RESEARCH PAPER

The role of hybridization and introgression in maintaining species integrity and cohesion in naturally isolated inselberg bromeliad populations

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ABSTRACT

- Hybridization is a widespread phenomenon present in numerous lineages across the tree of life. Its evolutionary consequences range from effects on the origin and maintenance, to the loss of biodiversity.
- We studied genetic diversity and intra- and interspecific gene flow between two sympatric populations of closely-related species, *Pitcairnia flammea* and *P. corcovadensis* (*Bromeliaceae*), which are adapted to naturally fragmented Neotropical inselbergs, based on nuclear and plastidial DNA.
- Our main results indicate a strong reproductive isolation barrier, although low levels of interspecific gene flow were observed in both sympatric populations. The low rates of intraspecific gene flow observed for both *P. corcovadensis* and *P. flammea* populations corroborate the increasing body of evidence that inselberg bromeliad species are maintained as discrete evolutionary units despite the presence of low genetic connectivity. Nuclear patterns of genetic diversity and gene flow revealed that hybridization and introgression might not cause species extinction *via* genetic assimilation of the rare *P. corcovadensis*.
- In the face of reduced intraspecific gene exchange, hybridization and introgression may be important aspects of the *Pitcairnia* diversification process, with a positive evolutionary impact at the bromeliad community level, and thus contribute to increasing and maintaining genetic diversity in local isolated inselberg populations.

INTRODUCTION

Hybridization has long captured the interest of biologists (*e.g.* Linnaeus 1760), but it was generally considered a rare event with little impact on evolutionary processes (Mayr 1963). In recent decades, ever-growing expertise in genome sequencing and statistical analysis has greatly facilitated gene flow detection, revealing that hybridization and introgression are more common than previously believed. Hybridization is now recognised as a widespread phenomenon present in most lineages across the tree of life, with significant evolutionary consequences (Abbott *et al.* 2016; Goulet *et al.* 2017; among others). By allowing gene flow and recombination between divergent lineages, hybridization can influence the creation, maintenance and loss of biodiversity. In several lineages, hybridization may have contributed to speciation and adaptation by generating genetic variation, functional novelty and even new species (Seehausen 2004; Abbott *et al.* 2016). However, detrimental consequences of hybridization such as outbreeding depression, genetic assimilation and gene swamping may push populations toward extinction (Gómez *et al.* 2015). For example, risk of extinction due to hybridization has been reported between common species and closely related but rare or endangered relatives (Lepais *et al.* 2009; Balao *et al.* 2015).

Hybridization occurs more frequently among closely related species and is a common process in recently diverged lineages, such as those experiencing rapid species radiation (Mallet *et al.* 2007). Recent adaptive radiations are good systems for studying the evolution of pre- and post-zygotic reproductive isolation barriers and for understanding how genetic drift and divergent selection may interplay to promote population differentiation, local adaptation and speciation (Schluter 2000; Seehausen 2004). Moreover, hybridization may facilitate adaptive radiation by promoting speciation events (Seehausen 2004) and accommodating adaptation *via* the creation of functional genetic variation or breaking of genetic correlations that constrained the evolvability of the parental lineages (Mallet *et al.* 2007; Meier *et al.* 2017).

The family *Bromeliaceae* is a well-characterised plant system that went through adaptive radiation in the Neotropics (Givnish *et al.* 2011; Silvestro *et al.* 2014; Palma-Silva *et al.* 2016). It is well known that hybridization and reticulate evolution played important roles in the evolution and diversification of this young plant family (Palma-Silva *et al.* 2016). Hybridization and reticulate evolution are often reported as challenges to phylogenetic reconstruction and species delimitation in the *Bromeliaceae* (Barfuss *et al.* 2016; Palma-Silva *et al.* 2016; Pinangé *et al.* 2016; Goetze *et al.* 2017). Many

bromeliads are partially interfertile in artificial crosses (Rôças *et al.* 2004; Wagner *et al.* 2015) and natural hybridization has been recorded in some species (Gardner 1984; Luther 1985; Wendt *et al.* 2001, 2008; Gonçalves & de Azevêdo-Gonçalves 2009; Schulte *et al.* 2010), but few studies have analysed bromeliad hybrid zones in detail and reported interspecific gene flow (introgression) estimates (*Pitcairnia*: Palma-Silva *et al.* 2011; *Alcantarea*: Lexer *et al.* 2016; *Vriesea*: Zanella *et al.* 2016; Neri *et al.* 2017). The amount of interspecific gene flow that actually occurs among closely related bromeliads and the consequences of this interspecific gene flow on speciation and adaptive diversification of species-rich bromeliad lineages remain to be determined.

The genus *Pitcairnia* is one of the largest and most widely distributed genera of *Bromeliaceae*. *Pitcairnia* began to diversify 10 Mya (Givnish *et al.* 2011), and today the genus possesses more than 450 species that are mainly found in Central and South America (Saraiva *et al.* 2015). Most of these species are endemics of naturally fragmented habitats, such as rock outcrops and inselbergs, which are described as 'terrestrial islands' (Porembski & Barthlott 2000), making this group a good model for studying the interactions of intra- and interspecific gene flow, genetic drift and selection during plant speciation and radiation. Our previous research showed that reproductive barriers among closely related and morphologically divergent *Pitcairnia* species were strong but permeable, allowing interspecific gene flow among Neotropical inselberg species (Palma-Silva *et al.* 2011, 2015). In *Pitcairnia*, reinforcement of mating system shifts (from outcrossing to selfing) was important (Palma-Silva *et al.* 2015), as were a variety of other prezygotic (flower phenology and pollinator selection) and post-zygotic (Bateson-Dobzhansky-Muller genetic incompatibilities) barriers for maintaining species integrity (Wendt *et al.* 2001; Palma-Silva *et al.* 2011). Here, we extend these previous works to investigate genetic diversity and intra- and interspecific gene flow between sympatric populations of two closely related species adapted to inselbergs within the Brazilian Atlantic Forest: *Pitcairnia flammea* Lindl. and *P. corcovadensis* Wawra (*Bromeliaceae*). In our previous paper, plastidial haplotype sharing between *P. corcovadensis* and *P. flammea* within an inselberg suggested hybridization and/or incomplete lineage sorting might occurring (Palma-Silva *et al.* 2011), although nuclear genetic patterns of gene flow were not investigated. Additionally, artificial cross experiments indicated that F_1 hybrids and backcrosses in both directions are partially fertile (Wendt *et al.* 2002; Rôças *et al.* 2004), suggesting that hybridization and interspecific gene flow may occur in wild sympatric populations.

Pitcairnia flammea is one of the most morphologically variable and widespread bromeliad species in the Brazilian Atlantic Forest, whereas *P. corcovadensis* is a rare species that is narrowly distributed on a few inselbergs, always sympatrically with *P. flammea*. *Pitcairnia corcovadensis* is a tiny plant with up to seven flowers per inflorescence (Wendt *et al.* 2000), which was once considered a morphological variant of *P. flammea* but has been re-established as a distinct species (Wendt *et al.* 2000). These two species have similar flower morphologies, with diurnal anthesis and large red tubular flowers, characteristic of hummingbird pollination (Wendt *et al.* 2000, 2002). *Pitcairnia corcovadensis* is mainly autonomously self-pollinated with no nectar production, while *P. flammea* produces nectar and has

pollinator-dependent seed set (Wendt *et al.* 2002). Moreover, the species are self-compatible and have slightly different peak flowering times (Wendt *et al.* 2002).

In this study, we aimed to contribute to elucidation of the role of hybridization and introgression in speciation and diversification of this recently radiated Neotropical family. We employed plastidial and nuclear markers to investigate the occurrence of hybridization and introgression in two sympatric populations of the rare narrow endemic *P. corcovadensis* and the more common and widely distributed *P. flammea* by evaluating the genetic diversity and structure of each species. Because these species have divergent mating system and pollination behaviour we hypothesise that pre-zygotic isolation barriers will help to keep species integrity in sympatry. We also discuss whether hybridization and introgression might reduce or increase the risk of extinction for the rare species *P. corcovadensis*. Specifically, we asked the following questions:

1 Do *P. corcovadensis* and *P. flammea* hybridise in the wild? If they do hybridise, what is the direction of genomic introgression between the rare and the common species?
2 What do patterns of plastidial and nuclear admixture tell us about current or ancient hybridization and introgression events in inselberg populations of these two species?
3 How do intra- and interspecific gene flow allow species to coexist in a naturally fragmented and highly diverse community?

MATERIAL AND METHODS

Species studied and sampling

Here, we studied two sympatric populations of these morphologically distinct *Pitcairnia* (*Bromeliaceae*) species. A total of 108 wild specimens of *P. flammea*, *P. corcovadensis* and their hybrids were randomly sampled at Teresópolis and Corcovado Mountain, Rio de Janeiro State, southeast Brazil (Table 1). Voucher specimens were deposited in the Herbarium Rioclarense (HRCB), Rio Claro, Brazil.

Table 1. Population names, localities, geographic coordinates and sample sizes of *Pitcairnia flammea*, *Pitcairnia corcovadensis* and their hybrids from the Atlantic Rainforest, southeast Brazil. Hybrid individuals were classified based on Bayesian admixture coefficients from STRUCTURE (see Results for details).

population/species	coordinates	altitude (m)	sample size (N)	
			nuclear	plastid
Teresópolis				
Pitcairnia flammea	S22 28.826	740	29	23
Pitcairnia corcovadensis	W42 59.937		13	10
Hybrids			2	2
Total			44	35
Corcovado				
Pitcairnia flammea	S22 56.976	530	23	21
Pitcairnia corcovadensis	W43 13.356		29	15
Hybrids			12	8
Total			54	44

Extraction of DNA and PCR amplification

From each individual plant, fresh leaf samples were collected, dried on silica gel and stored at $-20\ °C$ in a freezer until DNA extraction. Total genomic DNA was extracted using the DNeasy 96 Plant Kit (Qiagen, Germantown, MD, USA), according to the manufacturer's instructions.

We used eight nuclear microsatellite loci (E6B: Boneh *et al.* 2003; PaA05, PaA10, PaD07, PaB12: Paggi *et al.* 2008; VgA04: Palma-Silva *et al.* 2007; ngFos22: Wöhrmann *et al.* 2012; Acom12.12: Wöhrmann & Weising 2011; Table S1) and five plastidial microsatellites (PaCP01, PaCP02, PaCP03, PaCP04: Palma-Silva *et al.* 2011; VgCP04: Palma-Silva *et al.* 2009; Table S2) that were previously developed for other bromeliad species.

For each microsatellite locus, the forward primer was synthesised with a 19-bp M13 tail (5′-CACGACGTTGTAAAACGAC-3′), following a method that uses three primers: a forward SSR-specific primer with the M13 tail at its 5′-end, a reverse locus-specific primer and a universal M13 primer labelled with one of four fluorescent dyes: FAM, VIC, PET or NED (Applied Biosystems, Foster City, CA, USA). Polymerase chain reaction (PCR) amplifications of nuclear and plastidial markers were carried out in 10-μl reaction volumes comprising 1× GoTaq Colorless Master Mix (Promega, Madison, WI, USA), 10 pmol of each microsatellite primer, 1 pmol of M13 universal primer and 10 ng of template DNA. For nuclear markers, we used a 'touchdown' cycling programme, following the protocol described by Palma-Silva *et al.* (2007). For plastidial markers, we used a standard cycling programme consisting of 94 °C for 1 min, 30 cycles of 94 °C for 30 s, 58 °C for 40 s and 72 °C for 40 s, and a final elongation step at 72 °C for 10 min. All reactions were performed using the Veriti 96-well thermal cycler (Applied Biosystems). Amplifications of both nuclear and plastidial microsatellites were conducted on an ABI 3500 sequencer (Applied Biosystems, Warrington, UK) and compared with the GeneScan LIZ 500 molecular size standard (Applied Biosystems). The loci were visually analysed using GeneMarker version 1.95 Demo software (SoftGenetics, State College, Pennsylvania, USA).

Admixture analysis and assignment tests

To estimate the nuclear admixture proportion and patterns of introgression, we performed three complementary analyses. Each hybrid zone was analysed separately because allele frequencies are expected to be divergent in naturally fragmented inselberg populations (Barbará *et al.* 2007; Palma-Silva *et al.* 2011; Gonçalves-Oliveira *et al.* 2017; Hmeljevski *et al.* 2017). First, we conducted model-based Bayesian clustering using STRUCTURE 2.3.2 software (Pritchard *et al.* 2000) to obtain estimates of the admixture proportions between *P. flammea* and *P. corcovadensis*. We used a predefined model with genetic cluster (K) value = 2, corresponding to the gene pool of two species. Analysis were carried out under the admixture model assuming independent allele frequencies and using a burn-in period of 250,000 and run length of 1,000,000 with the Markov Chain Monte Carlo (MCMC) method. No prior population conditions were used for analysis. STRUCTURE was used to classify individuals into parental species and hybrids, using a threshold of $q > 0.90$ for pure individuals of *P. corcovadensis*, $q < 0.10$ for pure individuals of *P. flammea* and $0.10 < q < 0.90$ for hybrids (Burgarella *et al.* 2009).

Second, we employed Bayesian analysis using NewHybrids version 1.1 beta software (Anderson & Thompson 2002) to assign individuals into different genotypic classes (pure parental species, F_1 and F_2 early generation hybrids, and backcrosses). Analyses were performed without reference populations following Vähä & Primmer (2006). We performed 1,000,000 sweeps after a 100,000-sweep burn-in period, using a threshold value of $q = 0.75$, meaning that individuals with $q < 0.75$ remained unassigned (Burgarella *et al.* 2009).

Finally, we used a non-Bayesian approach implemented through the program FLOCK (Duchesne & Turgeon 2009) to allocate individuals into one of a defined number of genetic clusters (K). Each hybrid zone was analysed without reference populations using a partition scheme of $K = 2$ and the following parameters: initial random choice of samples, 50 runs, 20 re-allocations per run and a lower limit of detection (LLOD) threshold of zero for allocation. LLOD scores were then plotted to evaluate whether there were distinct clusters representing parental and hybrid classes.

Genetic diversity and structure analyses

The nuclear genetic diversity of microsatellite loci were characterised in *P. corcovadensis*, *P. flammea* and their hybrids based on the number of alleles (A), allelic richness (A_R), observed heterozygosity (Ho), expected heterozygosity (He) and inbreeding coefficient (F_{IS}) calculated for each locus using MSAnalyzer version 4.05 software (Dieringer & Schlötterer 2003). Departures from Hardy–Weinberg equilibrium (HWE) for each locus within each species were determined using Genepop on the Web software (Raymond & Rousset 1995). The populations of each species and hybrids were also characterised in terms of the same genetic parameter set described above, and departures from HWE were calculated for each population of each species and hybrids using Genepop on the Web. Each population was also characterised in terms of plastidial DNA diversity based on the number of haplotypes and gene diversity (H_K), as estimated using the program CONTRIB (Petit *et al.* 1998).

For both nuclear and plastidial microsatellite loci, we performed analyses of molecular variance (AMOVA) in ARLEQUIN 3.11 software (Excoffier *et al.* 2005) to assess patterns of nuclear and plastidial genomic differentiation. Three separate AMOVAs were carried out using distinct hierarchical models. We first evaluated partitioning of genetic variability 'between species', 'between populations within species' and 'within populations' using the entire dataset without hybrid individuals, as assigned by STRUCTURE. Then we performed two separate analyses using pure individuals of *P. flammea* or *P. corcovadensis* to test the distribution of genetic variance among and within populations of each species. We also assessed nuclear genetic differentiation using estimates of F_{ST} (Weir & Cockerham 1984) and G'_{ST} (Hedrick 2005) calculated in MSAnalyzer, including only pure individuals of *P. corcovadensis* and *P. flammea*.

For each individual, genetic variants for all plastidial microsatellite loci were combined into haplotypes. The relationship among haplotypes was then assessed with the program
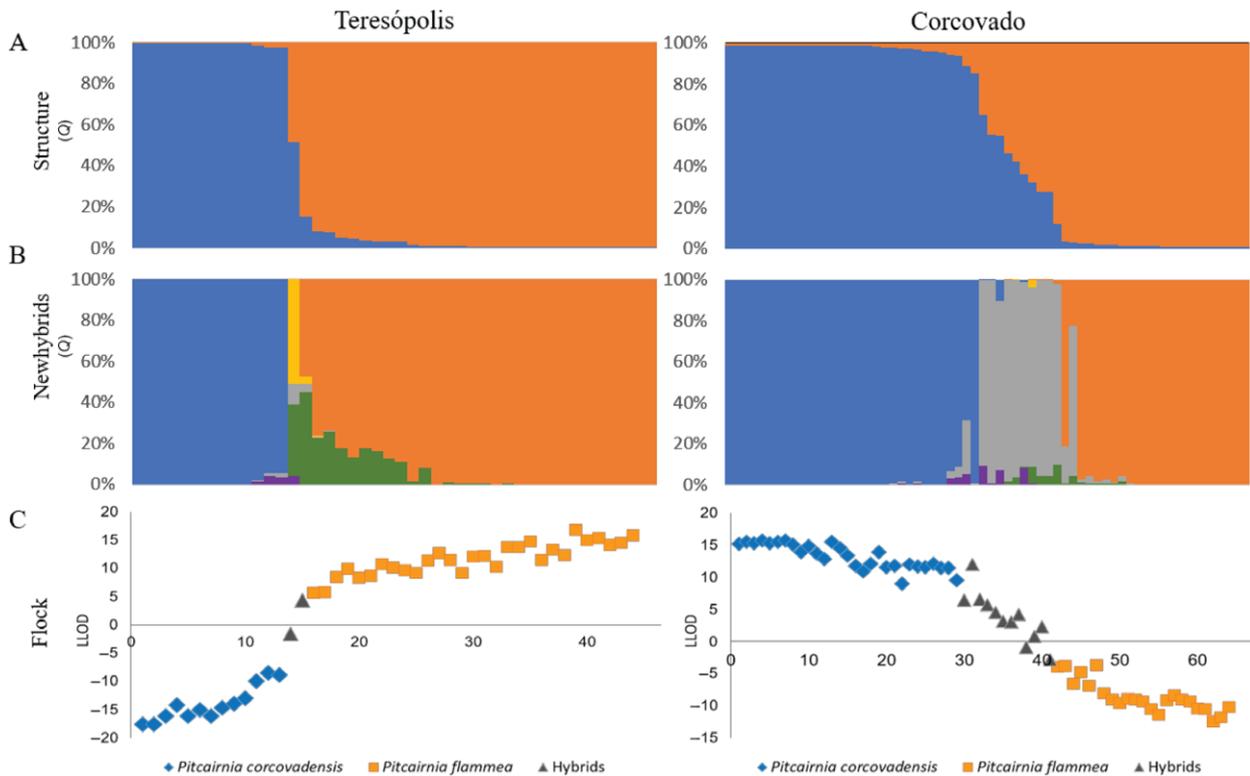
Fig. 1. Genetic admixture in two distinct hybrid zones between *Pitcairnia flammea* and *Pitcairnia corcovadensis* based on eight microsatellite markers according to Structure (A): orange for *P. flammea* and blue for *P. corcovadensis*; NewHybrids (B): orange for *P. flammea*, blue for *P. corcovadensis*, yellow for F_1 hybrids, grey for F_2 hybrids, green for *P. flammea* backcrossing and purple for *P. corcovadensis* backcrossing; and (C) FLOCK: orange squares for *P. flammea*, blue lozenges for *P. corcovadensis*; green triangles for hybrids. FLOCK graphic was coloured according to Structure results.

Network 4.5.1.6 (http: //www.fluxus-engineering.com) using the median-joining algorithm (Bandelt *et al.* 1999). Following the maximum parsimony model, we constructed one tree with the shortest and least complex phylogenetic relationships among haplotypes.

Demographic analyses

The intensity of historical intra- and interspecific gene flow is crucial to explaining the current patterns of genetic structure, particularly for small and/or isolated populations. Theta ($4N_e\mu$ for biparental inherited loci, with N_e = effective population size and μ = mutation rate) and the number of immigrants per generation (θM, with M = mutation-scaled effective immigration rate) were estimated using a coalescent framework in LAMARC 2.1.9 software (Kuhner 2006). To estimate N_e, we used a mutation rate of 0.00077, as detected in maize microsatellites (Vigouroux *et al.* 2002). Analysis was conducted with a Bayesian search method using four adaptively heated Markov chains (with temperatures of 1.0, 1.5, 3.0 and 1.0×10^6), 50,000 recorded trees (with the first 10,000 discarded as burn-in) and a sampling increment of 20. Starting values were calculated using F_{ST}, and we used model averaging to estimate migration rates and θ values. Stationarity of the Markov chain was assessed by examining the effective sample size for each parameter. We also estimated g (population growth) using the parameters described above.

RESULTS

Genetic composition of hybrid zones

Bayesian STRUCTURE results for each sympatric population, considering two genetic clusters, clearly discriminated *P. flammea* and *P. corcovadensis* samples and indicated that most of the samples were purebred, whereas only 14 individuals showed genetic mixture between the two clusters (STRUCTURE threshold q-value of $0.10 < q < 0.90$; Fig. 1). Nuclear admixture analyses performed for each hybrid zone identified 29 *P. flammea*, 13 *P. corcovadensis* and two hybrids in the Teresópolis population and 23 *P. flammea*, 29 *P. corcovadensis*, and 12 hybrids in the Corcovado population (Fig. 1). Using a q-value > 0.75, NewHybrids identified 12 hybrids, most of which were classified as early generation F_2 hybrids (Fig. 1), while only three individuals could not be classified. In Teresópolis, NewHybrids could not assign two hybrids to any class.

Further analysis using FLOCK identified clusters of *P. flammea* and *P. corcovadensis* individuals in both hybrid zones (mean LLOD = 11.39 ± 3.12 SD and -15.45 ± 0.48 SD in Teresópolis; mean LLOD = -8.99 ± 2.13 SD and 12.91 ± 2.25 SD in Corcovado; Fig. 1C). As expected, individuals classified as hybrids using STRUCTURE and NewHybrids analysis occupied an intermediate position, confirming their hybrid category (Fig. 1).

Table 2. Characterisation of *Pitcairnia flammea*, *Pitcairnia corcovadensis* and their hybrids, including sample size (N), allelic number per loci (A), allelic richness (AR), expected (H_E) and observed heterozygosities (H_O), and inbreeding coefficient (F_{IS}), based on eight nuclear microsatellite markers; and gene H_K based on five plastid microsatellites markers.

	nuclear microsatellites						plastid microsatellites			
	N	A	AR	H_E	H_O	F_{IS}	N	H_K	Pb	haplotypes
Pitcairnia flammea										
Teresópolis	29	54	2.158	0.545	0.403	0.196*	23	0	0	H7
Corcovado	23	51	2.241	0.542	0.377	0.244*	21	0.629	0.629	H1, H2, H3, H4
Overall/average	52	105	2.200	0.544	0.390	0.220*	44	0.315	0.315	
Pitcairnia corcovadensis										
Teresópolis	13	45	2.334	0.631	0.570	0.102	10	0	0	H7
Corcovado	29	26	1.584	0.28	0.192	0.199*	15	0.59	0.59	H2, H5, H6
Overall/average	42	71	1.959	0.456	0.381	0.151*	25	0.295	0.295	
Hybrids										
Teresópolis	2	24	3.000	1.000	1.000	−1.000	2	0	0	H7
Corcovado	12	40	2.382	0.609	0.543	−0.018*	8	0.75	0.75	H1, H2, H5
Overall/average	14	64	2.691	0.805	0.772	−0.509	10	0.375	0.375	

Inbreeding coefficients (F_{IS}) departing significantly from Hardy–Weinberg equilibrium (HWE) are indicated by asterisks (*$P < 0.05$).
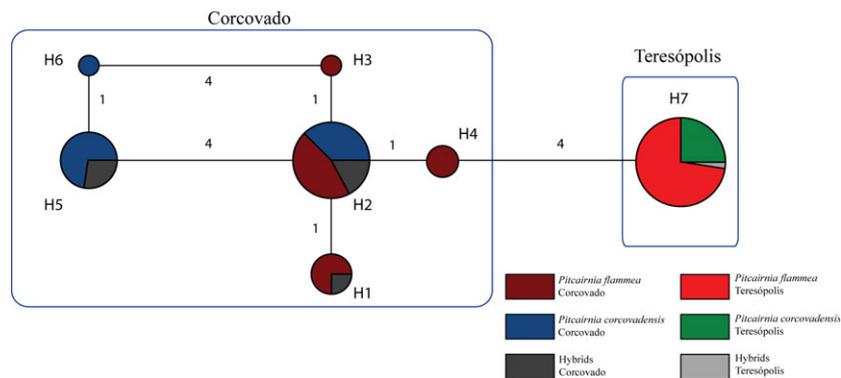

Fig. 2. Median-joining network of plastid DNA haplotypes of *Pitcairnia flammea* and *Pitcairnia corcovadensis*. Filled circles indicate the haplotype. The size of each circle being proportional to the observed frequency of each haplotype.

Nuclear genetic diversity and demographic patterns

Eight nuclear microsatellite loci were polymorphic in both species and their hybrids. The total number of alleles ranged from three to 24 per locus in *P. flammea* and from two to 13 in *P. corcovadensis*. The inbreeding coefficients (F_{IS}) were high and departed significantly from HWE at almost all loci ($P < 0.01$; Table S4), in accordance with self-pollination breeding system of both species.

Regarding diversity at the population level, *P. flammea* populations showed higher genetic diversity than *P. corcovadensis* populations in most of the parameters analysed (Table 2), which likely reflects differences in population size between the species (more in *P. flammea*; see Ne estimates and Fig. 2). As expected, we also detected higher genetic diversity in hybrids than in parental individuals in both sympatric populations (see Table 2). Apart from *P. corcovadensis* and hybrids in Teresópolis, all populations showed significant deviations from HWE ($P < 0.05$) due to deficiency of heterozygotes (Table 2). The population-level inbreeding coefficient (F_{IS}) was slightly higher for *P. flammea* (0.220) than for *P. corcovadensis* (0.151)

and was low and not statistically significant for hybrids (−0.509; Table 2).

Significant nuclear genomic differentiation was observed between *P. flammea* and *P. corcovadensis* (AMOVA $F_{CT} = 24.07\%$, $P < 0.001$; Table 3; G'_{ST} between species = 0.73, $P = 0.001$). Lower population genetic structure was detected in *P. flammea* ($F_{ST} = 0.179$, $P = 0.001$; and $G_{ST}' = 0.31$, $P = 0.001$) than in *P. corcovadensis* ($F_{ST} = 0.443$, $P = 0.001$; and $G_{ST}' = 0.39$, $P = 0.001$; Table 3).

The effective population sizes were small for both *P. corcovadensis* and *P. flammea* (Fig. 3, Table 4). However, lower effective population sizes were observed for *P. corcovadensis* (Corcovado: Nem = 5.51; Teresópolis: Nem = 7.79) than for *P. flammea* (Corcovado: Nem = 23.05; Teresópolis: Nem = 11.68). Divergent levels of interspecific gene exchange were identified between hybrid zones, with higher levels of introgression in Teresópolis (Nem = 0.33 and 0.25; Fig. 3) than in Corcovado (Nem = 0.09 and 0.06; Fig. 3). Asymmetric migration rates were observed between species in both sympatric populations, with lower introgression toward the more common species, *P. flammea* (Fig. 3, Table 4). Overall,

Table 3. Results of analysis of molecular variance (AMOVA) for *Pitcairnia flammea* and *Pitcairnia corcovadensis* using three different hierarchical models: a three-level model including all populations and species, and separate two-level models for each species.

model	source variation	nuclear microsatellites			plastid microsatellites		
		variation (%)	*F*-statistics	*P*-value	variation (%)	*F*-statistic	*P*-value
All populations and species	Between species	24.07	F_{CT}: 0.240	<0.001	−50.89	F_{CT}: −0.509	N.S.
	Among populations between species	22.55	F_{ST}: 0.466	<0.001	117.88	F_{ST}: 0.670	<0.001
	Within populations	53.38	F_{SC}: 0.297	<0.001	33.01	F_{SC}: 0.781	<0.001
Pitcairnia flammea	Between populations	17.96	F_{ST}: 0.179	<0.001	89.3	F_{ST}: 0.893	<0.001
	Within populations	82.03			10.69		
Pitcairnia corcovadensis	Between populations	44.33	F_{ST}: 0.443	<0.001	57.85	F_{ST}: 0.578	<0.001
	Within populations	55.67			42.14		
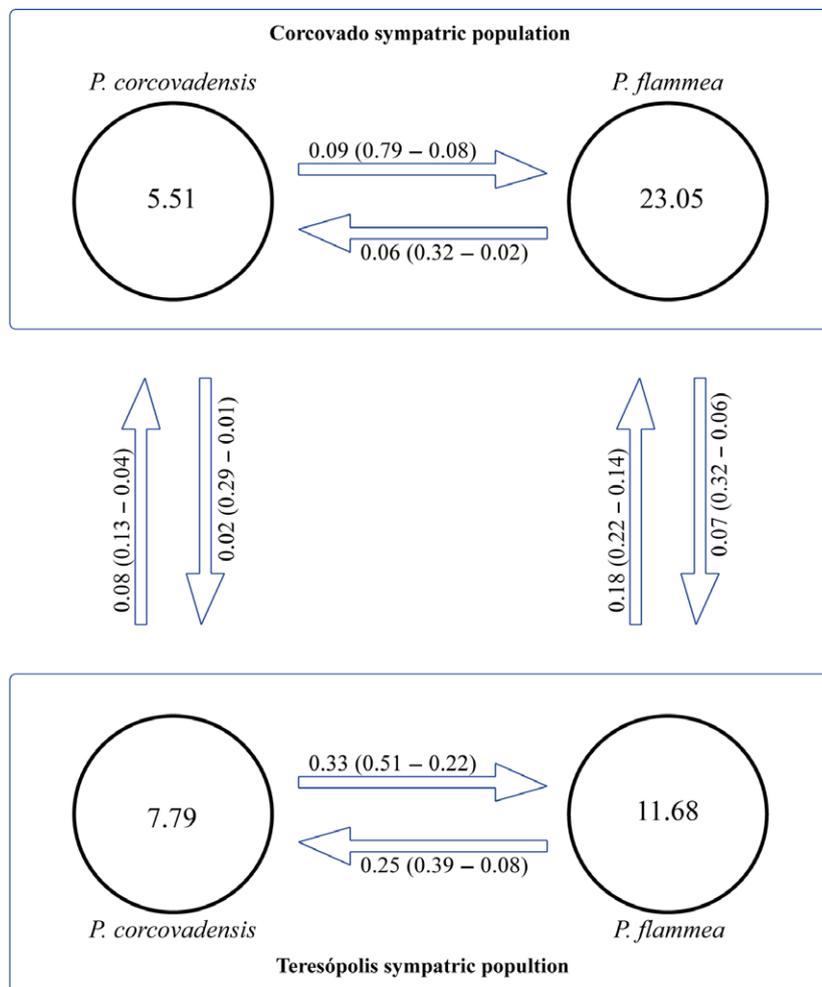
Fig. 3. Effective population size Ne (inside circles) and number of immigrants per generation θM (below and above arrows), including 95% confidence intervals, calculated for *Pitcairnia corcovadensis* and *Pitcairnia flammea* from two sympatric populations, Corcovado and Teresópolis. Arrows indicate the direction of gene flow.

intraspecific gene flow rates were lower than interspecific gene exchange rates (*P. corcovadensis* Nem = 0.08 and 0.02; *P. flammea* Nem = 0.18 and 0.17; Fig. 3). Population growth exhibited positive values for both species in all populations, suggesting a tendency of populations to increase over time (Table 4).

Plastidial DNA diversity and haplotype network

We identified a total of seven plastidial haplotypes among the two species and the hybrids. Haplotype sharing between species was observed in both sympatric populations. Median-joining analysis revealed a multi-species haplotype network with two

Table 4. Demographic estimates of *Pitcairnia corcovadensis* and *Pitcairnia flammea* populations, including the sample size (N), the effective population size (N_e) and estimates of population growth (g). The 95% confidence intervals for N_e and g are between parenthesis.

populations	N	N_ea	gb
Corcovado			
Pitcairnia corcovadensis	29	5.51 (7.46–2.59)	0.96 (0.95–1.14)
Pitcairnia flammea	23	23.05 (33.76–5.51)	0.91 (0.94–1.14)
Teresópolis			
Pitcairnia corcovadensis	13	7.79 (18.18–4.87)	0.82 (0.85–1.05)
Pitcairnia flammea	29	11.68 (29.54–8.44)	0.99 (0.97–1.22)

aEffective population sizes were calculated using the formula N_e = theta/4μ, where μ = mutation rate (0.00077, found at microsatellite loci in maize (Vigouroux *et al.* 2002).

bPositive values of g indicate population growth, and negative values indicate shrinkage, only if confidence intervals exclude zero.

distinct haplogroups for each sympatric population connected by four mutation steps (Fig. 3). Among a total of seven haplotypes, one was fixed and shared among species and hybrids in Teresópolis (H7), suggesting chloroplast capture or incomplete lineage sorting during the speciation process that separated these species (Fig. 3, Table S3). The other six haplotypes were exclusive to the Corcovado hybrid zone (H1, H2, H3, H4, H5 and H6; Fig. 3, Table S3). In this sympatric population, haplotypes H3 and H4 were exclusive to *P. flammea*, whereas H6 was only found in *P. corcovadensis*. The central haplotype H2 was shared among both species and hybrids, while H5 was shared between *P. corcovadensis* and hybrids, and H1 between *P. flammea* and hybrids. Because hybrids in the two hybrid zones carried haplotypes from both parental species (Fig. 3), it is likely that both species can act as both male and female parents (seed and pollen donors) during interspecific hybridization. The species could not be significantly differentiated using plastidial markers, as AMOVA results based on plastidial markers showed non-significant differentiation between species (F_{CT}: −0.509; not significant, Table 3). Population genetic differentiation of the plastidial genome was higher in *P. flammea* (F_{ST} = 0.893; P = 0.001) than in *P. corcovadensis* (F_{ST} = 0.578; P = 0.001; Table 3).

DISCUSSION

In this study, we investigated the levels of hybridization and introgression between *P. corcovadensis* and *P. flammea* and discuss their causes and evolutionary consequences. Our main results showed that these species have strong isolation barriers, although interspecific gene flow was observed in both sympatric populations with differing outcomes of hybridization and introgression. In the Teresópolis population, nuclear and plastidial genome analysis indicated higher levels of interspecific gene flow and more ancient hybridization and introgression patterns, while samples from Corcovado showed more limited but recent interspecific gene flow. Reproductive isolation barriers were strong but permeable, and pre-zygotic barriers such as spontaneous selfing and divergent pollination behaviour (Wendt *et al.* 2002) may prevent introgression. Post-zygotic barriers may also contribute to species integrity, in accordance with previously reported hybrid unviability due to lower seed

germination and seedling survival rates (Rôças *et al.* 2004). The low levels of intraspecific gene flow observed in *P. corcovadensis* and *P. flammea* corroborate the growing body of studies in which inselberg bromeliad species are maintained as discrete evolutionary units despite the presence of low levels of genetic connection. Patterns of nuclear genetic diversity and gene flow revealed that hybridization and introgression might not cause species extinction *via* genetic assimilation of the rare *P. corcovadensis*. Considering the reduced intraspecific gene exchanges, hybridization and introgression, may be important aspects of the *Pitcairnia* diversification process, with positive evolutionary impacts at the bromeliad community level, and may contribute to increasing and maintaining genetic diversity of isolated inselberg populations.

Patterns of hybridization and introgression between species

All analytical methods (STRUCTURE, NewHybrids and FLOCK) identified pure individuals of each species, *P. corcovadensis* and *P. flammea,* and intermediate admixtured individuals in both sympatric populations, confirming the hypothesis of hybridization between these species (Wendt *et al.* 2000; Rôças *et al.* 2004; Palma-Silva *et al.* 2011). The patterns of nuclear hybridization and introgression differed between hybrid zones, although both suggest interspecific gene exchange between *P. corcovadensis* and *P. flammea* (Fig. 3). In Teresópolis, we only detected two hybrids (note that the number of SSR loci might be insufficient to precisely identify their hybrid classes), but their genomic admixture proportions suggest that they might be late backcrosses rather than early generation F_1 or F_2 hybrids. Accordingly, coalescent-based estimates of migration rates (Fig. 3) indicated greater levels of interspecific gene flow in the Teresópolis population. In the Corcovado population, 12 individuals were identified as hybrids, most of which are early-generation F_2 hybrids, suggesting that backcrosses were uncommon, in agreement with the low levels of gene flow detected through coalescent migration rate analysis (Fig. 3). These results are in accordance with a large body of data on gene flow during the late stages of speciation, when hybridization may occur but introgression is a rare event (Twyford *et al.* 2015). These results will contribute to elucidating how speciation with gene flow might produce strong isolation and genome-wide differentiation. In fact, both species show high genomic divergence (AMOVA F_{CT} = 0.24, P < 0.001; Table 3), suggesting that strong reproductive isolation barriers have efficiently maintained species integrity even in sympatry.

A strong geographic pattern of haplotype sharing was observed for maternally-inherited plastidial DNA among species and hybrids in both sympatric populations (Fig. 2). Haplotype sharing can potentially be explained through several processes: local gene flow (recent introgressive hybridization); retention of ancestral polymorphism (incomplete lineage sorting) and homoplasy (evolutionary convergence) or a combination of these processes (Palma-Silva *et al.* 2011). In Teresópolis, where chloroplast capture or incomplete lineage sorting was detected, it is unlikely that hybridization between *P. corcovadensis* and *P. flammea* is a more recent event (Rieseberg 1997). On the other hand, in the Corcovado population, the only central haplotype (H2) that was shared among species and hybrids indicates that incomplete lineage sorting may also be responsible for haplotype sharing between species. Distinguishing

introgression from incomplete lineage sorting is a difficult task, and currently there is no effective approach for differentiating these processes. The geographic pattern of shared haplotypes observed in our study is expected to provide further evidence for hybridization as observed elsewhere (Heuertz *et al.* 2006; McKinnon *et al.* 2010; Pinheiro *et al.* 2010, 2014; Palma-Silva *et al.* 2011). Thus, our results suggest possible cooperation of these two processes in the evolution of reproductive isolation between the closely-related species *P. corcovadensis* and *P. flammea*. In Teresópolis, nuclear and plastidial genome analysis showed higher levels of interspecific gene flow and more ancient hybridization and introgression patterns, while in Corcovado, both plastidial and nuclear markers suggested more limited but recent interspecific gene flow.

Evolution of reproductive isolation–mating system variation and rewardless flowers

The differing patterns of gene introgression observed offer a great opportunity to study the diverse stages of the divergence continuum, speciation with gene flow, and the evolution of reproductive isolation barriers. Moreover, differences in reproductive ecology (*e.g.* mate choice, pollination biology, mating systems, dispersal) may account for discrepancies in gene exchange dynamics among sympatric populations (Sweigart & Willis 2003), constituting important pre-zygotic reproductive isolation barriers. Compared to the more common and widely distributed *P. flammea* (N_e = 23.05 and 11.68), *P. corcovadensis* is considered a rare species, in accordance with our observation of lower effective population sizes (N_e = 5.51 and 7.79). Furthermore, *P. corcovadensis* is rewardless, with no nectar production and few flowers per inflorescence (Wendt *et al.* 2002). Thus, these variations in pollination biology could act as a strong pre-zygotic isolation barrier and might have influenced the contrasting patterns of hybridization and introgression between hybrid zones revealed in our analysis. In the Teresópolis population, where few hybrids but older patterns of hybridization and introgression were reported, the two species have more balanced effective population sizes (N_e = 7.79 and 11.68). In this frequency-dependent case, when a rewardless species (*P. corcovadensis*) is as abundant as a rewarding species (*P. flammea*), pollinators may more easily identify the rewardless plants, promoting higher pollination fidelity. Thus, fewer deceptive visits to *P. corcovadensis* might ensure reproductive isolation from *P. flammea*. At the other site, in the Corcovado population, the rewarding *P. flammea* has a much larger effective population size (N_e = 23.05) than *P. corcovadensis* (N_e = 5.51), possibly allowing more mistaken pollination visits, and consequently lower pollinator fidelity would have favoured increased frequency of early-generation hybrid individuals (Fig. 1).

Despite the observed differences in gene flow between hybrid zones, pollination biology may also have influenced the gene flow asymmetries between species. In both hybrid zones, *P. corcovadensis* showed lower interspecific migration rates (θM: 0.06 and 0.25), while levels of gene flow toward *P. flammea* were higher (θM: 0.09 and 0.33). Such differences may be influenced by pollination behaviour, where occasional visits by a pollinator to a rewardless flower (*P. corcovadensis*) will likely precede a visit to a rewarding flower (*P. flammea*). In addition, spontaneous selfing reported in *P. corcovadensis* (Wendt *et al.* 2002)

may represent another important strategy for pre-zygotic reproductive isolation from heterospecific pollen, in particular because this species always occurs in sympatry with congeneric *P. flammea*. Thus, the asymmetric gene exchanges reported in both sympatric populations suggest that spontaneous selfing in *P. corcovadensis* might protect it from introgression by *P. flammea*. Indeed, an increasing number of theoretical (Cannon & Lerdau 2015; Hu 2015) and empirical (Martin & Willis 2007; Ruhsam *et al.* 2011; Palma-Silva *et al.* 2015; Wagner *et al.* 2015; Neri *et al.* 2017, 2018) studies have recognised that selfing rather than outcrossing rates hamper hybridization and introgression, and thus represent an important pre-zygotic reproductive isolation barrier. Hence, pollination behaviour and mating system variation are important pre-zygotic reproductive barriers that act in combination in both hybrid zones.

Post-mating barriers, such as pollen–stigma incompatibility, fruit abortion, hybrid unviability and hybrid sterility, may also influence hybridization patterns. In fact, lower seed viability and seedling survival were previously reported for interspecific crosses between these species (Wendt *et al.* 2002; Rôças *et al.* 2004). The large genomic divergence observed between *P. corcovadensis* and *P. flammea* (Table 3) suggests that Bateson-Dobzhansky-Muller genic incompatibilities, resulting from negative genetic interactions (Orr & Turelli 2001) might occur frequently when the species hybridise and contribute to maintenance of species integrity, as has been recognised in a number of other plant groups (Moyle *et al.* 2012; Briscoe Runquist *et al.* 2014; Johnson *et al.* 2015; Pinheiro *et al.* 2015; Matallana *et al.* 2016).

Intraspecific gene flow

The concordance of introgression patterns in both the nuclear and plastidial genomes of the two species highlights the importance of the geographic isolation of these island-like habitats for the speciation and diversification processes (Palma-Silva *et al.* 2011; Millar *et al.* 2013; Pinheiro *et al.* 2014; Tapper *et al.* 2014; Gonçalves-Oliveira *et al.* 2017). The phylogenetic relationships among haplotypes suggest closer genetic association among haplotypes belonging to the same inselberg population, independent of the species effect (Fig. 2). Despite the observed introgression in both sympatric populations, our results also revealed extremely low levels of gene flow, as measured through coalescent analysis between populations of the same species belonging to different inselberg populations: *P. flammea* (θM = 0.18 and 0.07) and *P. corcovadensis* (θM = 0.08 and 0.02). Accordingly, genomic divergence estimates based on nuclear (F_{ST} = 0.179 and 0.443, respectively) and plastidial markers (F_{ST} = 0.893 and 0.578, respectively) were significantly high for both species. The predominantly short-distance pollen and seed dispersal among inselbergs greatly reduces the chance of intraspecific gene flow for both the plastidial and nuclear genomes. The large, vibrant red flowers of most *Pitcairnia* species suggest that hummingbirds may be the main pollinators (Givnish *et al.* 2014). In fact, three different hummingbird species were observed pollinating *P. flammea* on Corcovado Mountain (Wendt *et al.* 2002). These hummingbird species usually exhibit aggressive behaviour, defending nectar sources from intruders, thus promoting low rates of long-distance pollen flow and consequently increasing endogamy. In rewardless *P. corcovadensis*, the scarcity of pollinator visits has promoted

lower overall levels of intraspecific gene flow (θM = 0.08 and 0.02). In addition, loss of the dispersal mechanism for *Pitcairnia*'s seeds (Benzing 2000) in highly fragmented habitats such as inselbergs and rock outcrops corroborates the low gene flow between populations being due to low dispersion rates of both pollen and seeds.

The low levels of intraspecific gene flow observed between *P. corcovadensis* and *P. flammea* populations corroborate the growing body of studies showing that inselberg bromeliad species are maintained as discrete evolutionary units, even with low intraspecific migration rates (Barbará *et al.* 2007; Boisselier-Dubayle *et al.* 2010; Palma-Silva *et al.* 2011; Gonçalves-Oliveira *et al.* 2017; Hmeljevski *et al.* 2017). Together, these studies suggest that gene flow might not be the most important evolutionary process in maintaining species cohesion (Mayr 1943). In species with limited gene exchange, natural selection can also act to maintain species cohesion, as populations may evolve in concert through the spread of advantageous alleles (Morjan & Rieseberg 2004; Kane *et al.* 2009; Palma-Silva *et al.* 2011). Thus, despite limited intraspecific gene flow at neutral loci, gene flow at specific loci would be critical to maintaining species integrity, even at low rates (Palma-Silva *et al.* 2011).

The outcomes of hybridization and introgression in *Pitcairnia*

Permeable but strong reproductive isolation barriers are commonly associated with rapid radiation of bromeliads (Palma-Silva *et al.* 2011, 2015; Zanella *et al.* 2016; Goetze *et al.* 2017; Neri *et al.* 2017, 2018), particularly among species found in geographically isolated habitats (Palma-Silva *et al.* 2011; Wagner *et al.* 2015; Lexer *et al.* 2016). In this context, we should also consider the evolutionary role of gene introgression in situations with extremely limited intraspecific gene exchange.

Historically, researchers have been concerned that hybridization and introgression may promote population extinction through outbreeding depression, genetic assimilation or gene swamping (reviewed by Gómez *et al.* 2015). These concerns are especially important for introgression of rare species that live in sympatry with more common, closely related species (Ellstrand & Elam 1993; Burgess *et al.* 2005; Lepais *et al.* 2009). On the other hand, current theoretical studies suggest that interspecific gene flow may reduce extinction risks and enhance local adaptation through introgression of adaptive genetic variation (Cannon & Lerdau 2015). Thus, hybridization and introgression might promote genetic rescue and demographic recovery in response to environmental changes (Hamilton & Miller 2016). Our results suggest extinction risks of *P. corcovadensis* and *P. flammea* populations are not increasing because of hybridization. Instead, coalescent analysis showed the effective populations sizes, although low, are not declining, suggesting that hybridization likely does not enhance the extinction risk for either of these species. Accordingly, the observed asymmetric introgression toward *P. flammea* was another unexpected result (Ellstrand & Elam 1993; Burgess *et al.* 2005; Lepais *et al.* 2009), which revealed that the rare species

P. corcovadensis might not have increased susceptibility to gene flow from the common *P. flammea*. In the Teresópolis population, the fixed haplotype H7 suggested that chloroplast capture had occurred. However, we could not identify whether this haplotype was originally from *P. flammea* or *P. corcovadensis*, or even whether it was due to incomplete lineage sorting. In fact, rare species are not always prompt to exhibit genetic assimilation by more common species, as has been reported in other low-abundance species (Worley *et al.* 2009; Neri *et al.* 2017).

Our study has implications for understanding species coexistence in lineages undergoing adaptive radiation as well as highly diverse communities dominated by highly unpredictable selective environments. Thus, interspecific gene flow might also be advantageous for long-term survival of species in the context of rapid global climate changes (Hamilton & Miller 2016).

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AUTHORS CONTRIBUTION

CPS, TW, FP participated in the study design and fieldwork; MRM carried out the molecular genetic data collection; MRM, BSSL, PF analysed data; and MRM, FB, BSSL drafted the study. All authors read and approved the final version of the manuscript.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Universal primers of nuclear microsatellites used in PCR amplification.

Table S2. Universal primers of plastidial microsatellites used in PCR amplification.

Table S3. Distribution and frequencies of plastid microsatellite haplotypes in the six analyzed populations.

Table S4. Species-level estimates of genetic diversity and genetic differentiation per locus. Allelic number per loci (A), allelic richness (AR), expected (H_E) and observed heterozygosities (H_O), within-population inbreeding coefficient (F_{IS}); fixation index (F_{ST}) and standardized measure of differentiation (G'_{ST}).

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