observed phylogeographic pattern is a recent evolutionary history with a number of bottleneck events in multiple refuges (between major rivers), followed by rapid populational and range expansion. Yet, phenomena like the sea-level drop and the high moisture periods during glaciations may have somehow influenced the evolution and assembly of xeric plants of the *Caatinga*, which possibly reached this region through the dry Atlantic coast.

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