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Intra and interspecific sequence variation in closely related species of *Cereus* (CACTACEAE)

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ABSTRACT

In comparative phylogenetic and population genetic studies, one of the most crucial steps is to select appropriate DNA markers, a decision based primarily on the estimated variation in markers in cross-taxonomic surveys. To assess whether genetic variation at the intraspecific level in one species predicts the variation in another closely related species we used two congeneric species of *Cereus* (Cactaceae: Cereeae). We screened and characterized eight noncoding plastid regions (*trnS-trnG*, *atpI-atpH*, *trnT-trnL*, *psbD-trnT*, *petL-psbE*, *3'rps16-5'trnk*, *trnG intron*, and *trnL intron*), and one nuclear gene (*PhyC*) in *Cereus fernambucensis* and *C. hildmannianus*. A total of 40 individuals from 15 populations were characterized according to nucleotide diversity, number of haplotypes, and number of potentially informative characters. The results revealed that nucleotide substitutions and indels are the main source of variation, with the largest divergence between species found in *trnS-trnG*. The *trnL* intron and *petL-psbE* showed intraspecific variability in both species. The *psbD-trnT*, *atpI-atpH*, *trnS-trnG*, and *trnT-trnL*, which are the most variable regions in one species, showed no variation in the other. Finally, the nuclear gene *PhyC* showed more resolution between *Cereus* species than within species. We thus found considerable heterogeneity among widely used plastid markers, even between closely related species, and suggest the use of *PhyC* as a marker for phylogenetic inference in these species. These results reinforce the need of screening as a preliminary step to conduct phylogeographic or phylogenetic studies in face of unpredictable sequence variation of molecular markers in plants.

1. Introduction

Historically, chloroplast DNA (cpDNA) has been considered the most suitable genome to use for phylogeographic studies in plants (Avise, 2009). The wide taxonomic spectrum reached by "universal primers" (Taberlet et al., 1991; Shaw et al., 2005) allowed the use of noncoding cpDNA sequences for molecular work in systematics at a higher taxonomic level, but also

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proved useful in population-level studies (e.g.*trnT-trnF*, Taberlet et al., 1991; *trnK-matK*, Johnson and Soltis, 1994). A large screening of universal primers used for plant taxa classified the utility of noncoding cpDNA regions according to their slow and fast mutation rates (Small et al., 1998; Shaw et al., 2005; Prince, 2015). In addition, low and single copy nuclear markers have also been included in phylogenetic and phylogeographic studies to enhance resolution (see Zimmer and Wen, 2012). However, a well-known phenomenon of substitution rate variation among plant lineages (Romeiro-Brito et al., 2016) has increased the search for universally informative regions that enables the researchers to select the most suitable markers for comparative studies in phylogeny, biogeography, and population genetics analyses.

Shaw et al. (2005, 2007; 2014) have identified a series of plastid regions that are likely variable, at least for species-level comparisons. These candidate regions have been employed routinely in phylogenetic studies. However, the usefulness of these markers is not phylogenetically constrained and thus largely unpredictable. In molecular dating analyses, early methods assuming rate auto-correlation (e.g. r8s, Sanderson, 2003) have been largely superseded by those that allow this assumption to be relaxed (e.g. BEAST, Drummond et al., 2006), in accordance with studies that indicate that lineage-specific substitution rates are in fact the rule rather than the exception (Gustafsson et al., 2010; Romeiro-Brito et al., 2016).

Although the degree of genetic variation within a marker bears directly on the power and reliability of all subsequent analyses, the usefulness of universal markers at low taxonomic levels remains largely unexplored. Our aim is to assess whether genetic variation at the intraspecific level in one species predicts the variation in a closely related species. To investigate this, we use two South American congeneric cacti species: *Cereus fernambucensis* and *C. hildmannianus* (Cactaceae: Cereeae).

2. Material and methods

We screened molecular markers from noncoding plastid regions described as showing different levels of Potentially Informative Characters (PICs; Shaw et al., 2005) across angiosperms, according to Shaw et al. (2005, 2007; 2014) these were categorized as low (*trnL* intron), medium (*trnG* intron), and high variation (*trnS-trnG*, *trnT-trnL*, *atpI-atpH, 3'rps16-5'trnk*, *psbD-trnT*, and *petL-psbE*) regions. We also included the low copy nuclear gene phytochrome C (*PhyC*), as nuclear genes complements plastid DNA in phylogenetic studies, especially in order to infer species trees from unlinked markers (Pamilo and Nei, 1988). The sampling included 40 individuals from *C. fernambucensis* and *C. hildmannianus* distributed among 15 populations in eastern South America (Table 1, Fig. 1).

Genomic DNA was extracted from root tissues using the DNeasy Plant Mini Kit (Qiagen). Total DNA was quantified on a 1% agarose gel through a comparison with the molecular weight marker Low DNA Mass Ladder (Invitrogen). A polymerase chain reaction (PCR) was run with the following reaction: 1x reaction buffer, 3.0 mM $MgCl_2$, 200 μM of each dNTP, 0.1 μM of each primer, 1.25u GoTaq® Flexi DNA Polymerase (Promega), 10–60 ng template DNA, and nuclease-free water to a total volume of 25 μL. Primer sequences are given in Table 2. In *C. fernambucensis*, different concentrations of $MgCl_2$ were used for the PCR amplification of the *trnL* intron and *trnS-trnG* (1.5 mM), and *psbD-trnT* and *petL-psbE* (4.8 mM). For *C. hildmannianus* it was necessary to use different concentrations of $MgCl_2$ and/or Taq DNA polymerase in the *trnG* intron (1.5 mM $MgCl_2$ and 0.4 U Taq), and *trnS-trnG* (1.5 mM $MgCl_2$).

PCRs were performed with a Mastercycler Gradient (Eppendorf AG, Hamburg, Germany), following Shaw et al. (2005, 2007), with annealing temperatures varying according to each marker, without ramp step. For *C. fernambucensis*: 50 °C for *3'rps16-5'trnk* and *trnT-trnL*; 52 °C for *psbD-trnT*, *petL-psbE* and *trnG* intron; 62 °C for *trnS-trnG* and *trnL* intron; and 56 °C for *atpI-atpH*. For *C. hildmannianus*: 50 °C for *3'rps16-5'trnk*, *trnT-trnL*, *psbD-trnT*, *petL-psbE*, *trnG* intron, *trnL* intron, *atpI-atpH* and 62 °C for *trnS-trnG*. For *PhyC* amplification, PCR conditions followed Helsen et al. (2009), with a modification of the annealing temperature of *C. hildmannianus* to 54 °C. The quality of the PCR products was evaluated on a 1% agarose gel, stained with SYBR safe and purified with ExoSAP-IT for the *C. fernambucensis* samples and with the GFXTM PCR and Gel Band Purification kit (GE Healthcare) for the *C. hildmannianus* samples.

Sequencing was performed with the Big Dye terminator, version 3.1 cycle sequencing kit (Applied Biosystems) and a Gene Amp PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA, USA). Chromas 1.5 (Technelysium Pty Ltd., Tewantin, Australia) was used for the assembly of forward and reverse DNA sequences. Sequences were aligned and edited using ClustalW (Thompson et al., 1994), employed in BioEdit (Hall, 1999).

The number of haplotypes (Hap) and the nucleotide diversity (π) were estimated using DnaSP v.5.1 (Librado and Rozas, 2009). PICs were computed for each molecular marker by adding the nucleotide substitutions, indels, and inversions (NS, ID, and IV, respectively). In order to obtain the proportion of variable sites (% variability), the PIC value was divided by the total length of the sequence (L) and multiplied by 100.

3. Results

A total of 90 novel DNA sequences from nine molecular markers were generated (Table 1). The index of nucleotide diversity and the number of haplotypes (Table 3) indicated that of the eight noncoding regions that were sampled, *trnS-trnG* and *psbD-trnT* had the highest variability in *C. fernambucensis*, whereas *atpI-atpH*, *petL-psbE*, and the *trnL* intron were the most variable markers in *C. hildmannianus* (in decreasing order of variation). For the nuclear gene *PhyC*, nucleotide diversity was lower, but the number of haplotypes was similar to the plastid regions.

Table 1
GenBank accession numbers and location codes of samples used in this study for eight noncoding plastid DNA markers and the nuclear gene *PhyC*.

Code[a]/Voucher[b]	N	Molecular markers								
		psbD-trnT	***atpI-atpH***	***trnG***	***trnL***	***petL-psbE***	***trnS-trnG***	***3′rps16-5′trnK***	***trnT-trnL***	***PhyC***
C. hildmannianus										
A01/SPFR11888	4	KT164977	KT164960	–	KT164954	KT164944	KT164938	KT164986	KT164929	KT164917
A02/SPFR12343	4	KT164978	KT164961	KT164970	KT164955	KT164945	KT164939	KT164987	KT164930	KT164918
A15/SPFR12333	1	–	–	KT164971	–	–	–	–	–	–
A16/SPFR12351	1	KT164979	–	KT164972	–	–	–	–	–	–
A17/SPFR12346	3	KT164980	KT164962	KT164973	KT164956	KT164946	KT164940	KT164988	KT164931	KT164919 KT164920
A18/SPFR12344	5	–	KT164963	–	KT164957	KT164947	KT164941	KT164989	KT164932	KT164921 KT164922
A23/SPFR12342	3	KT164981	KT164964	KT164974	KT164958	KT164948	KT164942	KT164990	KT164933	KT164923 KT164924
C. fernambucensis										
S68/CCTS2657	3	KT164975	–	KT164965	KT164949	–	KT164934	KT164982	KT164925	KT164912
S72/CCTS2658	2	–	KR998177	KT164966	–	KR998108	–	–	KT164927	–
S76/CCTS2662	3	KR998081	KR998178	KT164968	–	KR998115	KT164937	KR998171	KR998127	KT164913
S82/CCTS2665	1	–	–	KT164969	KT164951	–	–	–	–	–
S80/CCTS2663	4	KT164976	KT164959	–	KT164950	KT164943	KT164935	KT164983	KT164926	KT164914
S87/CCTS2668	1	–	–	KT164967	KT164952	–	–	–	–	–
S88/CCTS2749	2	KR998084	KR998183	–	KT164953	KR998116	KR998162	KT164984	–	KT164915
S89/CCTS2669	3	KR998085	KR998182	–	–	KR998117	KT164936	KT164985	KT164928	KT164916

N – Number of individuals. Dashes (–) indicate individuals not sampled for Code.
[a] The location codes are shown in Fig. 1.
[b] Vouchers are deposited for each location sampled in the Herbarium of São Paulo Faculdade Ribeirão Preto (SPFR), University of São Paulo, and Herbarium of Centro de Ciências e Tecnologias para a Sustentabilidade (SORO), Federal University of São Carlos.

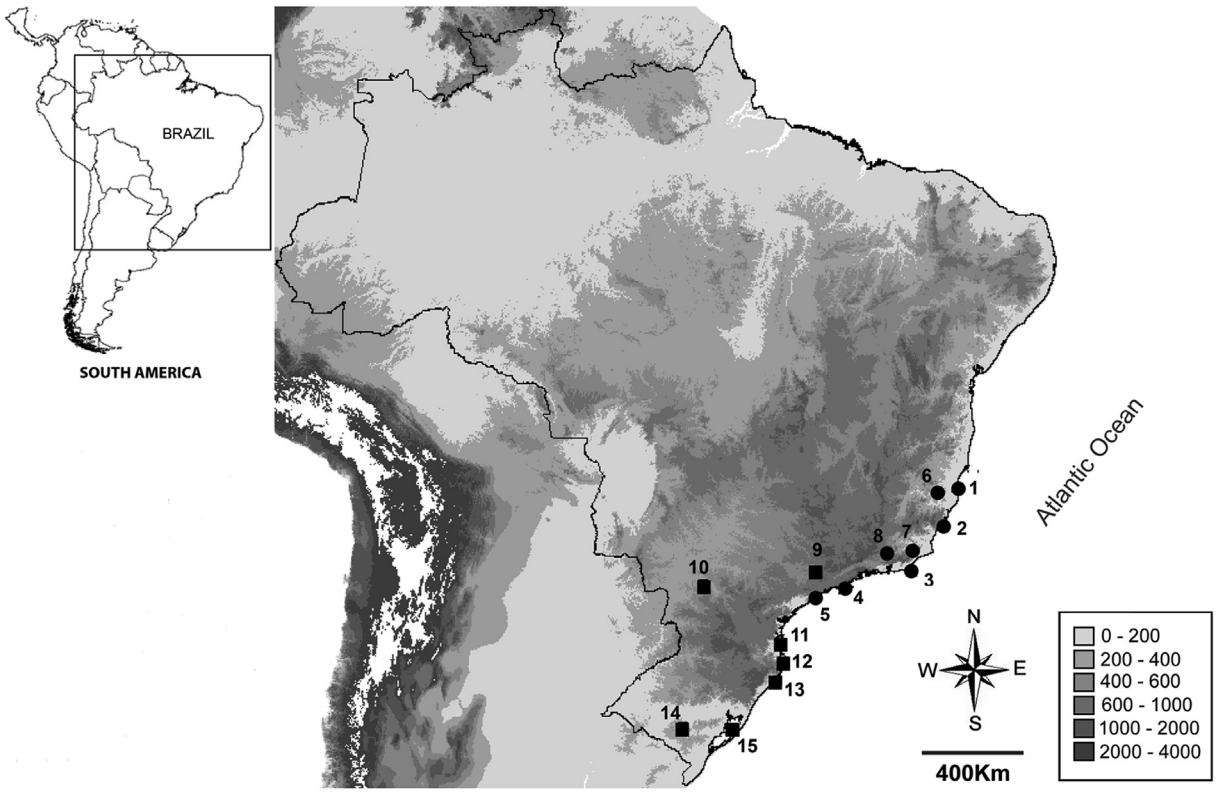

Fig. 1. Geographical sampling of *Cereus fernambucensis*(black circles), and *C. hildmannianus* (black square). Map showing Brazil, in eastern South America, with localities and codes: 1–São Mateus, ES (S89); 2–Guarapari, ES (S87); 3–Arraial do Cabo, RJ (S80); 4–Ubatuba, SP (S72); 5–Peruíbe, SP (S68); 6–Águia Branca, ES (S88); 7–Santa Maria Madalena, RJ (S82); 8–Três Rios, RJ (S76); 9–Itatiba, SP (A01); 10–Cianorte, PR (A23); 11–Penha, SC (A16); 12–Florianópolis, SC (A18); 13–Laguna, SC (A17); 14–Caçapava do Sul, RS (A02); 15–Mostardas, RS (A15). The bottom right legend indicates altitude (meters above sea level).

Table 2
Sequences of primers used to amplify noncoding plastid DNA markers and the nuclear gene *PhyC* in *Cereus fernambucensis and C. hildmannianus.*

Region	Primers	Reference
Nuclear		
PhyC	**phyC-R** TCCTCCACTTGACCACCTCT	Helsen et al. (2009)
	phyC-F AGCTGGGGCTTTCAAATCTT	
Plastid DNA		
trnG intron	**5′trnG2G** GCG GGT ATA GTT TAG TGG TAA AA	Shaw et al. (2005)
	trnG $^{(UUC)}$ GAA TCG AAC CCG CAT CGT TAG	Shaw et al. (2007)
trnS-trnG	**SG Rev 2** TCC GCT CAT TAG CTC TCC TC	Bonatelli et al. (2013)
	trnS$^{(GCU)}$ AAC TCG TAC AAC GGA TTA GCA ATC	Shaw et al. (2007)
	SG Fwd 2 CAC CCA TGG TTC CCA TTA GA	Bonatelli et al. (2013)
	5′trnG2S TTT TAC CAC TAA ACT ATA CCC GC	Shaw et al. (2005)
trnT-trnL	**5′trnL^{UAA}R (TabB)** TCT ACC GAT TTC GCC ATA TC	Taberlet et al. (1991)
	trnT^{UGU}F (TabA) CAT TAC AAA TGC GAT GCT CT	
trnL intron	**trnL5′^{UAA}F (TabC)** CGA AAT CGG TAG ACG CTA CG	Taberlet et al. (1991)
	3′trnL^{UAA}R (TabD) GGG GAT AGA GGG ACT TGA AC	
atpI-atpH	**atpI** TAT TTA CAA GYG GTA TTC AAG CT	Shaw et al. (2007)
	atpH CCA AYC CAG CAG CAA TAA C	
3′rps16-5′trnK	**rpS16x2F2** AAA GTG GGT TTT TAT GAT CC	Shaw et al. (2007)
	trnK$^{(UUU)}$x1 TTA AAA GCC GAG TAC TCT ACC	
petL-psbE	**petL** AGT AGA AAA CCG AAA TAA CTA GTT A	Shaw et al. (2007)
	psbE TAT CGA ATA CTG GTA ATA ATA TCA GC	
psbD-trnT	**psbD** CTC CGT ARC CAG TCA TCC ATA	Shaw et al. (2007)
	trnT(GGU)-R CCC TTT TAA CTC AGT GGT AG	

The main sources of variability were nucleotide substitutions and indels (Table 3), of which only two substitutions and three indels were found in seven or more mononucleotide repeats in the cpDNA sequences. Genetic variability ranged at the intraspecific level from 0.19% to 2.8% in *C. fernambucensis*, 0.17%–0.34% in *C. hildmannianus*, and from 0.28% to 3.9% at the interspecific level. Six of the tested regions presented intraspecific variability in *C. fernambucensis* (*trnS-trnG*, *trnL* intron, *trnT-trnL*, *psbD-trnT*, *petL-psbE*, and *PhyC*) and four presented intraspecific variability in *C. hildmannianus* (*atpI-atpH*, *petL-psbE*, *trnL* intron, and *PhyC*). Three markers were variable for both *Cereus* species (*trnL* intron, *petL-psbE*, and *PhyC*), of which only the nuclear gene exhibited similar PICs in both species. For noncoding regions, this estimate was different for each species. The region containing the highest PIC for *C. fernambucensis* (*trnS-trnG*) showed no variation in *C. hildmannianus*. Similarly, the most informative region for *C. hildmannianus* (*atpI-atpH*) showed no variation in *C. fernambucensis*. At the interspecific level, *trnS-trnG* was also the most variable marker. The only marker that showed no variability in either species was *3'rps16-5'trnk*.

4. Discussion

Of the six most informative noncoding plastid regions selected from Shaw et al. (2005, 2007), four revealed usefulness at low taxonomic level in *Cereus*. For *C. hildmannianus*, the *trnL* intron contained the lowest intraspecific information, in agreement with Shaw et al. (2005). However, this marker had a high PIC in *C. fernambucensis* and many informative characters that separate the two *Cereus* species. Similarly, for the nuclear gene *PhyC* we found low PIC values, haplotype numbers, and nucleotide diversity at the intraspecific level, but considerable information between species. Based on our results we suggest the use of *PhyC* and three plastid markers: *trnS-trnG*, the *trnL* intron, and *petL-psbE* for phylogenetic purposes among species of *Cereus*. We suggest using *trnS-trnG* for further intraspecific studies in *C. fernambucensis* and *atpI-atpH* in *C. hildmannianus*.

In Cactaceae, two of the variable plastid regions examined here (*trnS-trnG* and *atpI-atpH*) contained variation at both population and species level in *Pilosocereus aurisetus* (Bonatelli et al., 2013). The *trnS-trnG* region, which exhibited the highest PIC between closely related *Cereus* species (Table 3), is also useful for phylogenetic studies within the genus *Cereus* (Romeiro-Brito et al., 2016 – Appendix S1), although a high PIC does not necessarily imply that a region is phylogenetically informative (Korotkova et al., 2014).

Previous works have screened a number of noncoding regions in plants to find appropriate markers for various taxonomic levels (Särkinen and George, 2013; Korotkova et al., 2014; Shaw et al., 2014). The main picture that emerges from these results is that the evolutionary rates of plastid regions are highly variable and different markers are found to be the most appropriate for phylogeographic studies, according to the focal group. This highlights the unpredictable variability of markers, as is found here in the case of closely related *Cereus* species.

In angiosperms, eight consistently variable noncoding plastid regions are suggested for an initial screening at low taxonomic level, or alternatively, looking for some of the most variable plastid regions from the major lineage as closest as to the focal group (Shaw et al., 2014). Although this may be useful general advice, emerging evidence suggests that researchers should not rely exclusively on it. In the current study, the four regions (*psbD-trnT*, *atpI-atpH*, *trnS-trnG*, and *trnT-trnL*) that were among the most variable at the species level in one *Cereus* species showed no variation in the other species investigated, demonstrating the lack of predictability in sequence variation among these markers. Similar to the results reported here, Särkinen and George (2013) found that different levels of variation in plastid markers between two closely related *Solanum*

Table 3
Diversity indices based on noncoding plastid DNA and the nuclear gene *PhyC*.

	Cereus fernambucensis				*C. hildmannianus*				*C. fernambucensis* + *C. hildmannianus*			
Markers	N	π	Hap	PIC/L (SN+ID+IV) % variability	N	π	Hap	PIC/L (SN+ID+IV) % variability	N	π	Hap	PIC/L (SN+ID+IV) % variability
psbD-trnT	05	0.0012	3	2/675 (2+0+0) 0.29	05	0.0000	1	0/667 –	10	0.00306	4	6/676 (5+1+0) 0.88
atpI-atpH	05	0.0000	1	0/543 –	05	0.0017	3	2/591 (2+0+0) 0.34	10	0.00179	4	3/591 (3+0+0) 0.50
***trnG* intron**	05	0.0000	1	0/714 –	05	0.0000	1	0/476 –	10	0.00078	2	3/716 (1+2+0) 0.42
***trnL* intron**	05	0.0000	2	6/647 (0+6+0) 0.93	05	0.0011	2	1/581 (1+0+0) 0.17	10	0.00137	4	26/665 (2+24+0) 3.90
petL-psbE	05	0.0008	2	1/505 (1+0+0) 0.19	05	0.0011	2	1/531 (1+0+0) 0.19	10	0.00629	4	8/541 (7+1+0) 1.48
trnS-trnG	05	0.0026	3	34/1213 (7+27+0) 2.8	05	0.0000	1	0/961 –	10	0.00425	4	45/1213 (13+32+0) 3.71
3′rps16-5′trnK	05	0.0000	1	0/285 –	05	0.0000	1	0/221 –	10	0	1	0/285 –
trnT-trnL	05	0.0012	2	1/321 (1+0+0) 0.31	05	0.0000	1	0/348 –	10	0.00057	2	1/348 (1+0+0) 0.28
PhyC	05	0.0009	3	2/785 (2/0/0) 0.25	08	0.0007	3	2/998 (2/0/0) 0.20	13	0.00134	6	5/998 (5/0/0) 0.50

N – number of samples, **π** – Nucleotide diversity, **Hap** – number of haplotypes, **PIC/L** – ratio of the number of Potentially Informative Characters to the number of base pairs, **SN** – nucleotide substitutions, **ID** – indels, and **IV** – inversions.

clades (Solanaceae) indicated a narrower applicability of family-specific screening by demonstrating the unpredictable variability of these markers. Likewise, in *Pyrus* (Rosaceae), the variability of the fastest evolving plastid regions is also lineage-specific, suggesting that the mutational dynamics of plastid genomic regions may follow their own path in different lineages (Korotkova et al., 2014).

Nuclear markers were proven useful for inferring phylogenetic resolution at the interspecific level in other plant groups (Hughes et al., 2006). Whenever possible, the choice of low copy nuclear genes jointly with cpDNA markers may improve resolution for phylogeographic and phylogenetic analyses, since cpDNA markers only reflect the maternal lineages and might also fail to provide sufficient resolution (Sang, 2002; Hughes et al., 2006; Zimmer and Wen, 2012).

In Cactaceae, a higher resolution at deep nodes has been demonstrated when *PhyC* is combined with markers of uniparental inheritance (chloroplast and mitochondrial) (Butterworth and Edwards, 2008; Arakaki et al., 2011). However, low or absent variation for this region at the interspecific level in several genera of cacti (*Pereskia*—Butterworth and Edwards, 2008; *Opuntia*—Helsen et al., 2009; *Pilosocereus*—Bonatelli et al., 2014) shows the unpredicted nature of variability and phylogenetic information for this gene. Aside from the lack of predictability in noncoding plastid sequences and phylogenetic information from *PhyC*, we found relatively low PICs in both cactus species (Table 3). In plants, the lack of resolution among closely related species may be due to adaptive radiations resulting in short evolutionary histories and low sequence divergence even in rapidly evolving non-coding DNA (Small et al., 1998). Following this, the relatively low PICs for *C. fernambucensis* and *C. hildmannianus* could be due to a recent diversification in *Cereus* (Romeiro-Brito et al., 2016—Appendix S1). This may be a common pattern in the family Cactaceae, which—despite its present high diversity—did not begin to diversify extensively until relatively recently (c. 10–5 Ma, Arakaki et al., 2011).

In summary, for obtaining phylogenetic information at low taxonomic levels (among closely related species and populations) we emphasize the importance of thoroughly searching for informative molecular regions—rather than simply choosing a region proposed by cross-taxonomic surveys. This applies even at the interspecific level, as highlighted by the unpredictable sequence variation between *C. fernambucensis* and *C. hildmannianus*. Independently of methodological approach chosen (e.g., first or next-generation sequencing), this should be one of the first steps in experimental design for molecular studies. For building robust and well resolved phylogenetic and phylogeographic hypotheses it remains crucial to invest time and care on the choice of sequencing regions.

Conflict of interest

The authors declare that they have no conflict of interest.

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