

Genetic diversity and reproductive biology of the dioecious and epiphytic bromeliad *Aechmea mariaereginae* (Bromeliaceae) in Costa Rica: implications for its conservation

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Ecological traits and pollination mode associated with dioecious sexual expression in plants are likely to influence their reproductive success and levels of genetic diversity. Dioecy is an uncommon condition in Bromeliaceae. Currently, there is limited information on the reproductive ecology and genetics of dioecious and epiphytic bromeliads. Therefore, we studied the reproductive biology and genetic diversity of the epiphytic and dioecious bromeliad *Aechmea mariaereginae* in Costa Rica. Flowering of pistillate and staminate plants in one population showed high synchrony and were pollinated by non-hermit hummingbirds. Sex ratio was biased to males (3.5:1). Fruit set was relatively high (68%) but there was high female reproductive variance (C. V. = 59.7%). The species is capable of parthenocarpic fruit production. Using eight nuclear microsatellite markers and six populations, we found moderate to high levels of genetic variation ($H_E = 0.571–0.726$). Populations showed significant genetic structure ($G'_{ST} = 0.385$) and Bayesian population assignment grouped them into lowland and montane clusters. Isolated montane populations had slightly lower genetic diversity probably due to lower effective population size caused by biased sex ratios and recent habitat fragmentation that limits long-distance pollinator movements and results in isolation by distance. If habitat fragmentation and isolation persist, populations in montane habitats may be at higher risk of decline and extinction.

KEYWORDS: breeding system – genetic structure – hummingbird pollination – isolation by distance – microsatellites – parthenocarpy – sex ratios.

INTRODUCTION

Bromeliaceae have the highest diversification (c. 3500 species) among the flowering plant groups more or less endemic to the New World (Govaerts, Luther & Grant, 2018; one species is found in Africa). They have developed diverse physiological, morphological and ecological traits such as tank-forming rosettes, absorptive scale trichomes, crassulacean acid metabolism (CAM), an epiphytic life form and avian pollination among others that represent a notable example of an adaptive radiation (Givnish *et al.*, 2014). Sexual reproduction of bromeliads is less diverse, and species predominantly have bisexual flowers bearing both reproductive organs: stamens and pistils.

Diclinous species are present in three subfamilies, although they represent only a small percentage (c. 2%) of the family (Benzing, 2000). Among diclinous species, the reduced group of bromeliads with separate genders or dioecious species are confined to *Hechtia* Klotzsch (Hechtioideae) and *Catopsis* Griseb. (Tillandsioideae), the monotypic *Androlepis skinneri* (K. Koch) Brongn. ex Houlet (Bromelioideae) and *Aechmea mariaereginae* H. Wendl. (Bromelioideae), which are mainly distributed in southern Mexico and Central America (Benzing, 2000). Dioecious bromeliads have been mostly neglected from studies of genetic diversity and reproductive biology (reviewed by Matallana *et al.*, 2010; Zanella *et al.*, 2012; but see Ramírez-Morillo *et al.*, 2008).

Dioecy has evolved independently in several lineages of flowering plants and is present in c.

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6% of all species worldwide (175 families and 987 genera) (Renner, 2014). The evolution of dioecy is interpreted as a mechanism to increase outcrossing and reduce conflicts between male and female flower functions (Darwin, 1877; Lewis, 1942); consequently, the obligate allogamous breeding system of dioecious species should result in high levels of genetic variation (Loveless & Hamrick, 1984). Although empirical data are scarce for tropical dioecious plants, several studies have shown moderate to high levels of genetic diversity (e.g. Hardesty *et al.*, 2005; Cibrián-Jaramillo *et al.*, 2009; Riba-Hernández *et al.*, 2014). However, other studies have also found limited diversity and significant inbreeding coefficients in dioecious species due to reduced effective population sizes caused by clonality (Waycott, 1996), biased sex ratios (Vandepitte *et al.*, 2009) or variance in male or female reproductive success (Luna, Epperson & Oyama, 2005). Almost all dioecious plants show entomophilous pollination associated with small generalist insects (Bawa & Opler, 1975; Bawa, 1980). Limited gene dispersal by short-ranging pollinators reduces gene flow, which in turn increases drift and its diminishing effects on genetic diversity within populations (Loveless & Hamrick, 1984). Long-distance movements of pollen vectors may be affected by the pervasive effects of forest fragmentation that may increase geographical isolation between plant populations (Saunders, Hobbs & Margules, 1991).

Aechmea mariae-reginae is an epiphytic and dioecious species (Smith & Downs, 1979), a remarkable reproductive strategy in the diverse genus *Aechmea* Ruiz & Pav. (c. 280 spp.; Luther, 2014), as it is the only species in the genus with separate genders. This bromeliad has a narrow geographical distribution, being confined to southern Central America, and it has a wide ecological range, from warm and moist lowland forests near sea level to cool mountain humid forests up to 1800 m a.s.l. (Morales, 2003). Most of the original natural habitat of *A. mariae-reginae* has been severely reduced by deforestation in the last century and, at present, its current remaining habitat is highly fragmented (Sánchez-Azofeifa, Harriess & Skole, 2001). Such state of habitat fragmentation is expected to negatively affect genetic diversity (Fahrig, 2003; Aguilar *et al.*, 2008) and patterns of gene flow and genetic connectivity among remnant populations (Young, Boyle & Brown, 1996).

The pollination ecology and reproductive biology of *A. mariae-reginae* are almost unknown. Stiles (1978) reported pollination by non-hermit hummingbirds in a lowland forest population, but information about flowering phenology and reproductive success is still lacking. Information about the reproductive biology of dioecious species is contextual to genetic

diversity estimates, as it explains the observed patterns. Like several congeners, *A. mariae-reginae* is a valuable ornamental plant (Cheers, 2004), and from a conservation perspective knowledge of its genetic diversity and distribution is important as a tool for the identification of genetic resources for future propagation programmes and germplasm conservation (Heywood, 2004). Since life-history traits related to dioecy and epiphytic habitat are associated to higher extinction risk (Willis, 2017), research on population genetics and reproductive biology of this restricted group of bromeliads is necessary to properly evaluate its extinction risk (Frankham, 2005) and design effective conservation strategies.

In this study, we aimed at investigating genetic diversity and structure among populations of the dioecious *A. mariae-reginae* from lowland and montane habitats from Costa Rica using a set of nuclear microsatellite loci. We also studied the reproductive biology of this species in a montane forest population to examine its potential influence on the observed levels of genetic variation and population connectivity.

MATERIAL AND METHODS

STUDY SPECIES

Aechmea mariae-reginae was originally described from cultivated plants collected from the Caribbean lowlands of the Sarapiquí Valley in the Province of Heredia in Costa Rica. Plants can reproduce clonally by asexual side-ramets. Ramets form a robust tank-rosette, 100–170 cm in diameter; leaves are stiff and coriaceous, with spiny margins. The sessile and blue-purplish flowers are densely aggregated on spiked inflorescences that have showy foliaceous pink bracts on the erect peduncle of staminate and pistillate plants (Fig. 1A, B). Its geographical distribution extends from south-eastern Nicaragua (Morales, 2003) to north-western Panama (Wester *et al.*, 2011); Costa Rica represents its main distribution area.

GENETIC DIVERSITY ANALYSIS

We collected 106 adult plants of *A. mariae-reginae* from six populations in Costa Rica in 2017–2018 (Table S1). Individuals were located outside protected areas in disturbed habitats by anthropogenic activities associated to cattle farming and human settlements. In these sites, *A. mariae-reginae* survives on isolated remnant trees on pastures or small forest fragments interspersed in the agricultural landscape. Four populations from the Caribbean lowlands (25–260 m a.s.l.) and two populations from montane areas (1760–1880 m a.s.l.) near the Central Valley of the country

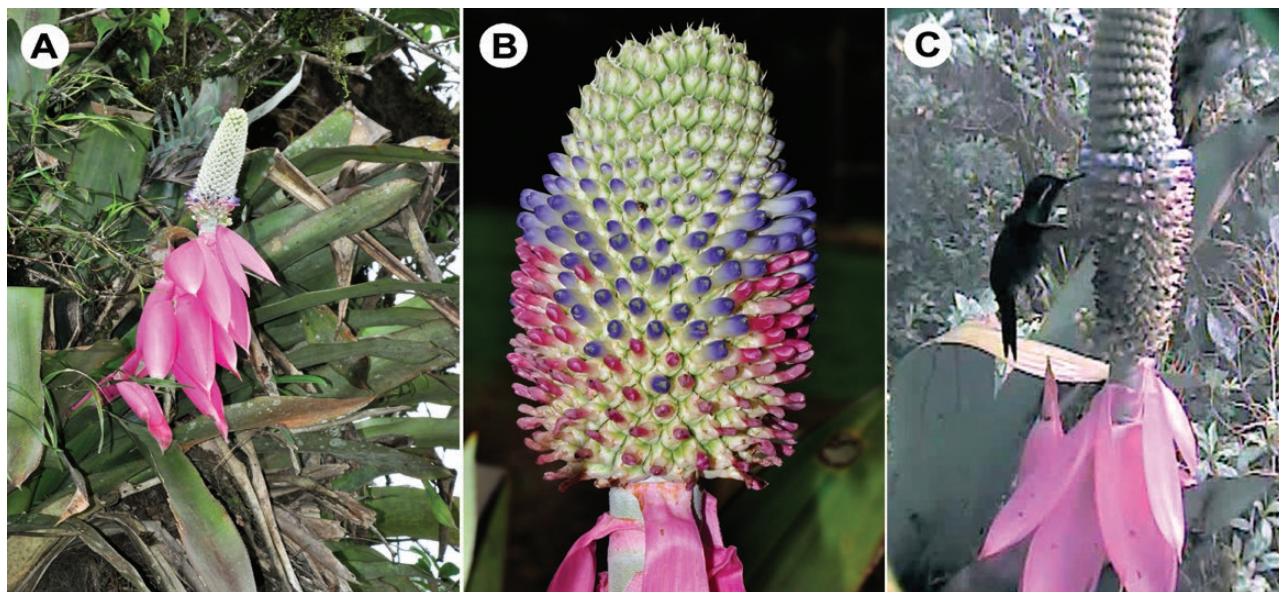


Figure 1. *Aechmea mariae-reginae* (Bromeliaceae): A, Staminate plant with inflorescence. B, Pistillate inflorescence. C, Staminate inflorescence visited by *Lampornis calolaemus* (Trochilidae). Cerros La Carpintera, Costa Rica. Photographs: A. Cascante-Marín.

were sampled. The geographical separation between populations ranged from 8 km (LC-SM) to 123 km (PV-BN) (Fig. 2). A similar congener (*A. mexicana* Baker) is sympatric with our study species; to avoid misidentifications only reproductive individuals (with inflorescences present) were sampled and to avoid collecting clonal ramets from the same genet we only sampled one individual per phorophyte. Voucher specimens were deposited at the USJ herbarium of the Universidad de Costa Rica. Tissue fragments from young leaves were collected and stored in silica gel until completely dry. Total genomic DNA was extracted using a modified version of the CTAB protocol (Doyle & Doyle, 1990).

Eight microsatellite loci were used: Ac01, Ac11, Ac25 and Ac55 (Goetze *et al.*, 2013); Acom_71.3, Acom_78.4, Acom_82.8 and Acom_109.6 (Wöhrmann & Weising, 2011). All PCR amplifications were performed in a Veriti96-Well Thermal Cycler (Applied Biosystems, Foster City, CA, USA) following the conditions described by Goetze *et al.* (2013). Microsatellite alleles were resolved on an ABI 3500 DNA Analyzer (Applied Biosystems) and sized against the GS500 LIZ molecular size standard (Applied Biosystems) using GeneMarker Demo v.1.97 (SoftGenetics, State College, PA, USA).

To describe the genetic diversity, we estimated the number of alleles (A), expected allele number (A_E), allelic richness (A_R), observed (H_O) and expected (H_E) heterozygosities and inbreeding coefficients (F_{IS}) for each population. We tested each locus for deviations

from Hardy–Weinberg equilibrium (HWE) using exact test implemented in Genepop software (Raymond & Rousset, 1995). The significance of the inbreeding coefficients was estimated based on 1000 bootstrap samples as implemented in the *mmod* library (Winter, 2012). We also estimated differences in allele frequencies among populations using the standardized genetic differentiation index G'_{ST} (Hedrick, 2005). Pairwise G'_{ST} between populations were estimated and visually analysed using a phenogram constructed with the neighbor-joining (NJ) algorithm in *ape* (Paradis & Schliep, 2018). We also tested for isolation-by-distance (IBD) using Euclidian distances among populations and the transformed fixation index ($G_{ST}/1-G_{ST}$) as suggested by Rousset (1997). A Mantel test was used to assess the significance of IBD. All diversity and structure estimates were computed using the *adegenet* (Jombart, 2008) and *poppr* (Kamvar, Tabima & Grunwald, 2014) libraries in the R computer language (R Core Team, 2015).

The differences in allele frequencies among populations were analysed via a discriminant analysis of principal components (DAPC) that maximizes between-group variability while ignoring within-group variance as implemented in the *adegenet* library in R. We retained 40 components and four discriminant functions. We estimated the partitioning of genetic diversity among lowland and montane regions and within and between populations with an analysis of molecular variance (AMOVA) using Arlequin 3.5 with 30 000 permutations (Excoffier & Lischer, 2010).

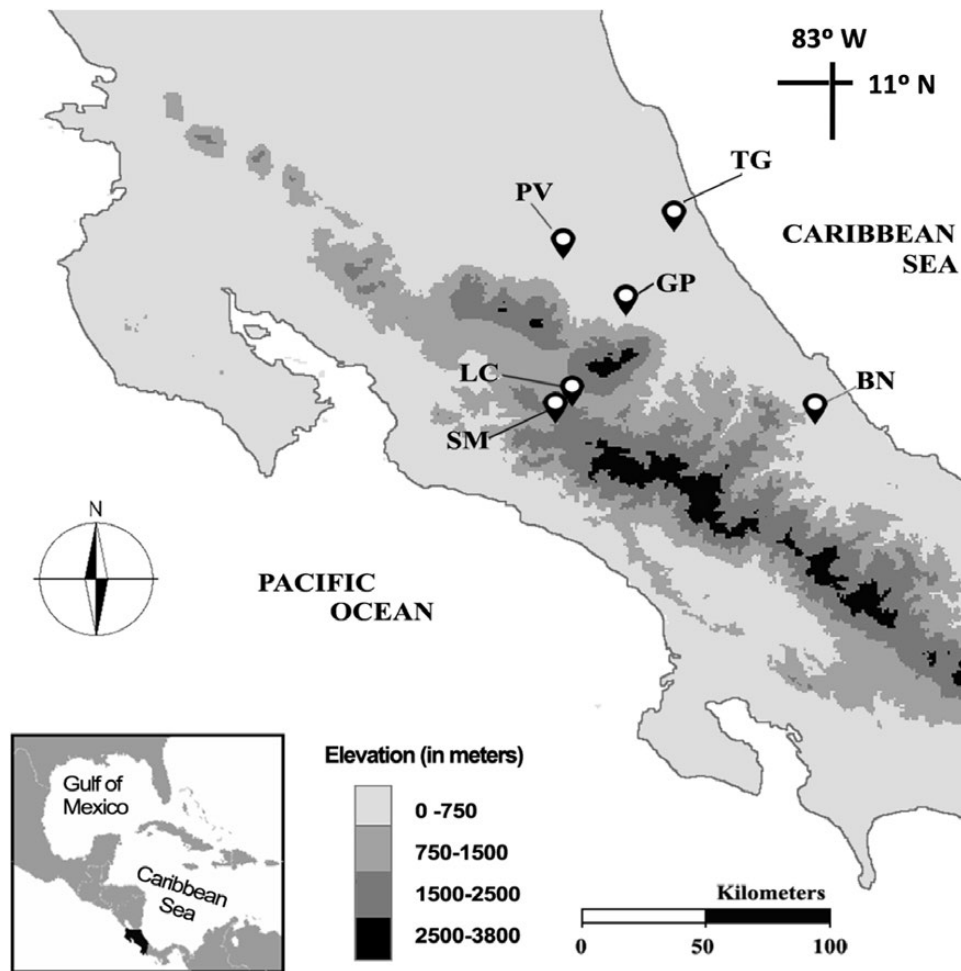


Figure 2. Map of Costa Rica indicating the location of the sampled populations for the genetic analyses of *Aechmea mariae-reginae* (Bromeliaceae). Refer to Supporting Information Table S1 for population codes.

We also used the Bayesian clustering algorithm in STRUCTURE to analyse the underlying structure of *A. mariae-reginae* populations (Pritchard, Stephens & Donnelly, 2000). Analyses were run using the Python pipeline StrAuto v.1.0 (Chhatre & Emerson, 2017) to parallelize STRUCTURE analyses on the computer cluster of the Laboratorio Nacional de Análisis y Síntesis Ecológica (LANASE) from Universidad Nacional Autónoma de México (UNAM). We tested for different cluster numbers ($K \in [2, \dots, 10]$) using 350 000 MCMC with an initial burning of 75 000 runs, correlated allele frequencies and the admixture model. The most likely number of clusters was determined using Evanno's method (Evanno, Regnaut & Goudet, 2005) by replicating analyses for each K value 20 times.

REPRODUCTIVE BIOLOGY STUDY

To understand the reproductive biology of *A. mariae-reginae* that determines genetic diversity patterns,

between 2011 and 2013 we studied the floral biology, reproductive phenology and pollination system in a population at Cerros La Carpintera (LC) located in the Central Valley of Costa Rica ($09^{\circ}53'20''$ N – $83^{\circ}58'10''$ W) at 1600–1800 m a.s.l. (Fig. 2).

We recorded the anthesis time and flower longevity in a sample of 20 pistillate and 25 staminate flowers from two or three inflorescences from each gender. Nectar volume was estimated using calibrated glass micropipettes and sugar concentration with a hand-held refractometer (Eclipse model; Bellingham & Standley, UK) on ten bagged flowers from three inflorescences of each gender 4–5 h after anthesis. We conducted an organoleptic test to determine the emission of any noticeable floral scent. Differences in floral display between genders were determined by counting the number of flowers from 15 pistillate and 15 staminate inflorescences.

Visitors to inflorescences were recorded with video Bushnell Trophy (model 119436) cameras positioned

1–2 m away from target inflorescences. We followed four inflorescences from each gender throughout their flowering period between March and June 2012. Video recording was carried out at daytime, from 05.00 to 18.00 for a total of 220 monitoring hours. For each visitor type, we determined its identity, number of visits per hour and flowers visited per inflorescence.

The flowering phenology and reproductive synchrony between genders was determined by carrying out bi-weekly censuses to record the reproductive condition of 274 individuals in 2011 and 330 plants in 2012. We estimated the flowering synchrony between female and male populations with Schoener's temporal niche overlap index, O_{ij} (Schoener, 1970). Sex ratios (male:female) were determined for both reproductive seasons.

Fruit set in female plants was estimated as the percent of developed fruits in a sample of 50–60 fruits per inflorescence from 17 pistillate plants. We also recorded mean seed number per fruit. The ability to produce seeds by agamosperous means (apomixis) was tested by excluding pollinators in six female inflorescences with a mesh bag at anthesis. Fruits and seeds were counted 4–6 months after flowering.

RESULTS

GENETIC DIVERSITY AND STRUCTURE

We found moderate to high levels of genetic diversity in this dioecious bromeliad ($H_E = 0.571$ – 0.726) (Table 1; see Table S2 for per locus estimates). All loci departed significantly from HWE ($P < 0.05$). Three lowland populations showed a significant deficit of heterozygotes (Table 1). Genetic diversity was significantly structured among all populations ($G'_{ST} = 0.385$, $P < 0.001$). The NJ tree (Fig. 3) shows a clear genetic separation of montane populations (SM, LC) in the Central Valley from those found in the Caribbean lowlands (GP, PV, TG). The southernmost

Caribbean population, BN, is placed in a separate branch in the NJ tree. The DAPC analysis suggests similar results (Fig. S1). Congruently, the AMOVA showed that there is a greater proportion of the genetic variance partitioned among regions (i.e. lowland versus montane; $F_{CT} = 0.107$, $P < 0.001$) than among populations within regions ($F_{SC} = 0.082$, $P < 0.001$) and significant structure among populations ($F_{ST} = 0.180$, $P < 0.001$; Table 2). We found significant IBD ($r = 0.492$, $P = 0.044$), populations that are further apart have stronger differences in allele frequencies compared to nearby populations (Table S3).

Evanno's method suggested that the six populations of *A. mariae-reginae* should be grouped into three ($K = 3$) separate clusters (Fig. 4). STRUCTURE grouped montane populations in one cluster, whereas individuals in lowland Caribbean populations were placed into the two remaining clusters (Fig. 4). The admixture plot (Fig. 4) suggests recent gene flow among BN and TG populations.

REPRODUCTIVE BIOLOGY

Flowers of *A. mariae-reginae* are functionally unisexual, but atrophied reproductive organs (staminodes and pistilloids) of the opposite sex are present. In both genders, anthesis occurred before dawn (04.00–05.30) and flowers had a life span of c. 12 h, senescing by the end of the day (c. 18.00). On senescence, the blue-purple corolla turns pink (Fig. 1B). Nectar volumes were similar between floral types, but sugar concentration was higher in staminate flowers (t -test = 7.27; $P < 0.01$) (Table 3). We did not perceive any floral scent. Pistillate and staminate inflorescences produced similar amounts of flowers; however, pistillate plants opened more flowers per day (t -test = 3.74; $P < 0.01$; Table 3), resulting in a shorter flowering period for female inflorescences compared to male inflorescences (mean = 13.9 vs. 29.8 days, respectively).

Table 1. Genetic diversity estimates for the dioecious and epiphytic bromeliad *Aechmea mariae-reginae* from Costa Rica. Data from six populations from montane and lowland habitat and eight nuclear microsatellite markers

Population	Habitat	N	A	A_E	A_R	H_O	H_E	F_{IS}
LC	montane	20	3.62	2.35	3.32	0.625	0.571	–0.097
SM	montane	20	5.12	2.54	4.60	0.544	0.599	0.083
BN	lowland	15	5.62	3.68	5.55	0.467	0.676	0.261*
GP	lowland	16	5.50	3.18	5.30	0.523	0.609	0.119*
PV	lowland	15	6.50	3.71	6.41	0.604	0.646	0.037
TG	lowland	20	6.75	4.12	6.25	0.478	0.726	0.339*
	Overall	106	10.75	2.89	5.25	—	0.638	0.153*

N, sample size; A, number of alleles; A_E , effective allele number; A_R , allelic richness; H_O , observed heterozygosity; H_E , expected heterozygosity; F_{IS} , inbreeding coefficient. * $P < 0.01$. H_O at the species level is omitted because it includes Wahlund effect.

Hummingbirds were the exclusive visitors to inflorescences of both genders. The most frequent visitor (76% of all recorded visits) was the non-hermit hummingbird *Lampornis calolaemus* (Aves: Trochilidae), followed by the non-hermits *Amazilia rutila*, *Colibri thalassinus* and *Eugenes fulgens*. Hummingbirds visited the inflorescences from around 05.00 and were more active during the morning and around mid-day, with visitation rates of 1.80 and 2.29 visits per hour for pistillate and staminate inflorescences, respectively. At each visiting event birds introduced their bills in several (8–17) flowers. We also observed sporadic visits by butterflies and wasps that were not present in our video recordings.

Flowering of staminate and pistillate populations showed a marked peak in the early rainy season (May–June) (Fig. 5A) and showed a relatively high synchrony, $O_{ij} = 0.70$ and $O_{ij} = 0.78$ for the first and second reproductive year, respectively. The population of staminate plants flowered for a longer period (Fig. 5A). Operational sex ratios significantly deviated from 1:1 and were biased toward staminate plants (3.5:1) in both reproductive seasons ($\chi^2 = 73.8$, $P < 0.01$ and $\chi^2 = 91.1$, $P < 0.01$).

Fruit set in female plants was apparently high, all naturally pollinated pistillate flowers turned into

fleshy berry-type fruits (fruit set = 100%; Fig. 5B). However, after dissecting a sample, several fruits contained no seeds. In a total sample of 640 dissected fruits, an average of 68.6% (± 41.0 SD) fruits per inflorescence contained at least one seed. Fertile fruits contained from one to 694 seeds (mean = 188 ± 166 SD). Seedless or sterile fruits developed normally and were morphologically indistinctive from fertile fruits. Bagged pistillate flowers excluded from pollinators developed and matured all fruits. However, none of the 2608 dissected fruits from six examined inflorescences developed any seeds. Thus, fruit development by parthenocarpy (without pollination), instead of apomixis, best explains these results. Fruits of *A. mariae-reginae* were eaten by brown jays, *Psilorhinus morio* (Corvidae; Fig. 5C).

DISCUSSION

Dioecy in plants is correlated with ecological attributes related to woody growth form, small or inconspicuous flowers and pollination by wind or small insects (Bawa, 1980; Renner & Feil, 1993; Renner & Ricketts, 1995; Vamosi, Otto & Barrett, 2003). Consequently, most research on the reproductive biology and genetics of dioecious tropical plants has centred around tree species (Armstrong & Irvine, 1989; House, 1992, 1993; Riba-Hernández et al., 2014; Paschoa et al., 2018). Here, we present results about the reproductive biology and genetic diversity of an atypical dioecious plant, *A. mariae-reginae*, a herbaceous, epiphytic species with conspicuous hummingbird-pollinated flowers.

REPRODUCTIVE BIOLOGY

Pollination studies in *Aechmea* point to hummingbirds as the main pollen vectors (e.g. Canela & Sazima, 2003; Lenzi, Matos & Orth, 2006; Scrok & Varassin, 2011) and, in some cases, bees as secondary pollinators (Schmid et al., 2010). In *A. mariae-reginae*, floral morphology and field observations clearly support pollination by hummingbirds. The diurnal and apparently scentless flowers that produce nectar as

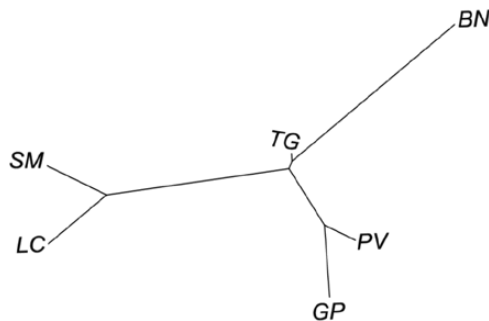


Figure 3. Neighbor-joining tree based on pairwise differences of allele frequencies among six populations of *Aechmea mariae-reginae* (Bromeliaceae) in Costa Rica. Refer to Supporting Information Table S1 for population codes.

Table 2. Analysis of molecular variance (AMOVA) for six populations of *Aechmea mariae-reginae* (Bromeliaceae) in Costa Rica. Populations were grouped into montane (LC, SM) and lowland regions (BN, GP, PV, TG)

Source of variation	d.f.	Sum of squares	Variance components	Percentage of Variation	Phi statistics
Among regions	1	43.69	0.328	10.7	$F_{CT} = 0.107$ ***
Among populations within regions	4	41.284	0.225	7.33	$F_{SC} = 0.082$ ***
Within populations	206	518.86	2.519	81.98	$F_{ST} = 0.180$ ***
Total	211	603.835	3.072		

***: $P < 0.001$.

reward fit the trochilophily pollination syndrome (Faegri & van der Pijl, 1979; Willmer, 2011). Moreover, the colourful pink bracts of inflorescences in both genders are readily noticeable and stand out amidst the green forest canopy, attracting the local guild of hummingbirds from a distance. In the lowland wet forest of the Sarapiquí region in Costa Rica, plants of *A. mariae-reginae* are also visited by non-hermit hummingbirds (Stiles, 1978).

Flowering showed a distinctive annual pattern with a pronounced peak in both genders and with a relatively high synchrony between the staminate and pistillate populations. However, the abundance of flowering plants from both genders was markedly different and resulted in a significantly biased male:female ratio in the studied population. In the two reproductive seasons studied, more staminate plants flowered (3.5:1 in both years). Sex ratios in dioecious species may depart from the expected 1:1 value, and when it happens it is frequently biased to males (Opler & Bawa, 1978; Sinclair, Emlen & Freeman, 2012; Field, Pickup & Barrett, 2013). In *Hechtia schottii* Baker a dioecious and terrestrial bromeliad with insect pollination, a similar male bias (3.1:1) has been reported (Ramírez-Morillo *et al.*, 2008). The specific reasons for our results are unknown, in general, biased sex ratios in plants can be the result of genetic or environmental factors,

such as differential mortality or resource availability (Lloyd & Webb, 1977; Dellaporta & Calderón-Urrea, 1993; Sinclair *et al.*, 2012). From a pollination perspective, a higher abundance of pollen donors is expected to reduce the effects of pollen limitation on female plants of dioecious species (Knight *et al.*, 2005). However, the high variation (mean = 68.6%, C. V. = 59.7%) in fruit set (seeded fruits) among female individuals and in seed number among fertile fruits (mean = 188, C. V. = 88.3%) is indicative of pollen limitation. Although unconfirmed, this variation in female reproductive output is possibly the result of variation in male density and distance to reproductive conspecifics and flowering synchrony with males (Flanagan & Moser, 1985; House, 1993; Torimaru & Tomaru, 2006; Anderson *et al.*, 2015).

The production of seedless fruits (parthenocarpic or sterile fruits) has been documented in other dioecious plants (Ramos-Ordoñez, Márquez-Guzmán & Arizmendi, 2008) and in hermaphroditic species of Bromelioideae, e.g. *Aechmea lindenii* (E.Morren) Baker (Lenzi *et al.*, 2006), *Bromelia hieronymi* Mez (Godoy *et al.*, 2018) and the cultivated *Ananas comosus* (L.) Merr (Bartholomew, Paull & Rohrbach, 2003). Parthenocarpy is considered a strategy to reduce predation on seeded fruits or to attract seed dispersers by increasing the visual signal (Verdú & García-Fayos, 1998, 2001). On average, one-third of the fruits developed by our study species was sterile and morphologically indistinguishable from fertile fruits, and therefore sterile fruits could increase the conspicuousness or attractiveness of the pineapple-like infructescence of *A. mariae-reginae* to frugivores (Fuentes, 1995).

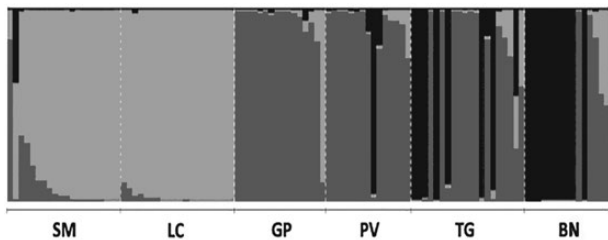


Figure 4. Population structure in *Aechmea mariae-reginae* (Bromeliaceae) using Bayesian assignment analysis. Evanno's method suggested grouping into three ($K = 3$) clusters. Data based on eight nuclear microsatellite markers from six populations from Costa Rica. Refer to Supporting Information Table S1 for population codes.

GENETIC DIVERSITY AND STRUCTURE

Obligate outcrossing in dioecious flowering plants is commonly associated with higher genetic diversity (Hamrick & Godt, 1996; Glémin, Bazin & Charlesworth, 2006) and, as expected, we found moderate to high levels of genetic diversity in *A. mariae-reginae*, with slightly lower estimates in montane populations (LC and SM). Our overall genetic diversity estimates are

Table 3. Floral traits of pistillate and staminate plants in the dioecious and epiphytic bromeliad *Aechmea mariae-reginae* (Bromeliaceae), Cerros La Carpintera, Costa Rica. Mean values \pm 1 S.D. Sample sizes in parentheses.

Trait	Pistillate plants	Staminate plants
Flowers per inflorescence	389 \pm 114 (15)	388 \pm 128 (15)
Open flowers per day/ inflorescence **	28 \pm 17 (10)	13 \pm 6 (15)
Floral nectar volume (μ l)	51.1 \pm 4.2 (10)	57.5 \pm 14.4 (10)
Sugar concentration of nectar ($^{\circ}$ Bx) **	11.3 \pm 1.0 (10)	16.3 \pm 2.0 (10)

** $P < 0.01$ after a t -test.

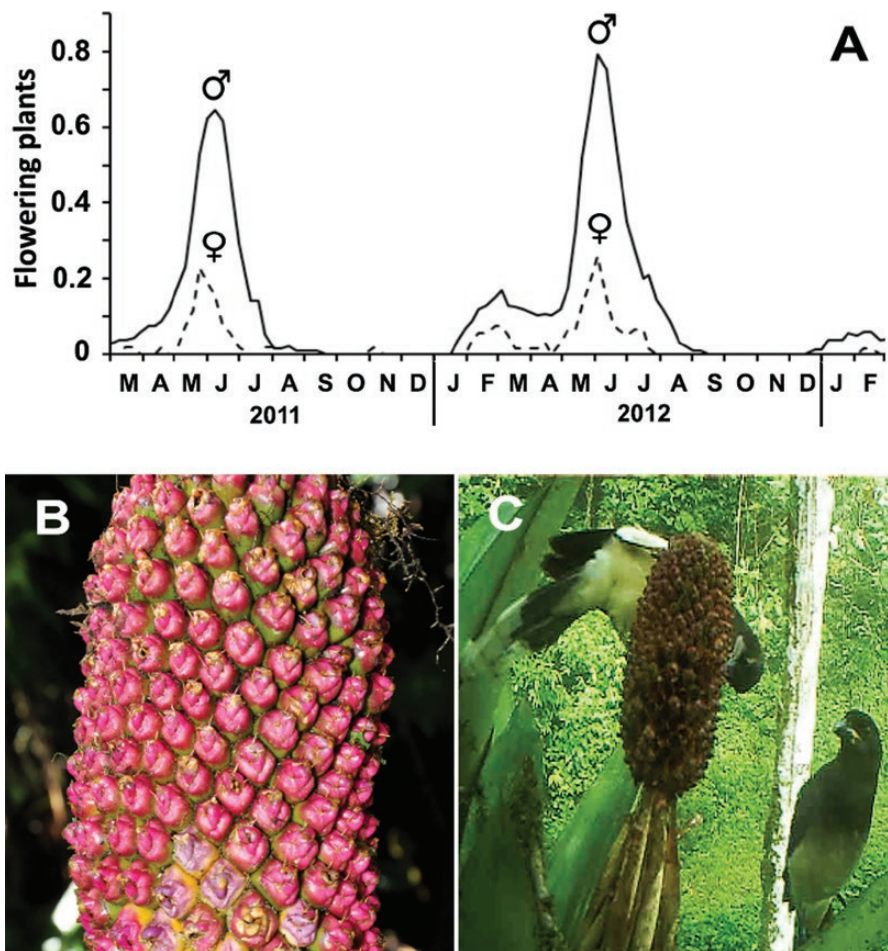


Figure 5. Flowering pattern of *Aechmea mariae-reginae* (Bromeliaceae) at Cerros La Carpintera, Costa Rica. A, Data are the proportion of flowering male (continuous line) and female plants (dashed line) from March 2011 to February 2013. B, Mature infructescence, note all fruits developed. C, Individuals of *Psilorhinus morio* (Corvidae) eating fruits. Photographs: A. Cascante-Marín.

comparable to those found for hermaphroditic and obligate outcrossing *Aechmea* spp. (Goetze *et al.*, 2016, 2018) and similar to other tropical dioecious species ($H_o = 0.470\text{--}0.860$, $H_e = 0.560\text{--}0.890$: Hardesty *et al.*, 2005; Cibrián-Jaramillo *et al.*, 2009; Riba-Hernández *et al.*, 2014). The relatively lower genetic diversity observed in montane population of *A. mariae-reginae* may be related to other factors associated with dioecious life-history traits, which may lower effective population sizes and genetic diversity. For example, biased sex ratios and variance in fertilization rates both decrease effective population sizes in dioecious species (Luna, Epperson & Oyama, 2007; Vandepitte *et al.*, 2009). Our reproductive data from the LC population of *A. mariae-reginae* indicated a significant male-biased sex ratio and important variance in female reproductive success, both of which reduce effective population sizes and may partially explain the observed lower genetic diversity in that

population. Thus, we may conclude that moderate to high levels of genetic diversity among populations of *A. mariae-reginae* are the result of a balance between forces that increase genetic diversity such as obligate outcrossing and demographic factors associated with dioecious life form that reduce effective population size such as biased sex ratios and variance in male/female reproductive success.

Our data showed significant inbreeding coefficients due to deficit of heterozygotes in three lowland populations; since *A. mariae-reginae* is an obligate outcrosser, inbreeding is most likely the result of biparental inbreeding. For bromeliads, in general, clonal reproduction by means of asexual ramets is a common reproductive strategy (Benzing, 2000) and short-range seed dispersal has been observed in some species (Cascante-Marín *et al.*, 2009; Paggi *et al.*, 2010); both characteristics are likely to increase consanguineous mating between genets and related individuals, either

on the same phorophyte or between nearby trees. Since our sampling was specifically conducted to avoid collecting genets or related individuals on the same tree, we do not have data on relatedness within trees. However, the higher likelihood of observing biparental inbreeding in the lowland populations may be associated with lower density of individuals caused by greater habitat loss and fragmentation in this region of the country (Cascante-Marín *et al.*, 2002; Fuchs, Lobo & Quesada, 2003). An increase in inbreeding due to urbanization and recent deforestation was also suggested for *A. winkleri* Reitz, an endemic congener from Brazil (Goetze *et al.*, 2015).

Populations of *A. mariae-reginae* in Costa Rica are genetically structured. Our estimates of Hedrick's (2005) population genetic differentiation are slightly higher than those reported for other hermaphroditic *Aechmea* spp. using SSR markers: $G'_{ST} = 0.236$ in *A. calyculata* (E. Morren) Baker (Goetze *et al.*, 2016) and $G'_{ST} = 0.106$ in *A. kertesziei* Reitz (Goetze *et al.*, 2018), but they are somewhat lower when compared to other members of Bromeliaceae based on F_{ST} values (Lavor *et al.*, 2014). Most studies attribute patterns of genetic structure in Bromeliaceae to gene vector movement patterns, and pollen movement is regarded as the main factor responsible for gene flow among bromeliad populations (Barbará *et al.*, 2008; Palma-Silva *et al.*, 2009; Paggi *et al.*, 2010). *Aechmea mariae-reginae* is pollinated mostly by territorial hummingbirds; the main pollinator at LC was *Lampornis calolaemus*, a non-migratory species restricted to cloud montane forests of Central America (Peters, 2011). Such territorial pollinators have short foraging routes, and thus pollen dispersal occurs commonly at short distances (Webb & Bawa, 1983). Seed dispersal of the berry-type fruits of *A. mariae-reginae* and congeners fit the ornithochory syndrome; however, seed dispersal dynamics of bird-dispersed bromeliads and the role in gene flow are less known (Benzing, 2000). For *A. mariae-reginae* the role of brown jays as potential long-distance seed dispersers requires further study. In some tillandsioid wind-dispersed bromeliads previous works have shown that fine-scale structure due to limited seed dispersal is common (Cascante-Marín *et al.*, 2009; Paggi *et al.*, 2010) and can easily limit gene flow among populations. Further, for dioecious plants seed dispersal is restricted by the fact that only half the population disperses seeds and they disperse around female plants creating a clumped spatial distribution compared to an equivalent co-sexual population, a phenomenon referred to as the seed-shadow handicap (Heilbuth, Ilves & Otto, 2001; Barot & Gignoux, 2004); this may limit gene flow while increasing genetic isolation among populations.

The DAPC results and the neighbor-joining tree congruently showed that montane populations

are grouped into a separate cluster from lowland populations. Moreover, these analyses also indicated that BN, the southernmost population, is genetically structured from other Caribbean sites. However, our analyses suggest that gene flow may still occur between TG and BN, since individuals in both populations were grouped within the same cluster by STRUCTURE (Fig. 4). These two lowland populations are separated by nearly 100 km but have moderate structure among them, $G'_{ST} = 0.315$ (Table S3). Even if significant genetic structure and IBD exist in *A. mariae-reginae*; STRUCTURE results suggest the potential for gene flow over relatively long distances, particularly between TG and BN. The original habitat of *A. mariae-reginae* in the Caribbean lowlands of the country was severely reduced during the expansion of the banana industry in the early 20th century (Jones & Morrison, 1952) and largely contributed to the present fragmented distribution of remnant populations of this epiphytic bromeliad. However, these sites are still interconnected by interspersed forest fragments, which may explain occasional gene flow events through intermediate individuals or small populations that we were unable to locate and thus were not included in our sampling scheme. Tropical forest cover outside protected areas is constantly being reduced (Achard *et al.*, 2014) and in the Caribbean lowlands of Costa Rica is more recently related to the pineapple industry (INEC, 2015). In addition, the frequent tilling of large remnant trees associated with urbanization or agricultural expansion, may limit phorophyte availability for epiphytic bromeliads lowering population sizes and removing key individuals that connect fragmented habitats and facilitate gene flow (i.e. stepping-stones; Cascante-Marín *et al.*, 2002; Fuchs & Hamrick, 2011). Therefore, it is likely that occasional long-distance gene-flow events that link distant populations will become rare, increasing the negative effects of drift on within-population genetic diversity. Our data showing lower genetic diversity and stronger genetic differences between montane and lowland populations support these predictions.

Montane populations in the Central Valley of Costa Rica are not only at a relatively greater distance from lowland populations in the Caribbean coast, but they are also topographically isolated by the Central Volcanic mountain range and occupy a habitat with different environmental conditions (e.g. lower temperature and different rainfall regime). Habitat variation may generate ecotypic differences in flowering times among populations and limit gene flow. In LC we recorded the flowering peak of *A. mariae-reginae* in May–June, whereas Stiles (1978) documented that a population from the Caribbean lowlands, near our PV population, had its flowering peak in February–March. These different phenological patterns may have arisen

as a result of adaptations to local climatic conditions and interactions with pollinator communities (Rathcke & Lacey, 1985) and are likely to disrupt effective gene flow due to asynchrony in reproductive schedules between populations in different habitats (e.g. isolation-by-environment; Sexton, Hangartner & Hoffmann, 2013). Therefore, our results may indicate that isolation-by-environment, in which gene flow occurs preferentially among similar environments (i.e. among sites at similar elevation *sensu* Sexton *et al.*, 2013) is also limiting gene flow between montane and lowland populations of *A. mariae-reginae*.

CONSERVATION REMARKS

Aechmea mariae-reginae has a restricted distribution to the southern part of Central America and represents an interesting evolutionary adaptation as it is the only dioecious species in the genus; preserving populations of this species may allow future insights into the evolution of dioecy and the effects of inbreeding. Montane populations had lower genetic diversity estimates compared to lowland populations possibly caused by reduced effective population sizes. These two populations are at a significantly different elevation and thus are isolated from lowland populations by distance and environmental conditions. Additionally, forest cover surrounding SM and LC has been greatly reduced and fragmented due to urbanization and transformation of montane forests into coffee plantations and cattle pastures (Sánchez-Azofeifa *et al.*, 2001), limiting populations to a few remnant forested areas (our sampling sites). This reduction has had an impact on genetic diversity in contrast to lowland populations that span greater area and were probably connected in the recent past as suggested by our clustering results. Although our results show that lowland populations of this bromeliad in Costa Rica have higher levels of genetic diversity, they still highlight the importance of maintaining connectivity among remnant forests that allows gene flow to preserve diversity. Inbreeding in lowland populations may be the result of habitat loss and fragmentation (Aguilar *et al.*, 2008). Lowland tropical habitats are constantly in peril of habitat loss through urbanization or agricultural expansion (Achard *et al.*, 2014), which may further reduce population sizes of plants and pollinators, increasing isolation and reducing gene flow even further; this will have a toll on genetic diversity in these populations that presently have the most diversity in this species.

Montane populations (SM and LC) from the Central Valley represent a distinctive group of genotypes different from lowland populations. Our results show that these populations are genetically less diverse and, given the prominent sex bias and the variance in

reproductive success, effective population size may be small and thus highly susceptible to further reductions by stochastic events or anthropogenic effects. In general, dioecious species are more sensitive to extinction risk (Vamosi & Vamosi, 2005; Willis, 2017), and conservation efforts and future studies on long-term viability should focus on montane populations of *A. mariae-reginae* as they are probably at greater risk.

CONCLUSIONS

Our results revealed moderate to high levels of genetic diversity in *A. mariae-reginae* as expected for an obligate allogamous and dioecious plant. The genetic variation is structured in three clusters that reflect an environmental pattern that comprises populations from lowland and montane habitats. Although some recent gene flow was detected among populations and clusters, the studied populations showed significant genetic differentiation and further reduction and fragmentation of the natural habitat of *A. mariae-reginae* is likely to reduce inter-population gene flow and increase genetic isolation. Conservation of this geographically restricted and dioecious epiphytic bromeliad is contingent on the preservation of its habitat and pollinators.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

TABLE S1. Sampled populations for the study of the genetic variation in *Aechmea mariae-reginae* (Bromeliaceae) in Costa Rica. Montane populations (LC and SM) from the Central Valley and lowland populations (BN, GP, PV and TG) from the Caribbean coast.

TABLE S2. Genetic diversity estimates per locus and per population for six populations of *Aechmea mariae-reginae* in Costa Rica. H_O : Observed heterozygosity; H_E : Expected heterozygosity; F_{IS} : Inbreeding coefficient; A : Observed number of alleles; A_E : Expected allele number. All stats were estimated using the *hierfstat* library in R.

TABLE S3. Geographic distances in kilometers (upper diagonal) and pairwise G'_{ST} statistics (lower diagonal) between all studied populations of *Aechmea mariae-reginae* (Bromeliaceae) in Costa Rica.

Figure S1. Discriminant analysis using principal component axis one (PC1) and axis two (PC2) based on multilocus genotype data of *Aechmea mariae-reginae* (Bromeliaceae) from six populations from Costa Rica. Data from eight nuclear microsatellite markers. Each population is coded with a different colour; refer to Table S1 for population information.