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BIODIVERSITY RESEARCH

Evolutionary history of the endangered shrub snapdragon (*Galvezia leucantha*) of the Galápagos Islands

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ABSTRACT

Aim The endangered Galápagos shrub snapdragon (*Galvezia leucantha*, Antirrhineae, Plantaginaceae) is restricted to small populations on four islands. In this study, we appraised results from taxonomy, genetics, phylogenetics, phylogeography and pollination ecology to reconstruct the evolutionary history of the genus *Galvezia*.

Location Peru, continental Ecuador and Galápagos.

Methods We sequenced the nuclear ribosomal ITS and two plastid regions, *ndhF* and *ndhF-rpL32*, to infer the origin of *Galvezia* and patterns of colonization to and across the Galápagos archipelago, based on Bayesian inference and statistical parsimony analyses. To investigate genetic diversity and differentiation within *G. leucantha*, we screened the genome of six populations and obtained 194 AFLP fingerprints. Autogamy tests and pollination network analyses were performed to evaluate the colonization potential and to investigate the structure of the pollinators' assemblage of *Galvezia*.

Results Relationships of seven nucleotide-substitution haplotypes and 11 nucleotide-substitution ribotypes of Galvezia revealed monophyly for the Galapagos species. Dating estimates indicated divergence of the insular *Galvezia* lineage in the Middle-Upper Pleistocene (0.66–0.09 Ma). In addition, distribution of genotypes (seven haplotypes, eight ribotypes) across the three continental species showed geographical differentiation, while low differentiation and distribution of *G. leucantha*. AFLP genetic diversity is relatively high (H_T = 0.109), but a low proportion of the total allelic variance is attributed to variation among subspecies/islands (H_b = 0.035, hierarchical AMOVA: 3.77% of total variance). The endemic bee (*Xylocopa darwinii*) accounted for 87.30% of the floral visits to *G. leucantha*.

Main conclusions We inferred a single origin for an insular lineage that colonized the Galápagos Islands from northern Peru in the Pleistocene. Recent colonization of the archipelago, Pleistocene land bridges between islands and active gene flow promoted by *X. darwinii* may account for the low-moderate genetic differentiation of *G. leucantha* subspecies. An unusual pollination shift from ornithophily (hummingbirds on the continent) to entomophily (*Xylocopa* in the Galápagos Islands) is supported.

Keywords
genetic diversity, geographical speciation, island colonization, ornithophily, pollination network, secondary melittophily.

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INTRODUCTION

The Galápagos Islands were formed by the emerging tops of submarine volcanoes rising from the sea floor and currently forming 13 large islands and about 100 small islets. Located on the equator, the Galápagos Islands (hereafter Galápagos) are known worldwide for their highly diverse forms of life, rich ecosystems and their unique geological composition (Gillespie & Clague, 2009). Unfortunately, insular endemic plants are vulnerable to extinction in the Galápagos due to the direct negative impact of humans, habitat degradation, competition and hybridization with alien species (Rieseberg & Swensen, 1996; Maunder *et al.*, 1998). Nearly one-fifth of the endemic plant species are Endangered and *c.* 12% are Critically Endangered (www.galapagos.org/conservation/conservation). The Galápagos shrub snapdragon, *Galvezia leucantha,* is one of such species included in the red list of the endemic plants of Ecuador as Endangered (León-Yánez *et al.*, 2011) probably as a consequence of intense goat herbivory in the past (Tye & Jäger, 2000). A conservation programme helped to protect the *Galvezia* populations of Santiago and Rábida islands (Atkinson *et al.*, 2008), on which goats were exterminated in 2000, but herbivory by feral livestock is still a major threat on the larger islands (Carrión *et al.*, 2011).

Although the Galápagos shrub snapdragon has been recognized since its original description, the continental species of *Galvezia* has been subject to scientific discussion (Dillon & Quipuscoa, 2014). A new taxonomic account proposes that *Galvezia* Dombey ex Juss. (Antirrhineae, Plantaginaceae) is a small-sized genus of four perennial shrub species distributed in arid habitats near the Pacific coast of Peru and Ecuador (*G. elisensii*, *G. fruticosa*, *G. grandiflora*) and the Galápagos (*G. leucantha*) (Elisens, 1989; Dillon & Quipuscoa, 2014). Mainland species of *Galvezia* are morphologically similar (Fig. 1a–d), with differences typically confined to corolla size, anther filament pubescence and leaf shape (Sutton, 1988). In addition, the Galápagos shrub snapdragon has several unique morphological characters (Wiggins, 1968) (Fig. 1e–g), whereas Ecuadorian and Peruvian species have red flowers with long corolla tubes, *G. leucantha* has shorter and wider corollas and spans a broad colour range (whitish-to-purple) (Fig. 1). *Galvezia leucantha* exhibits

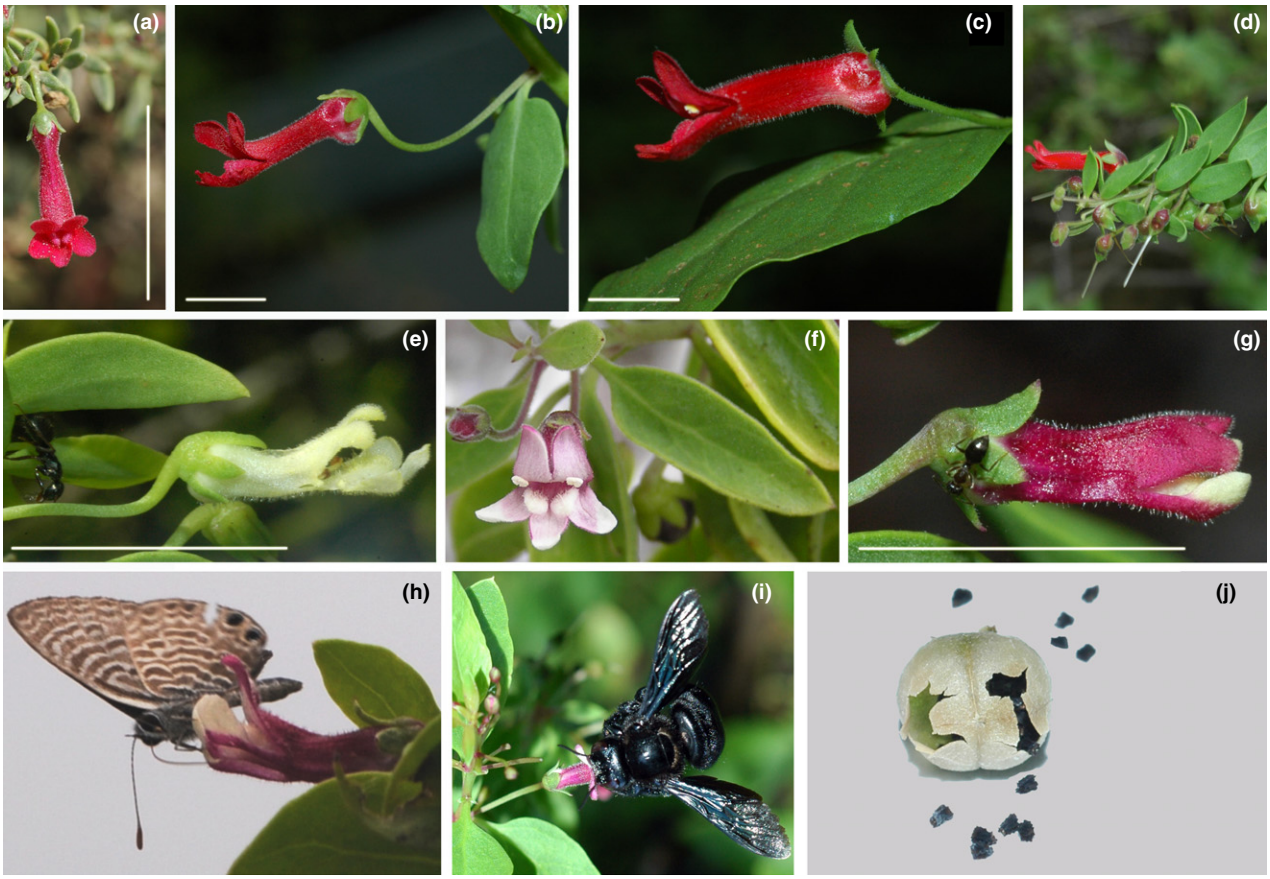

Figure 1 Floral diversity of *Galvezia* species. *G. elisensii* (a), *G. fruticosa* (b), *G. grandiflora* (c & d), *G. leucantha* subsp. *leucantha* (e), *G. leucantha* subsp. *pubescens* (f), *G. leucantha* subsp. *porphyrantha* (g), *Leptotes parrhasioides* foraging in *G. leucantha* subsp. *porphyrantha* from population SAJam (h), *Xylocopa darwini* foraging in *G. leucantha* subsp. *porphyrantha* from population SAJam (i), open capsule (j). The scale bars correspond to 1 cm. Photographs by M.O. Dillon (a), P. Vargas (b–e, g, i, j), W. Simbaña (f), A. Traveset (h). [Colour figure can be viewed at wileyonlinelibrary.com]

morphological variation leading to the historical recognition of three subspecies (Wiggins, 1968; Wiggins & Porter, 1971; Tye & Jäger, 2000). Subspecific taxa within this species are delimited by young branches, pedicels and calyx pubescence between subspp. *pubescens* and *leucantha*/*porphyrantha*, while corolla colour delimits subspp. *leucantha* and *porphyranta* (Fig. 1e–g). All subspecies are geographically restricted to cliffs, lava flows and old craters of the four westernmost islands (Fig. 2): subsp. *leucantha* occurs on Isabela and Fernandina; subsp. *pubescens* is endemic to Rábida Island; and subsp. *porphyrantha* has only a few populations scattered on Santiago Island.

Genetic diversity is essential for reconstruction of the evolutionary history of the species and conservation programmes. However, studies on plant genetic diversity of Galápagos plants are extremely scarce (but see Jaramillo & Atkinson, 2011). In particular, former studies based on allozyme/isozyme variation of *Galvezia* found genetic links and minimal variation between Galápagos populations, which suggested a genetic bottleneck that prevented description of inter- and intra-island movements and population differentiation after a single colonization event of *Galvezia* arriving in the Galápagos (Elisens, 1992). Most of the Galápagos flora has long been assigned to the Americas (Hooker, 1847). Indeed, floristic (Wiggins & Porter, 1971) and phylogenetic (e.g. Moore *et al.*, 2006; Trusty *et al.*, 2012) studies have shown that most species of the Galápagos originated from South America and the Caribbean. *Galvezia* fruits are dehiscent capsules (Dillon & Quipuscoa, 2014) (Fig. 1j). Both fruits and seeds lack structures that favour long-distance dispersal, which leads to interpreting a single colonization event (Elisens, 1985; Vargas *et al.*, 2012). Nevertheless, a high number of early colonists appear to have arrived in the Galápagos irrespective of their diaspore

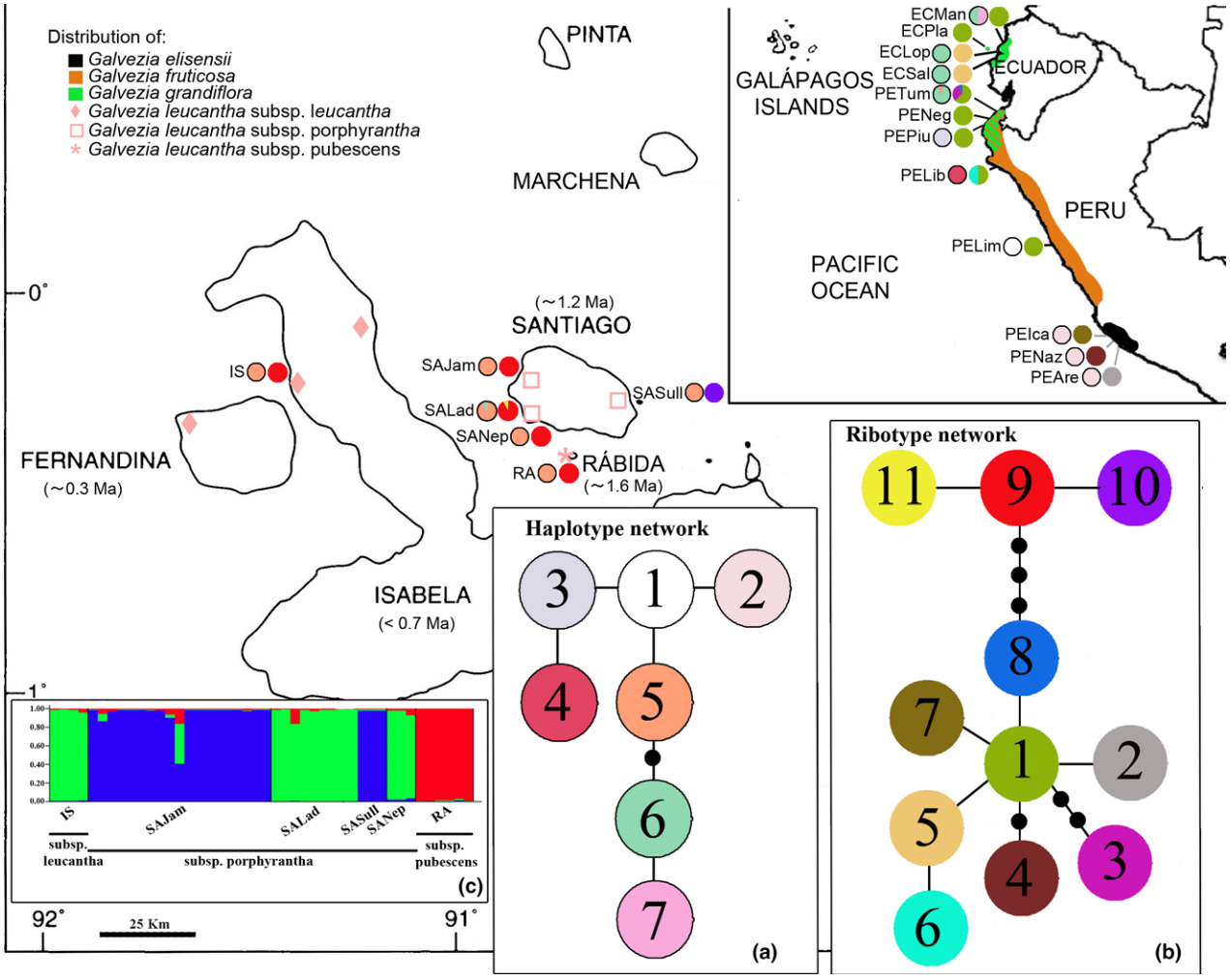

Figure 2 Distribution map and geographical range of seven haplotypes and 11 ribotypes of six *Galvezia* taxa from Peru, continental Ecuador and the Galápagos Islands. Statistical parsimony network based on (a) *ndhF-rpL32* and (b) ITS sequences of *Galvezia* taxa. STRUCTURE results for *Galvezia leucantha* (n = 52) using 194 AFLP markers and K = 3 population clusters (c). Haplotypes (1–7) and ribotypes (1–11) are indicated by numbers, lines indicate a single nucleotide substitution, and dots (●) represent extinct or not detected haplotypes/ribotypes. [Colour figure can be viewed at wileyonlinelibrary.com]

dispersal syndromes (Vargas *et al.*, 2012). Both single and multiple independent colonization events have historically been proposed, based simply on taxonomy (Wiggins & Porter, 1971). Indeed, several lineages sampled at the species (*Darwinothamus*, Andrus *et al.*, 2009) and population (*Croton scouleri*, Rumeu *et al.*, 2016) levels indicate that multiple colonization events may have occurred in spite of the isolation of the archipelago (*c.* 1000 km off the coast of Ecuador). Although a genetic link between *G. leucantha* (Galápagos) and South America (*G. fruticosa*) has already been proposed based on allozymes (Elisens, 1992), lack of accurate molecular markers, such as those from DNA, hindered more detailed inferences about the spatio-temporal history of *Galvezia* in the Galápagos.

Community-based conservation strategy is necessary to protect endangered plants because pollinators are essential for the reproductive success of the plants. A decrease in availability of pollinators may lead to a suppression of outcrossing and a decrease in genetic variability. Elisens (1992) suggested that red flowers with narrow corolla tubes would be adaptations for pollination by hummingbirds in mainland species. Shorter and wider whitish-to-purple corollas are pollinated by carpenter bees (*Xylocopa darwini*) in the Galápagos species. However, the pollinator fauna is still poorly documented for the entire genus. Over 65 families of angiosperms display ornithophilous floral traits (Cronk & Ojeda, 2008); that is, they offer numerous cases of recurrent convergent evolution involving certain flower features (tubular flowers, red corollas, flower orientation, among others) related to pollination by birds. This co-evolutionary association is well known in the Americas, where historical presence of hummingbirds is related to pollination shifts primarily derived from insect (entomophily) to bird (ornithophily) pollination (Rauscher, 2008). The loss of bird pollination seems to be a rare evolutionary pattern (Wilson *et al.*, 2007; Rauscher, 2008). In the Galápagos, the role of birds as pollinators has been historically underestimated given the absence of specialized nectarivorous birds (Traveset *et al.*, 2015a,b).

Our working hypothesis is that a single introduction to the Galápagos occurred in the lower Pleistocene, most probably from northern coastal Peru (Elisens, 1989), followed by changes in floral traits related to bee-pollination. In this study, we performed phylogenetic and phylogeographic analyses using nuclear (ITS sequences, AFLPs) and plastid (*ndhF* and *ndhF-rpL32* sequences) DNA variation from the four species and three subspecies of *Galvezia* to: (1) test the monophyly of the Galápagos populations; (2) find out the sister group of *G. leucantha*; (3) pinpoint a source area from the continent linked to Galápagos populations; (4) determine population structure of *G. leucantha* across the Galápagos; and (5) characterize their reproductive biology, floral visitors and pollination communities to help understand the evolutionary history of *Galvezia* lineages. The ultimate goal is to consider the evolutionary process of *Galvezia* differentiation in conservation planning of the Galápagos shrub snapdragon.

METHODS

Sampling strategy and DNA sequencing

Leaf samples were collected from 18 populations (87 individuals) of *Galvezia* species across Peru (10 populations/30 individuals), continental Ecuador (4/5) and the Galápagos (6/52) (Fig. 2, Table 1). Between 1 and 21 individuals per population were sampled depending on population abundance (Table S1 in Appendix S1 in Supporting Information). All known populations of the three subspecies of *G. leucantha* were sampled, except for subsp. *leucantha* from Fernandina, where three expeditions (2008, 2010, 2014) failed to find the population suggested by a herbarium specimen deposited in the CDF herbarium (Cabo Douglas, leg. T. Luong), and from eastern Isabela, where we also failed in 2014 (Volcán Alcedo, leg. T. Luong). Total genomic DNA was extracted from dried leaves using the Dneasy Plant Mini Kit (Qiagen, California) according to the manufacturer's instructions. A pilot study was performed to search for the most variable nuclear (AGT1, AroB, ATI03, EIF3E, ITS) and plastid (*atpI-atpH, matk, ndhF, ndhF-rpL32, psbC-trnS, trnD-trnT, trnDguc, trnH-trnK, trnS-trnfM, trnS-trnG, trnT-L-F*) DNA sequences within *Galvezia* (Table S2). The pilot study revealed the sequences of the nuclear ribosomal ITS region and the plastid intergenic spacer *ndhF-rpL32* to be the most variable (Table S2). Therefore, the ITS (84 *Galvezia* samples) and the *ndhF-rpL32* (83 *Galvezia* samples) regions were sequenced.

In addition, to infer phylogenetic relationships within Antirrhineae, two data sets were built based on previously published data plus newly generated sequences (Table S1). The nuclear ribosomal ITS (32 newly generated Antirrhineae sequences) and the plastid *ndhF* gene sequences (34 newly generated Antirrhineae sequences) helped infer phylogenetic relationships among 166 and 163 Antirrhineae taxa, respectively. In addition, phylogeographic patterns were inferred using ITS (87) and *ndhF-rpL32* (83) newly generated sequences of six *Galvezia* taxa. Methods for DNA extractions, PCR amplifications and sequencing were described in two recent studies of Antirrhineae for ITS (Fernández-Mazuecos *et al.*, 2013) and *ndh*F (Vargas *et al.*, 2014). See Appendix S2 for *ndhF-rpL32* sequencing details and for clarification of three incongruent results identified in previous Antirrhineae phylogenies (Ghebrehiwet *et al.*, 2000; Vargas *et al.*, 2004) according to Guzmán *et al.* (2015a).

Molecular dating

The nuclear ITS and the plastid *ndhF* gene were analysed to estimate divergence times of *Galvezia* and related lineages. The analysis was implemented in BEAST v.1.8.2 (Drummond & Rambaut, 2007) on the CIPRES portal teragrid (www.phylo.org; Miller *et al.*, 2010). We used a birth–death model for the tree prior, an uncorrelated relaxed molecular clock, a random starting tree, and the models selected by jModelTest (Posada, 2008) for each DNA region (ITS:

Table 1 Accession data, haplotype and ribotype number of 18 populations of *Galvezia* taxa (87 individuals). Taxonomy follows that of Dillon & Quipuscoa (2014).

Population code	Taxon	Locality (number of individuals)	Voucher number	Haplotype number	Ribotype number
PEAre	*G. elisensii*	Peru, Arequipa (1)	F1940835	2	2
PENaz	*G. elisensii*	Peru, Nazca (1)	F2144517	2	4
PEIca	*G. elisensii*	Peru, Departamento Ica (1)	F2184045	2	7
PELib	*G. fruticosa*	Peru, La Libertad, San Pedro de Lloc (2)	WE825 (OKL)	4	1, 6
PELim	*G. fruticosa*	Peru, Lima, Valle del río Chillon (5)	MA763175	1	1
PEPiu	*G. grandiflora*	Peru, Departamento Piura (2)	WE828 (OKL)	3	1
PETum	*G. grandiflora*	Peru, Tumbes (16)	MA763174	5, 6	1, 3, 8
PENeg	*G. grandiflora*	Peru, Negritos (2)	MA763173	–	1
ECMan	*G. grandiflora*	Ecuador, Manabí, Manta (2)	WS610/WS619 (MA)	6, 7	1
ECSal	*G. grandiflora*	Ecuador, Manabí, Salango (1)	WS612 (MA)	6	5
ECPla	*G. grandiflora*	Ecuador, Manabí, Plata Island (1)	WS613 (MA)	6	1
ECLop	*G. grandiflora*	Ecuador, Manabí, Puerto López (1)	WS615 (MA)	6	5
IS	*G. leucantha* subsp. *leucantha*	Ecuador, Galápagos Islands, Isabela, Tortuga Negra (6)	33/35/37/39/41BGA14 + 130PV08	5	9
SANep	*G. leucantha* subsp. *porphyrantha*	Ecuador, Galápagos Islands, Santiago, Cabo Nepean (3)	11100 CDRS	5	9
SASull	*G. leucantha* subsp. *porphyrantha*	Ecuador, Galápagos Islands, Santiago, Bahía Sullivan (3)	11874 CDRS	5	10
SALad	*G. leucantha* subsp. *porphyrantha*	Ecuador, Galápagos Islands, Santiago, Bahía Ladilla (13)	29PV10/41-45PV14/47PV14 (MA)	5, 6	9, 11
SAJam	*G. leucantha* subsp. *porphyrantha*	Ecuador, Galápagos Islands, Santiago, Bahía James (21)	27PV10 (MA)	5	9
RA	*G. leucantha* subsp. *pubescens*	Ecuador, Galápagos Islands, Rábida (6)	17871 CDRS/17872 CDRS	5	9

GTR+G, *ndhF*: GTR+I+G). We did two independent runs; each one for 120 million steps sampled every 12,000th. Convergence to stationarity and effective sample size (ESS) of model parameters were assessed using TRACER 1.5 (Rambaut & Drummond, 2007). Samples from both independent runs were pooled after removing a 10% burn-in using Log Combiner 1.8 (Drummond & Rambaut, 2007). We used a previous molecular-estimated crown group age of Antirrhineae as a calibration point (Vargas *et al.*, 2014) and applied a normally distributed calibration point prior with a mean of 30.22 ± 4 (SD) million years (Ma).

Phylogeographic data analysis

A cpDNA (*ndhF-rpL32*) and a nDNA (ITS) sequence network were constructed using the software TCS 1.21 (Clement *et al.*, 2000), which implements a statistical parsimony approach using the algorithm described in Templeton *et al.* (1992). The maximum number of differences among sequences, as a result of single substitutions, was calculated with 95% confidence limits and treating gaps as missing data.

AFLP fingerprinting of *G. leucantha* and data analysis

An AFLP approach (see for details Guzman *et al.*, 2015b) was used to find out genetic variability and structure across *G. leucantha* accessions. Primer sequences for AFLPs are shown in Table S3. Initially, 32 selective primer combinations were analysed in a subset of five samples comprising the three subspecies of *G. leucantha*. One replicate was included to test for reproducibility.

AFLPs profiles were analysed using GENEMAPPER 4.1 software (Applied Biosystems, Foster City, CA, USA). Six primer combinations (EcoRI-ACC/MseI-CTT, EcoRI-AGA/MseI-CAC, EcoRI-AAC/MseI-CAA, EcoRI-ACC/MseI-CAC, EcoRI-ACC/MseI-CAT and EcoRI-AAC/MseI-CAC) were chosen based on the number of polymorphic markers and the level of reproducibility. Markers < 100 bp in length were removed from the data as these showed some evidence of size homoplasy using the method of Vekemans *et al.* (2002), implemented in the software AFLP-SURV 1.0 (distributed by the author. Laboratoire de Génétique et d'Ecologie Végétales. Université Libre de Bruxelles. Belgium). All ambiguous markers and singletons were excluded from the data set prior to analyses. See Appendix S2 for a full description of genetic diversity and structure analyses.

Autogamy test

We carried out autogamy experiments in two species of *Galvezia* to test whether self-fertilization occurs in the genus. The experiment was performed on the individuals cultivated in the glasshouse of the *Real Jardín Botánico* in Madrid:

seven individuals of *G. grandiflora* (four from Tumbes (PETum) and three from Negritos (PENeg), Fig. 2) and one individual of *G. fruticosa* (from Lima, PELim). Between 2 and 309 flowers per individual (mean = 84.33) were examined. As the glasshouse was closed, we did not bag flower buds, but marked 1000 flowers of *G. grandiflora* and 12 of *G. fruticosa*. At the end of the experiment, we checked whether fruit developed from marked flowers. Variation in fruit set between (1) two populations of *G. grandiflora* (PETum and PENeg) and (2) two species of *Galvezia* (*G. fruticosa* and *G. grandiflora*) was analysed. A nonparametric k-sample median test was performed with IBM SPSS Statistics v. 21.

Floral visitors

One population of *G. grandiflora* [Isla de la Plata, continental Ecuador, February 2011; see also Guzmán *et al.* (2015a)] and two populations of *G. leucantha* (subspp. *leucantha*, Isabela Island, February 2011 and May 2014; and subspp. *porphyrantha*, Santiago Island, February 2011) displaying different numbers of individuals and flowers were chosen for floral visitor surveys. All surveys were performed during the flowering peak by direct observation of flowers at *Galvezia* locations (for 15-min intervals, followed by 5-min pauses). Diurnal and nocturnal surveys were performed on all populations for a total of 400 min (Table S4), reaching a maximum time of 240 min for Isabela and a minimum time of 75 min for Santiago. During the surveys, the identities of all insect visitors making contact with the reproductive organs of the flowers were recorded (Vargas *et al.*, 2010). One specimen per species was captured for identification and deposited at the insect collection at the Charles Darwin Research Station, Puerto Ayora (Santa Cruz Island).

Pollination network analysis

To characterize floral visitors to *G. leucantha* and to evaluate how the structure of pollination interactions of this species differs from that of other coexisting plant species, we assembled a community-level pollination network based on flower visitation censuses. The pollination network was constructed in the arid zone of Bahía James (Santiago) encompassing the coastal community of Santiago described in Traveset *et al.* (2013) and the nearby community of *G. leucantha* subsp. *porphyrantha* present on a rocky outcrop *c.* 1 km away. Flower visitors were quantified by performing timed censuses (125 h) on all flowering species on both sites during the peak of the flowering season (February) in 2010 and 2011. All animals contacting the reproductive organs of the flowers within the observation period were identified, and the interactions compiled on a large quantitative interaction matrix (Traveset *et al.*, 2013). The pollination network was visualized using the 'bipartite' package (Dormann *et al.*, 2009) for R (R Core Team, 2015), and three key descriptors of species interactions patterns were calculated, namely plant linkage level, that is number of flower visitor species per plant; plant specialization index d'_p measuring the selectiveness of each species as the deviation from a visitation pattern based exclusively on visitor abundance (Blüthgen *et al.*, 2006); and plant strength, quantifying the importance of each plant species to the pollinator community (Bascompte *et al.*, 2006).

RESULTS

Phylogenetic relationships and divergence times

A chronogram based on posterior means from MCMC tree of both ITS and *ndh*F sequences is shown in Fig. S1 (Antirrhineae) and Fig. 3 (*Galvezia-Pseudorontium*). Our well-supported results indicated (1) monophyly of *Galvezia* species and populations of each of the four species (1.00 PP), except for a sample of *G. fruticosa*; (2) the genus *Galvezia* diverged from *Pseudorontium* primarily during the Miocene-Pliocene (8.88–2.28 Ma, median 5.11 Ma, 95% HPD). Each analysis revealed one lineage of *G. fruticosa* plus *G. elisensii* populations as sister to the *G. leucantha* lineage (Fig. 3), but with low support. Monophyly is also inferred for *G. leucantha* populations with high support values (1.00 PP). Differentiation of Galápagos populations occurred in the Middle-Upper Pleistocene (0.66–0.09 Ma, median 0.30 Ma, 95% HPD).

Phylogeographic data analysis

The *ndhF-rpL32* sequences varied in seven nucleotide-substitution haplotypes within *Galvezia* (Table 1, Table S5). There were no haplotypes exclusive to the Galápagos (Fig. 2). Haplotype 5 was distributed in six Galápagos populations and one population from Peru (38.88% of all populations), whereas haplotype 6 was found in five populations from Peru, continental Ecuador and Galápagos (31.25% of all populations; Table 1) (Fig. 2). Four (1, 3, 4 and 7) of the seven haplotypes were each found exclusively in a single population of the continent. At the species level, the same haplotype was detected in all populations within each species, except for three haplotypes found exclusively in each of the three populations of *G. fruticosa* (Table 1; Fig. 2). A geographical pattern of haplotype distribution is observed because three haplotypes (5, 6 and 7) are exclusively found in the northern distribution (*G. grandiflora*), while one haplotype (1) is the only one found in the southern distribution (*G. elisensii*) of the continental *Galvezia*. Haplotype 5 was shared by all subspecies of *G. leucantha* (Table 1), while haplotype 6 was found exclusively in one individual of *G. leucantha* subsp. *porphyrantha*. At the population level, the same haplotype was detected in all individuals within each population, except for two haplotypes in two populations of *G. grandiflora* (ECMan, PETum) and two in the population of *G. leucantha* subsp. *porphyrantha* SALad (Table 1; Fig. 2).

The ITS sequences distinguished 11 nucleotide-substitution ribotypes in *Galvezia* (Table 1, Table S6). Ribotype 1 was

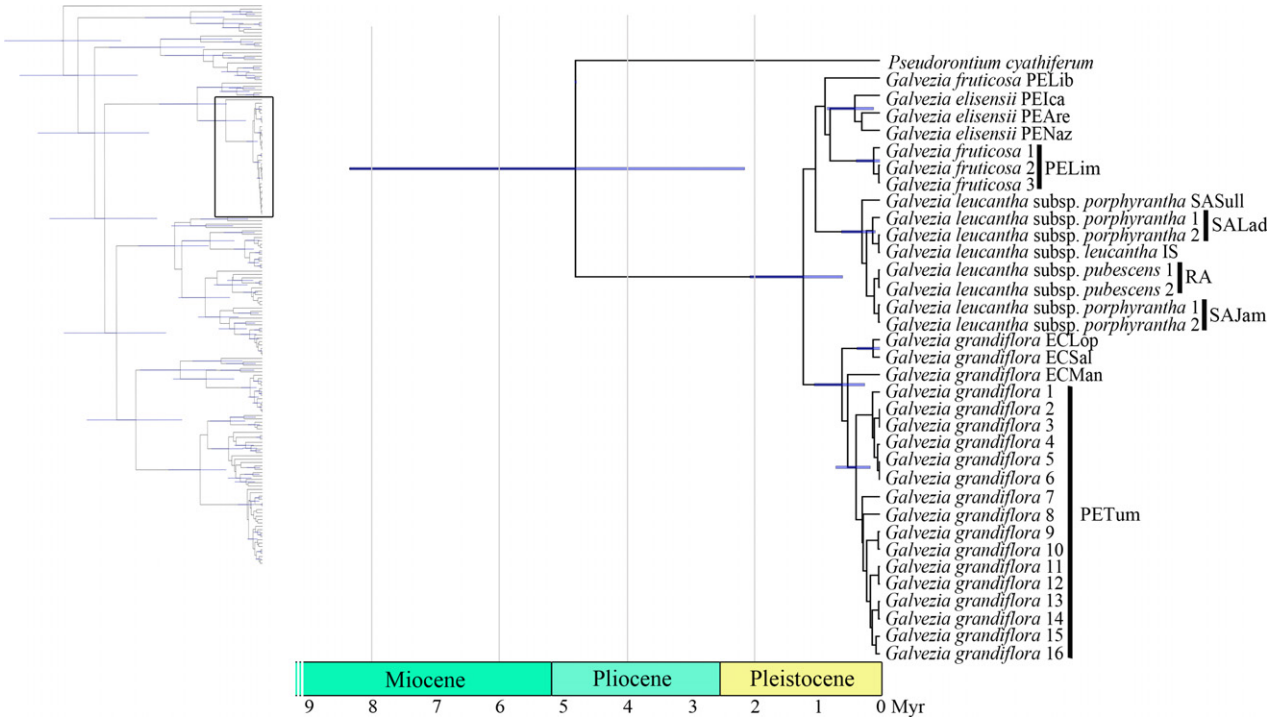

Figure 3 Detail of the Maximum Clade Credibility chronogram of the nuclear ribosomal ITS and the plastid *ndhF* gene sequences inferred using BEAST to establish *Galvezia* lineages. Time scale in millions of years before present (Ma). Error bars (blue) represent 95% posterior credibility intervals and are given only for nodes present on more than 95% of sampled trees. The whole MCC chronogram is shown in Fig. S1. [Colour figure can be viewed at wileyonlinelibrary.com]

widely distributed in seven populations from Peru and continental Ecuador (38.88% of all populations; Table 1), whereas ribotype 9 was distributed exclusively in the five Galápagos populations (22.22% of all populations) (Fig. 2). Eight of the 11 ribotypes (2, 3, 4, 6, 7, 8, 10 and 11) were each found exclusively in a single population. Ribotype 9 was shared over all subspecies of *G. leucantha* (Table 1), while ribotypes 10 and 11 were each found exclusively in one population of *G. leucantha* subsp. *porphyrantha.* At the population level, the same ribotype was detected in all the individuals within each population, except for two ribotypes in the population of *G. fruticosa* PELib, three in *G. grandiflora* (PETum) and two in *G. leucantha* subsp. *porphyrantha* SALad (Table 1; Fig. 2).

TCS constructed a single network of seven *Galvezia* haplotypes with no loops (Fig. 2a). Only two haplotypes (5 and 6) were found in the Galápagos, which were connected by a missing haplotype. Interestingly, these two haplotypes are also present in the continent. The network detected the interior haplotype 1 (continent) as the one with the highest number of mutational connections (three connections; Fig. 2a).

TCS constructed a single network of 11 *Galvezia* ribotypes with no loops. Continental ribotypes (1–8) were connected to Galápagos ribotypes by three absent (extinct or not found) ribotypes (Fig. 2b). Nevertheless, the continental ribotype 8 (only found in PETum) is the closest one to the Galápagos lineage. The network detected the interior ribotype 1 (continent) as the one with the highest number of mutational connections (six connections; Fig. 2b). Ribotype 9 is also interior in the Galápagos lineage and widespread across *G. leucantha* accessions.

AFLP genetic diversity in *G. leucantha*

The pilot study for the selection of primer combinations yielded a percentage of polymorphic markers between 0 and 50%. The reproducibility of the observed markers ranged between 66.60 and 100% (Table S7). Six AFLP selective primer combinations (EcoRI-ACC/MseI-CTT, EcoRI-AGA/MseI-CAC, EcoRI-AAC/MseI-CAA, EcoRI-ACC/MseI-CAC, EcoRI-ACC/MseI-CAT and EcoRI-AAC/MseI-CAC) resulted in 194 unambiguous fragments when extended to the successfully genotyped sample of 52 individuals (six populations). All these fragments were polymorphic. The reproducibility of the used AFLP fragments was above 94% for the six primer combinations (Table S7).

Table 2 summarizes the genetic diversity among six populations of *G. leucantha*. Individual populations had a mean polymorphic loci percentage of 20.17%, ranging from 6% in population from Bahía Sullivan to 33% in population from Bahía Ladilla. Per-population Nei's gene diversity (*Hj*) (= expected heterozygosity), under a model assuming no deviation from Hardy–Weinberg genotypic proportions, ranged

Table 2 Genetic diversity within six populations of *Galvezia leucantha* based on AFLP (194 markers).

	N	NPL	A*	Ap*	PLP 1%†	Hj	DW
Populations							
IS	6	19	1.03	0.05	3.87	0.08	117.80
SANep	3	14	1.13	0.01	0.82	0.08	122.06
SASul	3	6	1.01	0.01	0.82	0.04	107.43
SALad	13	33	1.04	0.02	6.29	0.10	104.00
SAJam	21	28	1.03	0.02	3.14	0.07	140.02
RA	6	21	1.05	0.05	1.13	0.08	137.17
Mean (SD)	–	20.17 (9.66)	1.05 (0.04)	0.03 (0.02)	2.68 (2.19)	0.07 (0.02)	121.41 (14.88)
Subspecies							
leucantha	6	19	1.05[a]	0.05[a]	3.87[a]	0.08[a]	117.80[a]
porphyrantha	40	40	1.03[a]	0.02[b]	0.73[a]	0.10[a]	120.55[a]
pubescens	6	21	1.05[a]	0.05[a]	1.13[a]	0.08[a]	137.51[a]

N, number of individuals; NPL, number of polymorphic loci; A, allelic richness; Ap, private allelic richness; PLP, percentage of polymorphic loci at 1% level; Hj, Nei's gene diversity (= expected heterozygosity); DW, rarity index (expressed as ratio of means).

*A and Ap were calculated using HP-Rare (Kalinowski, 2005) with rarefaction to three samples per population and one population per subspecies.

†PLP calculated using AFLPDIV (Coart *et al.*, 2005; Petit *et al.*, 1998) with rarefaction to three and six when analysing populations and subspecies.

[a]Medians not significantly different from each other (nonparametric k-sample median test for A, Ap, DW and PLP, $P < 0.05$).

[b]Medians significantly different from each other (nonparametric k-sample median test for A, Ap, DW and PLP, $P < 0.05$).

from 0.04% (Bahía Sullivan) to 0.10% (Bahía Ladilla), with an average of 0.07 ± 0.02%. The proportion of rare AFLP markers did not significantly differ between subspecies, and it was the highest in subspecies *pubescens* (DW = 137.51) (Table 2).

Population genetic structure and relationships within *G. leucantha*

There was low to moderate genetic differentiation among the studied *G. leucantha* populations ($F_{ST} = 0.2965$, $P < 0.01$; Table 3). Significant F_{ST} estimates suggested that the populations did not form a single panmictic unit and that there are significant genetic differences between some populations. This is despite the fact that the low-to-moderate value of the estimate proposes that the levels of differentiation are not very high.

The two-dimensional PCoA and the AFLP-based neighbour-joining tree revealed weak population differentiation and the absence of a clustering pattern either by subspecies (Fig. S2). The hierarchical AMOVA indicated that 'subspecies' was not a significant source of variability (Table 4) and that most of the variance was among individuals within populations (77.95%) and among populations within subspecies (18.28%). The largest the ΔK value the most probable the *K* value of 3 (Fig. S3). At $K = 3$, *Galvezia leucantha* subsp. *pubescens* is separated from subspp. *leucantha* and *porphyrantha* (Fig. 2c). The analysis also reveals admixture between subspp. *leucantha* and *porphyrantha* (Fig. 2c). *Galvezia leucantha* subsp. *porphyrantha* is a heterogeneous subspecies with two groups including one shared with subsp. *leucantha* (Fig. 2c).

Autogamy test

The three populations of *G. grandiflora* and *G. fruticosa* were found to be selfers (Table S8). A high variability in fruit set was found within individuals of *G. grandiflora* population PENeg (0–100%, Table S8), whereas intrapopulation variability was lower in PETum (59.22–36.76%, Table S8). The single individual of *G. fruticosa* showed *c.* 60% of selfing. No significant differences in fruit set were

Table 3 Genetic differentiation between populations based on 194 AFLP markers found in 52 individuals (six populations) of *Galvezia leucantha* of the Galapagos.

	H_T	H_W (SD)	H_b (SD)	F_{ST}	Lower 99% F_{ST}	Upper 99% F_{ST}
All populations	0.1092	0.0767 (0.0084)	0.0326 (0.0025)	0.2965	−0.0373	0.1179
Among subspecies	0.1235	0.0884 (0.0065)	0.0351 (0.0000)	0.2833	−0.0477	0.1129

H_T, total gene diversity; Hw, average gene diversity within populations; H_b, average gene diversity between populations; F_{ST}, Wright's fixation index, that is differentiation between populations; Lower 99% F_{ST} and Upper 99% F_{ST}, critical values at 99% in the randomization distribution of F_{ST}, assuming no genetic differentiation between populations, based on 10,000 random permutations. Standard deviations (SD) are shown in brackets.

Table 4 Hierarchical AMOVA based upon AFLP variation surveyed in a total of six populations (52 individuals) of three subspecies of *Galvezia leucantha* (subspp. *leucantha*, *porphyrantha* and *pubescens*).

Source of variation	d.f.	SS	Variance components	Total variance (%)	*P*-value
Among subspecies	2	81.078	40.539	3.772	0.182
Among populations within subspecies	3	111.958	37.319	18.282	0.004
Within populations	51	591.985	12.869	77.946	0.001

P-value estimates are based on 9999 permutations. d.f., degrees of freedom and SS, sum of squared deviations.

found among species (median = 53.38, $\chi_1^2 = 1.14$, $P = 0.28$) and among *G. grandiflora* populations (median = 53.38, $\chi_2^2 = 1.33$, $P = 0.51$).

Floral visitors

No visitors were observed during the 85-min survey of *G. grandiflora* in Isla de la Plata (but see Guzmán *et al.* 2015a for additional results). In contrast, *G. leucantha* showed a high number of visits by the Galápagos carpenter bee *X. darwinii* (187 visits in 315 min; 87.26% of visits) (Table S4). Only three other species, two ants (*Nylanderia* sp. and *Camponotus zonatus*) and the endemic lycaenid butterfly (*Leptotes parrhasioides*), were additionally observed visiting the population of Santiago.

Pollination network analysis

Overall, 125 h of visitation census were performed in the whole community of Bahía James (Traveset *et al.*, 2013), resulting in the observation of 2182 visits including 87 animal species and 32 plant species (Table S9, Fig. 4 and Fig. S4). Regarding diversity of pollination visits, *G. leucantha* showed a lower linkage level (*G. leucantha* = 4; community mean = 7.10, min = 1, max = 26), lower specialization (*G. leucantha* = 0.39; community mean = 0.53, min = 0.062, max = 0.97) and lower species strength (*G. leucantha* = 2.20; community mean = 3.20, min = 0.003, max = 15.30) than the average for all plant species in its community. Nevertheless, none of these differences were significant, due to the high dispersal of the data (Fig. S5).

DISCUSSION

Multidisciplinary studies, addressing several key issues (taxonomy, phylogenetics, population genetics, reproductive biology, pollination ecology, threat assessments), are essential to reconstruct evolutionary processes and to support more useful management programs (Silva *et al.*, 2015). The present study of *G. leucantha* is still a rare case in literature of a

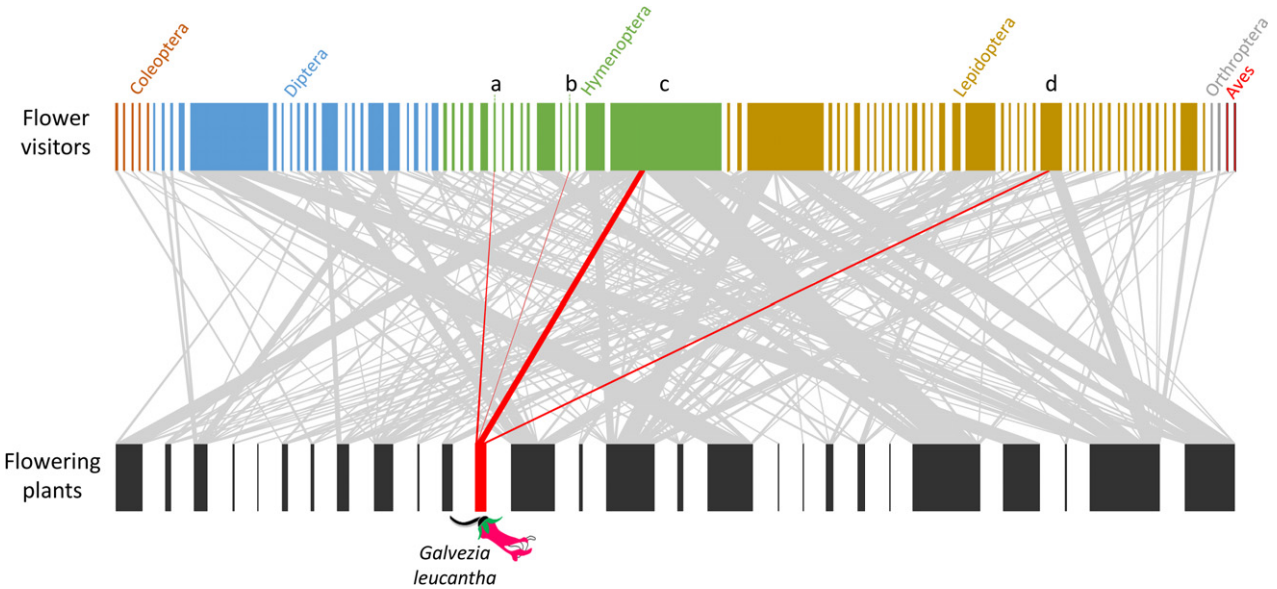

Figure 4 Pollination network of Bahía James (Santiago Island, Galápagos). This network quantifies the contacts between flower visitors (top level) with the reproductive organs of flowers (bottom level) in the community of Bahía James. A total of 7500 min of focal visitation census were performed in February 2010 and 2011, during which 2182 visits were recorded. The width of each box representing plants, animals and the interactions between the two is proportional to the number of visits observed. Colours represent different taxonomic groups of flower visitors. The four visitors to *Galvezia leucantha*, represented in red, are as follows: (a) *Nylanderia* sp., (b) Hymenoptera – small black wasp, (c) *Xylocopa darwini*, (d) *Leptotes parrhasioides* (Table S4). [Colour figure can be viewed at wileyonlinelibrary.com]

holistic conservation approach including evolutionary history of the species.

The origin of *Galvezia* may have occurred somewhere between the current distributions of *Pseudorontium* (SW North America) and *Galvezia* (NW South America) species in the Miocene (< 10 Ma). Rates of diversification in *Galvezia* are low (differentiation of four species during the Pleistocene) and distribution narrow (Fig. 2). Interestingly, one lineage found its way to colonize the remote Galápagos also during the Pleistocene.

Geographical speciation

Recent classification of *Galvezia* has included the Galápagos species (*G. leucantha*) together with four or three species, depending on circumscription of continental populations: *G. fruticosa*, *G. ballii*, *G. lanceolata* and *G.* sp. nov. (Elisens, 1992) or *G. elisensii*, *G. fruticosa*, *G. grandiflora* (Dillon & Quipuscoa, 2014). Our phylogenetic results revealed that: (1) *Galvezia* is a well-defined genus within the Antirrhineae; (2) *Galvezia* and *Pseudorontium* are sister groups; and (3) the recognition of four *Galvezia* species is supported by primarily monophyletic groups of populations (Dillon & Quipuscoa, 2014). The distribution of the continental species and haplotypes (and ribotypes at some extent) along the coast of Ecuador and Peru (Fig. 2) is congruent with a geographical pattern of latitudinal speciation during the Pleistocene. Specific geomorphic formations called *lomas* are common along the NW cost of South America, where a mild, uniform climate is characterized by regular formation of thick cloud banks below 1000 m during winter, as a result of the cold Humboldt sea current (Dillon *et al.*, 2011). Similar lowland climate conditions are found on the Galápagos, involving *lomas* occupied by the Galápagos *Galvezia*. Taxonomic delimitation of *G. leucantha* is also congruent with a pattern of geographical isolation and speciation after a single colonization event of the Galápagos archipelago (Elisens, 1992).

The colonization history of Galápagos by *Galvezia*

The few phylogenies published for Galápagos plants suggest that continental ancestors may have colonized from many different areas including the Caribbean, Ecuador, Peru and Chile (Tye & Francisco-Ortega, 2011; Trusty *et al.*, 2012). Phylogeographic and phylogenetic analyses revealed a single, independent dispersal event by *Galvezia* from the mainland to the Galápagos archipelago (Figs 2 and 3). This result is consistent with the hypothesis proposed by Elisens (1992), who interpreted a single introduction to the Galápagos during the Pleistocene based on isozyme and chorological data. Phylogeography and genotype distribution of *Galvezia* suggested that the Peruvian population of Tumbes (PETum) was directly connected to the Galápagos lineage (Fig. 2). Interestingly, Tumbes is the closest continental area to Galápagos (*c.* 950 km) and retains the highest diversity in terms of number of both haplotypes (two) and ribotypes (three) in a narrow area of *Galvezia* distribution. However, it is difficult to identify centres of dispersal for plants given the geographical and climatic changes resulting in potential waxing and waning processes during the Pleistocene (Cain, 1943). Moreover, our genotype networks of continental populations show a complex scenario of population relationships and geography (Fig. 2). Irrespective of whether Tumbes area is the cradle of the *Galvezia* ancestor that colonized Galápagos, it is indeed puzzling to understand how fruits or seeds with no traits related to long-distance dispersal (Fig. 1j) originally dispersed to Galápagos from any mainland area (but see Vargas *et al.*, 2012). Nevertheless, some other plant traits, such as habitat similarity, the strong selfing pattern displayed in this genus (see high values for Tumbes population in Table S8), and the high germination rates of seeds obtained by selfing (B. Guzmán & P. Vargas pers. obs.; Elisens, 1985) may have determined the successful colonization of Galápagos by *Galvezia*. Indeed, not only most of the Galápagos flora shows some level of autogamy and autonomous selfing (Chamorro *et al.*, 2012) but also does other insular floras (New Zealand: Webb & Kelly, 1993 and Juan Fernandez: Anderson *et al.*, 2001; Bernardello *et al.*, 2001).

The divergence time analysis of *Galvezia* lineages estimated a relatively recent colonization of the archipelago (< 600 kyr), followed by dispersal across four western islands. Some isolation of *G. leucantha* on these four western islands is reflected by some genetic differentiation of the populations and subtle flower traits currently used in subspecific taxonomy: *leucantha* on Isabela and Fernandina, *pubescens* on Rábida and *porphyrantha* on Santiago. However, our genetic analyses failed to find three well-defined lineages congruent with the three subspecies (Fig. 3; Fig. S1). Recent reconstruction of the archipelago's geodynamics based on sea level changes (eustasy) revealed that the four western-most islands have been recurrently connected by land bridges since 700 kyr (Ali & Aitchison, 2014). Both, land bridges and high interisland gene flow mediated by pollinators (*X. darwinii*; see Vargas *et al.*, 2015), have likely been responsible for the poor geographical structure of the genetic variation observed in *G. leucantha*.

Genetic variation and population demise in *G. leucantha*

Total genetic diversity of *G. leucantha* is relatively high (H_T = 0.11) (Table 2). One more endangered species (*Calandrina galapagosa*) in the Galápagos displays high genetic diversity (Jaramillo & Atkinson, 2011). This pattern of high levels of genetic diversity in endemic and narrowly distributed plants is not exclusive of the Galápagos. Endemisms of the Canary Islands, for instance, also showed relatively high levels of average species-level total genetic diversity (H_T = 0.19; Francisco-Ortega *et al.*, 2000). Although genetic diversity of narrow endemics is higher for some insular species than expected from historical predictions, the causes of variation of genetic diversity on islands appear to be species specific (Fernández-Mazuecos *et al.*, 2014). Care should be

taken when comparing data between unrelated species as genetic diversity depends on numerous factors, namely life history, breeding system, growth life forms, geographical range and type of molecular method used (Powell *et al.*, 1996; Nybom, 2004). The fact that genetic differentiation is relatively moderate in *G. leucantha* (F_{ST} = 0.2965, $P < 0.01$; Table 3), despite being narrowly distributed, is compatible with the distribution of extensive populations after speciation, followed by isolation and decline (Simbaña, 2007). In Galápagos, the most important factor causing population and species demise is human-mediated impacts, particularly goat herbivory and introduction of invasive plants (Cruz *et al.*, 2009; Carrión *et al.*, 2011). In the last decades, active conservation programmes undertaken by the Galápagos National Park Service succeeded in eradicating goats from several islands, including Santiago and Rábida (Carrión *et al.*, 2011). Goats are still one of the main threats to endemic flora in larger islands, such as Isabela, where only two populations with extremely few individuals of the subsp. *leucantha* are known (Fig. 2) (Tye & Jäger, 2000; Atkinson *et al.*, 2008). One more endangered species (*Calandrina galapagosa*) displaying high genetic diversity is fastly recovering after the implementation of goat-proof fences in San Cristóbal (Jaramillo & Atkinson, 2011).

Loss of ornithophilous traits

The scenario of recent colonization of the Galápagos archipelago from a northern Peruvian-southern Ecuadorian lineage of *Galvezia* was apparently accompanied by rapid differentiation in floral morphology (Fig. 1) and pollination biology. While there are some observations of hummingbirds visiting mainland *Galvezia* species with long, tubular, red flowers (Fig. 1a–d) (Guzmán *et al.*, 2015a), the Galápagos shrub snapdragon shows paler, shorter flowers (Fig. 1e–g) that are primarily pollinated by the endemic carpenter bee (*X. darwini*; Table S4; Elisens, 1989, 1992). A shift in pollinator system from bird to bee-pollination is scientifically perceived as a rare pattern (Wilson *et al.*, 2007; Rauscher, 2008); however, the Galápagos endemic *G. leucantha* offers an interesting case of secondary entomophily (melittophily). Given that a split between continental and Galápagos lineages of *Galvezia* occurred recently (Fig. 3), our phylogenetic results do not help reconstruct the ancestral condition of this evolutionary change. Nevertheless, the reconstruction of ancestral flower phenotypes of the Antirrhineae suggests that the most common recent ancestor of *Galvezia-Pseudorontium* had partially occluded corollas, which are related to bird pollination (Guzmán *et al.*, 2015a).

Our pollinator survey additionally shows that *G. leucantha* is an entomophilous species that has insects of three groups (ants, bees, butterflies) as flower visitors. Particularly, the carpenter bee (*X. darwinii*) is primarily mediating pollen transfer between flowers of this plant species, and it is actually the most generalist species (pollinator hub) of the entire Galápagos flora (Traveset *et al.*, 2013). Our surveys agree with preliminary observations and expectations for a strong bee–flower interaction (Elisens, 1989), although additional visitors have been observed (Table S4). Low insect specialization is interpreted when comparing results of *G. leucantha* to those from the other co-occurring plants of Bahía James (Santiago Island).

Despite the greater effort being dedicated to observing flower visitors on the Galápagos shrub snapdragon (630 min) compared to the other plant species in the community (240 min), *G. leucantha* flowers were never visited by birds, unlike those of two plant species (*Acacia rurodiana* and *Lantana peduncularis*) at Bahía James visited by two Galápagos finches (*Geospiza fuliginosa* and *Camarrhynchus parvulus*, Fig. 4 and Table S4). The role of birds as pollinators in the Galápagos has been historically underestimated given the absence of specialized nectarivorous birds. Indeed, estimation of pollen harvesting through inspection of pollen load on birds' beaks revealed at least 19 bird species visiting plants in Galápagos lowland communities (Traveset *et al.*, 2015a,b). Although this interesting phenomenon (coined 'interaction release') appears to be generalized in the Galápagos (including Bahía James), no pollination attributable to birds seems to have occurred in the insular *Galvezia* lineage (see Traveset *et al.*, 2015a,b). Indeed, birds were spotted perching on *G. leucantha* branches but never observed visiting its flowers (P. Vargas pers. obs.). Further evidence that *G. leucantha* is not visited by birds comes from the high number of pollen grains (over 1000 per sampled bird) identified from 17 plant species in Traveset *et al.* (2015a,b), of which none belonged to *Galvezia*.

CONCLUDING REMARKS

Conservation of endangered species has become a global concern in nature management, which requires the collation of information from different disciplines. In this study, we appraised results from taxonomy, genetics, phylogenetics, phylogeography and pollination ecology to reconstruct the evolutionary history of the Galápagos shrub snapdragon from a single colonization in the last 600.000 years. Causes for high morphological and genetic diversity include short island isolation in the past and long-range pollination primarily by a large and common bee (*X. darwinii*). Most recent population demise caused by goat hervibory over the last two centuries have contributed to its current conservation status. In addition, a rare case of evolutionary shift from bird- to bee-mediated pollination in *Galvezia* and Galápagos is hypothesized. Increasing field efforts to find new populations and to census and sample individuals from known populations, combined with *ex situ* conservation programs, will help prevent the extinction of the Galápagos shrub snapdragon.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Tables S1–S9, Figures S1–S5.

Table S1. Voucher reference and GenBank accession number of 296 samples (196 taxa) of the tribe Antirrhineae and *Lafuentea rotundifolia* as outgroup.

Table S2. Summary of the results from a pilot study performed to select DNA regions with the highest variation among six *Galvezia* populations.

Table S3. Sequence of AFLP primers used in this study.

Table S4. Visitors of *Galvezia grandiflora* and *Galvezia leucantha* subspp *leucantha* and *porphyrantha*.

Table S5. Summary of seven haplotypes based on the plastid spacer *ndhF-rpL32* variation of *Galvezia* spp.

Table S6. Summary of 11 ribotypes based on the nuclear ribosomal ITS variation of *Galvezia* spp.

Table S7. Summary of the results from a pilot study performed to select the best AFLP primer combination in *Galvezia leucantha* and the range of markers for the six selected primer.

Table S8. Summary of the results of the autogamy experiment in *Galvezia fruticosa* and *Galvezia grandiflora* from Peru.

Table S9. Floral visitors observed at the community of Bahía James (Santiago I., Galápagos), in February 2010 and February 2011.

Fig. S1. Maximum Clade Credibility chronogram of the nuclear ribosomal ITS and the plastid *ndhF* gene sequences inferred using BEAST.

Fig. S2. Phylogenetic relationships among 52 individuals (six populations, three subspecies) of *Galvezia leucantha* from AFLP data using a neighbor-joining analysis of Nei-Li distances and midpoint root.

Fig. S3. Delta K values for STRUCTURE analysis of *Galvezia leucantha* accessions.

Fig. S4. Pollination network of Bahía James (Santiago I., Galápagos).

Fig. S5. Comparison between the linkage level, specialization and strength of *Galvezia leucantha* against the mean values observed for the other plants at the community.

Appendix S2. Supplementary Methods and Results.

Fig. S6. Phylogeny of Antirrhineae based on the nuclear ribosomal ITS and the plastid *ndhF* sequences, using Bayesian Inference (BI).

BIOSKETCH

A multidisciplinary team of researchers from Spain, Portugal and Ecuador has been studying the threat of invasive species, conservation of endangered plants and plant–animal interactions in the Galápagos Islands since 2006.

Author contributions: P.V. conceived the study; B.G., R.H., M.N., W.S., A.T. and P.V. collected material and data; B.G., R.H. and P.V. analysed the data; B.G. and P.V. wrote the manuscript; and B.G., R.H., M.N., W.S., A.T. and P.V. read and corrected different versions.

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