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ORIGINAL PAPER

Species delimitation and conservation genetics of the Canarian endemic *Bethencourtia* (Asteraceae)

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Abstract

Bethencourtia Choisy ex Link is an endemic genus of the Canary Islands and comprises three species. *Bethencourtia hermosae* and *Bethencourtia rupicola* are restricted to La Gomera, while *Bethencourtia palmensis* is present in Tenerife and La Palma. Despite the morphological differences previously found between the species, there are still taxonomic incongruities in the group, with evident consequences for its monitoring and conservation. The objectives of this study were to define the species differentiation, perform population genetic analysis and propose conservation strategies for *Bethencourtia*. To achieve these objectives, we characterized 10 polymorphic SSR markers. Eleven natural populations (276 individuals) were analyzed (three for *B. hermosae*, five for *B. rupicola* and three for *B. palmensis*). The results obtained by AMOVA, PCoA and Bayesian analysis on STRUCTURE confirmed the evidence of well-structured groups corresponding to the three species. At the intra-specific level, *B. hermosae* and *B. rupicola* did not show a clear population structure, while *B. palmensis* was aggregated according to island of origin. This is consistent with self-incompatibility in the group and high gene flow within species. Overall, the genetic diversity of the three species was low, with expected heterozygosity values of 0.302 (*B. hermosae*), 0.382 (*B. rupicola*) and 0.454 (*B. palmensis*). Recent bottleneck events and a low number of individuals per population are probably the causes of the low genetic diversity. We consider that they are naturally rare species associated with specific habitats. The results given in this article will provide useful information to assist in conservation genetics programs for this endemic genus.

Keywords Canary Islands · Conservation genetics · Endemism · Genetic diversity · Microsatellites

Introduction

Insular endemics account for 25% of the described vascular plant species (Kreft et al. 2008). It has been estimated that 5–10% of these endemics may be highly threatened and that 3–4% could be in critical danger of extinction (Caujapé-Castells et al. 2010). The unique characteristics of island organisms, due to isolation and small population sizes, make them vulnerable to anthropogenic change (Whittaker and Fernández-Palacios 2007). The Canarian archipelago, in the Macaronesian biogeographic region, is composed of seven main islands located next to the north-western part of Africa. The Canary Islands are part of the Mediterranean biodiversity hotspot (Médail and Quézel 1997), containing around 1300 species of vascular plants, 44.3% of which are endemic, and 22 endemic plant genera (Whittaker and Fernández-Palacios 2007; Reyes-Betancort et al. 2008). Indeed, with only 1.5% of the national territory, the Canary Islands hold more than 50% of the Spanish endemic vascular plants. Many of these endemics are restricted to a single island or specific habitats. Moreover, 26% of the Canarian flora is threatened, with a high density of threatened species per area (Moreno-Saiz et al. 2015).

Conservation Genetics of endangered species is becoming a major component of Conservation Biology. Currently,

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conservation decisions often rely on the determination of species' boundaries, an area where advances in genetic studies are playing a crucial role (DeSalle and Amato 2004; González-Pérez et al. 2009b; Crawford and Stuessy 2016). Also, understanding the genetic variation within species helps to detect signs of inbreeding, genetic diversity loss and risks of extinction. This knowledge is of great importance for restoration and conservation programs of endangered populations (Frankham et al. 2002; Sosa et al. 2011).

The Canarian archipelago has a significant representation of the tribes of Asteraceae compared to other oceanic islands (Crawford et al. 2009), including the Senecioneae tribe. Within the Senecioneae tribe, *Pericallis* contains many single-island endemics in the Macaronesia, and *Bethencourtia* is the only endemic genus of the Canary Islands (Nordenstam et al. 2009). *Bethencourtia* Choisy ex Link (Asteraceae) includes three species: *Bethencourtia hermosae* (Pit.) G. Kunkel, *Bethencourtia rupicola* (B. Nord.) B. Nord. and *Bethencourtia palmensis* Choisy. *B. hermosae* and *B. rupicola* are island-exclusive to La Gomera. *B. hermosae* can be found in Vallehermoso with three main localities and *B. rupicola* is located in the "Monumento Natural de Los Roques" within the Garajonay National Park, on the steep slopes of the phonolitic outcrops of "Agando", "Ojila", "Carmona" and "Las Lajas" (Fig. 1). *B. palmensis* is present in Tenerife in the "Barranco de El Río" and is more widespread in La Palma, including at the summit of the "Caldera de Taburiente" National Park.

Bethencourtia palmensis and *B. hermosae* were considered to be under the genus *Senecio*, where *Senecio palmensis* (C. Sm. in Buch) Link and *S. hermosae* Pit, constituted the sect. *Bethencourtii* (Jeffrey 1992). Nevertheless, enough morphological differences were found to propose their inclusion in a separate genus by some authors, based on the caudate anthers and the obtuse style-branches that characterize the group (Kunkel 1975; Nordenstam 2006a). The description of this separate genus, *Canariothamnus* B. Nord., was published by Nordenstam (2006a), although he changed the nomenclature later on due to the legitimacy of the synonym *Bethencourtia* Choisy (Nordenstam 2006b). This separation of *Bethencourtia* was confirmed with a phylogeny of the Senecioneae tribe, and the genus appeared closely related to *Jacobaea* sp. from the Iberian Peninsula (Pelser et al. 2007).

Moreover, Nordenstam (2006a) found enough morphological differences between the populations in the 'Monumento Natural de Los Roques' and 'Vallehermoso' in the island of La Gomera to describe the new species *B. rupicola* as a different species from *B. hermosae*. He also emphasized

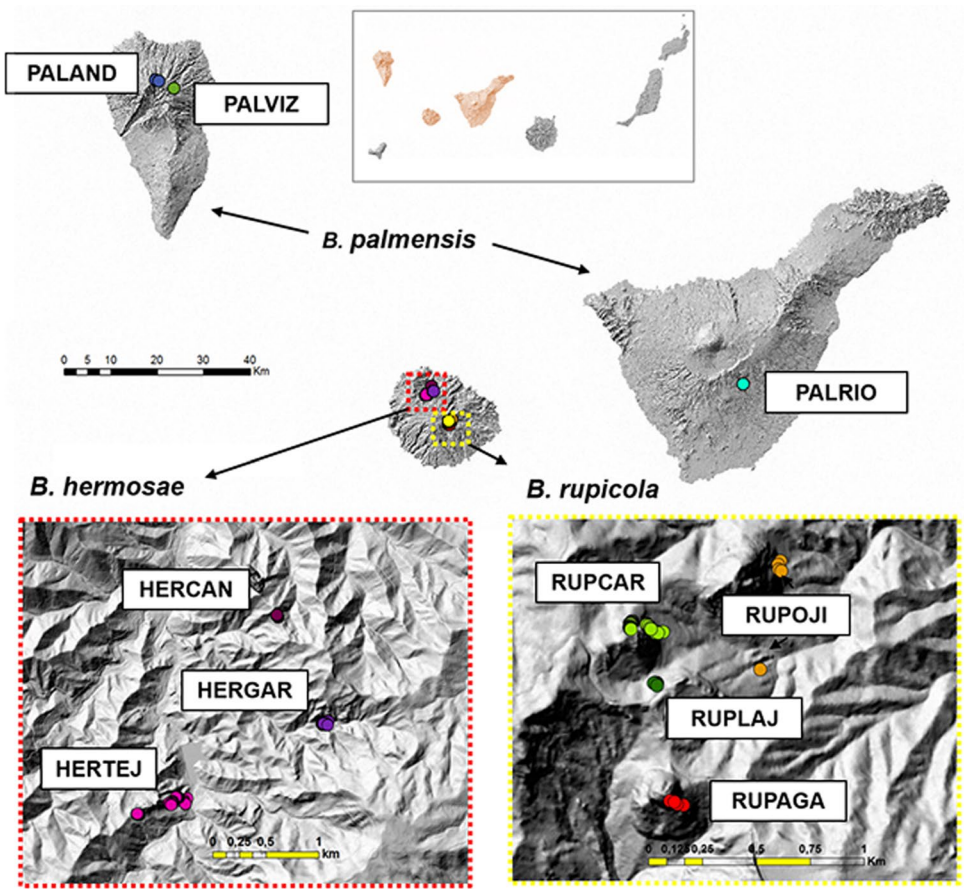

Fig. 1 Map of the distribution of *Bethencourtia* localities sampled for this study. The maps at the bottom correspond to *B. hermosae* (left) and *B. rupicola* (right). Population codes are detailed in Table 1

the low number of individuals per locality of *B. rupicola* that he found. The morphological differences between these three species are clear, the especially obvious dissimilarity being the length of the leaf lobes. Leaf lobes are 1–5 mm long in *B.* palmensis, 5–10 mm in *B. rupicola* and 10–25 mm in *B. hermosae* (Nordenstam 2006a). In addition, the breeding system of these species is not completely understood (Crawford et al. 2009). The dispersal syndrome is anemochorous and the pollinators are still unknown (Fernández-López and Velázquez-Barrera, unpublished report).

Bethencourtia palmensis and *B. hermosae* are considered within the genus *Senecio* in the official lists of species (Arechavaleta et al. 2010; BOC 2010), but *B. rupicola* has not been included as a taxonomic unit separate from *B. hermosae* (=*S. hermosae*). Thus, the reports on the demography and distribution of *B. hermosae* and *B. rupicola* consider all localities as a single species, which might result in an underestimated evaluation of the threat status for each of them separately. As management policies state that a species is the minimum unit for legal protection, this may become an important issue from a conservation standpoint (IUCN 2012). In the IUCN Red List, *B. hermosae* and *B. rupicola* (=*Canariothamnus hermosae*) are listed as "Vulnerable" due to their extremely narrow distribution, being affected by competition with exotic species and grazing (Martín Osorio et al. 2011). *Bethencourtia hermosae* is also cited as "Vulnerable" in the Spanish Red List of Vascular Flora (Moreno-Saiz 2010), and it is catalogued within the category "de interés para los ecosistemas Canarios" ("of interest for the Canarian ecosystem") in the regional list (BOC 2010). *Bethencourtia rupicola*, despite the low number of individuals, and possibly due to its recent description, is not considered in any list as a single taxon, but its populations are usually included together with *B. hermosae*. Unfortunately, after our sampling season in 2012, a fire occurred in the Garajonay National Park, which affected the populations of *B. rupicola* in the "Monumento Natural de los Roques". Nevertheless, a significant recovery of the individuals has been detected (Fernández-López et al. 2014). *Bethencourtia palmensis* is not currently threatened, as it presents a wider distribution with higher population sizes (Arechavaleta et al. 2010). Moreover, there are no detailed studies, either molecular or morphological, of the species delimitation in the genus *Bethencourtia* with a thorough sampling of its distribution.

The aims of this research were to (1) assess with molecular evidence the differentiation between *B. hermosae, B. rupicola* and *B. palmensis*; (2) estimate the intra- and inter-population genetic diversity and structure; (3) better understand traits of their reproductive biology such as the selfing rate and (4), propose conservation actions. To accomplish these objectives, we have developed 10 microsatellite markers for the three species, which are described in this article for the first time. These results will be relevant for taxonomical issues, as well as for the management and conservation of *Bethencourtia*.

Materials and methods

Sample collection

Leaf samples were collected in 2012 and 2013 from La Gomera in all the previously known locations for *B. hermosae* and *B. rupicola*, and three localities of *B. palmensis* in La Palma and Tenerife. The collections were made with the legal permissions granted by either the "Cabildo" administration of each island and the Garajonay National Park. Number of individuals per population, geographic coordinates and population codes are listed in Table 1. For the collection of the individuals in La Gomera the assistance of a professional climber was necessary, especially for the *B. rupicola* populations, due to the height and gradient of the phonolitic outcrops and cracks which they inhabit. All specimens were georeferenced in ArcGIS (ESRI) (Fig. 1). Leaf samples were stored in plastic bags with silica gel, and herbarium voucher specimens were brought to the TFC Herbarium at the University of La Laguna. Herbarium specimens were only collected from those populations with individuals in bloom during the sampling period.

Microsatellite development

We describe the characterization of 10 SSR markers in the endemic genus of the Canary Islands *Bethencourtia* Choisy ex Link, indicating their effectiveness in identifying patterns of genetic diversity.

Genomic DNA for the development of markers was extracted from leaf tissue using the Dellaporta et al. (1983) protocol. For subsequent analysis, the whole set of samples was extracted with Invisorb DNA Plant HTS 96 KIT INVISORB.

Microsatellite loci were selected from an Illumina paired-end shotgun library developed by Savannah River Ecology Laboratory (University of Georgia) using 3 samples of *B. hermosae*. We initially chose 38 primer pairs of this library of which 19 yielded some product and were labelled. Fifteen samples per species were used for cross-amplification testing. Finally, 10 primer pairs amplified consistently in the three species and were used for further analysis (Online Resource 1). For the initial testing, PCR was conducted individually with each primer pair in a 25 uL total reaction volume, which contained approximately: 20 ng of DNA, 10 pmol of each primer, as well as PCR Master Mix until 25 uL were completed (Reddy-Mix, ABgene, Surrey, UK). Reverse primers were color-labelled at the 5′-end with

Table 1 *Bethencourtia hermosae, B. rupicola* and *B. palmensis* localities sampled and included in this study

Species	Island	Location	Population code	Voucher	UTM	N
B. hermosae	La Gomera	Lomo Las Tejas	HERTEJ	-	28R 0277767; 3117351	41
		Presa del Garabato	HERGAR	TFC-50.736	28R 0279161; 3118030	49
		Roque Chico-Roque Cano	HERCAN	-	28R 0278698; 3119068	31
Subtotal						121
B. rupicola	La Gomera	Roque Carmona	RUPCAR	TFC-50.735	28R 0282494; 3111602	59
		Roque Las Lajas	RUPLAJ	-	28R 0282494; 3111363	11
		Roque Ojila	RUPOJI	-	28R 0283066; 3111932	5
		Roque Agando	RUPAGA	TFC-50.731	28R 0282615; 3110799	4
Subtotal						79
B. palmensis	La Palma	Los Andenes	PALAND	TFC-50.713	28R 0220035; 3184906	20
		Fuente Vizcaína	PALVIZ	TFC-50.712	28R 0223213; 3183373	22
	Tenerife	Barranco de El Río	PALRIO	TFC-50.714	28R 0345846; 3119572	34
Subtotal						76
Total						276

Voucher = Reference from TFC Herbarium in La Laguna University, *N* sample size

6-FAM, PET, NED or VIC. Amplifications were performed using the following conditions: 3 min denaturation at 95 °C, 35 cycles of 30 s denaturation at 95 °C, 30 s annealing at 60 °C, and 72 °C for 1.5 min; followed by 5 min elongation at 72 °C.

Once primers pairs known to generate products were labelled, we conducted the subsequent multiplex amplifications using the QIAGEN Multiplex Kit (QIAGEN). PCR were performed in 15 µL reaction volumes: 7.5 µL of Multiplex PCR Master Mix, 1.5 µL primer mix (containing 0.2 µM of each primer in TE), 1.5 µl of Q-solution, 20–40 ng of DNA and dH_20. Multiplexing was carried out in two primer groups as indicated in Online Resource 1. Following the instructions of the manufacturer, PCR consisted of a Touchdown protocol with the thermal conditions: 15 min at 95 °C, 10 cycles of 30 s at 94 °C, annealing for 90 s at 65 °C with a decrease of 0.5 °C per cycle and 60 s at 72 °C, following by 20 cycles of 30 s at 94 °C, annealing for 90 s at 55 °C and 60 s at 72 °C, with a final extension of 30 min at 60 °C.

All the products from both simple PCR and multiplex PCR were detected on an ABI 3730 Genetic Analyzer and fragments were sized against the LIZ (500-250) size standard (Applied Biosystems, Inc.) and visualized using Genemapper 4.0 (Applied Biosystems, Inc.). We identified allele peak profiles at each locus and assigned a genotype to each individual.

Statistical analysis

Linkage disequilibrium and deviation from the Hardy–Weinberg equilibrium (HWE) were calculated using GENEPOP version 4.2 (Rousset 2008). For all tests, a sequential Bonferroni correction for multiple comparisons was applied (Rice 1989). Estimation of null alleles for each population was carried out with MICROCHECKER 2.2.3 (Van Oosterhout et al. 2006). Additionaly, the neutrality of all microsatellites used in this study was tested with BAYESCAN 2.1 (Foll and Gaggiotti 2008) considering the datasets per species and setting prior odds at 100.

Basic genetic diversity indices such as number of alleles (*NA*), allelic richness (*A*); observed (H_o), and unbiased expected (H_e) heterozygosities for each locus were estimated with GENALEX version 6.5 (Peakall and Smouse 2012). Measures of allelic and private allelic richness with a hierarchical method between and within species were calculated using HP-RARE 1.0 (Kalinowski 2005), which uses rarefaction to correct for sampling error.

Estimates of selfing (David et al. 2007) were calculated for each species and implemented in SPAGeDi 1.5 (Hardy and Vekemans 2002). BOTTLENECK 1.2.02 software was used to identify any recent genetic drift events in the natural populations. (Cornuet and Luikart 1996). The two-phase mutation model (TPM), which is a modification of the stepwise mutation model (SMM), was implemented as is shown to be a better fit for most microsatellite data sets (Piry et al. 1999). In the TPM model, to optimize the most sensitive values for microsatellites, a proportion of SMM in the TPM = 0.000 and a variance of the geometric distribution for TPM = 0.36 were chosen.

Allele frequency information was analyzed using a nested analysis of molecular variance (AMOVA) (Excoffier et al. 1992) with ARLEQUIN software. The analyses were conducted with two different data sets: (1) all populations grouped by species (3 species, 9 populations); and (2) the data sets for each species individually. Matrices of Pairwise

F_{ST} values (Weir and Cockerham 1984) were also obtained from ARLEQUIN. Significance values were estimated over 100 permutations.

In addition, a principal coordinate analysis (PCoA), using the covariance standardized method of pairwise codominant genotypic distances among individuals, was implemented with GENALEX version 6.5 (Peakall and Smouse 2012). At the population level, a genetic distance matrix (Nei et al. 1983) between localities, and the resulting UPGMA tree, were estimated using POPULATIONS software (Langella 2002), with 100 bootstraps on each locus. The tree was visualized and edited on FigTree (Rambaut 2009).

To check if the taxonomic status of this endemic genus is in concordance with the genetic structure, all the genotypes were screened using a Bayesian admixture procedure with the software STRUCTURE (Pritchard et al. 2000). The model was assumed to be of population admixture and correlated allele frequencies. 10 independent runs were conducted for each value of K (from 1 to 15) and analysis consisted of a 10^5 burn-in period replicates and a run length of 10^6 replicates. The optimal number of clusters was found by the ΔK method (Evanno et al. 2005) visualized with STRUCTURE HARVESTER (Earl and vonHoldt 2012). Results of 10 replicates of the best fit K were processed using CLUMPP 1.1.2 (Jakobsson and Rosenberg 2007) to determine the optimal clustering. The STRUCTURE HARVESTER results for the election of the optimal K are presented in the Online Resource 1 for all analysis. These analyses were carried out at the interspecific level, including the whole set of samples, and at the intraspecific level to test the genetic structure within species with 1–10 values of K.

Results

The ten tested primer pairs amplified consistently and were polymorphic for the whole set of samples. Specifically, all the loci showed polymorphism in *B. palmensis*, but BetPen-6 was monomorphic for both *B. hermosae* and *B. rupicola* with different allele sizes in each species. BetPen-5 was monomorphic only for *B. rupicola*. RUPCAR in *B. rupicola* was the only population that deviated from HW after a Bonferroni correction and there was no significant linkage disequilibrium for all the pairwise tests analyzed. We detected the presence of null alleles in *B. rupicola* in the populations RUPCAR (Hex3 = 0.11; Hex4 = 0.30, Tet3 = 0.13; Tet5 = 0.23) and *B. palmensis* in the populations PALAND (Pen2 = 0.21, Tet3 = 0.31), PALVIZ (Tet3 = 0.31) and PALRIO (Pen2 = 0.20) with the Oosterhout algorithm implemented in MICROCHECKER. The neutrality test carried out with BAYESCAN showed evidence of selection only for the locus BetPen-6.

The highest values for all the genetic diversity parameters calculated were found in *B. palmensis*, with the highest value of H_e for PALAND (0.420). On the other hand, *B. hermosae* presented the lowest genetic diversity in HERGAR (H_e = 0.212). The average of rarefied allelic richness over all loci ranged from 1.57 in *B. hermosae* (HERCAN, HERGAR) to 2.34 in *B. palmensis* (PALAND). The highest presence of rarefied private allelic richness was found in *B. palmensis* equaling 0.240 (PALRIO) and *B. hermosae* with the value of 0.180 (HERTEJ) (Table 2).

The estimates of the selfing rate (from 0 to 1) based on the distribution of multilocus heterozygosity, showed low and similar values for *B. hermosae* (0.093 ± 0.248), *B. rupicola* (0.000 ± 0.109) and *B. palmensis* (0.000 ± 0.027). These results, in accordance with the Hardy Weinberg equilibrium for all the populations within species, corroborate the lack of selfing reproduction in this group.

The results from BOTTLENECK (Table 2) showed significant values for heterozygosity excess only in two *B. hermosae* populations (HERCAN and HERTEJ) under TPM ($P < 0.05$), presenting evidence of recent bottleneck events.

The analysis of molecular variance (AMOVA) at the species level indicated that 44.2% of the variation was found between species, while 10% was explained by the variation between populations within species and 45.8% within populations. The AMOVA analysis at the intraspecific level showed a similar pattern for the three species, with most of variation found within populations (86.3%, 82.3% and 77.3%) for *B. hermosae, B. rupicola* and *B. palmensis* respectively (Table 3).

Pairwise F_{ST} values (Online Resource 3) ranged from 0.056 (between PALAND and PALVIZ) to 0.669 (between HERGAR and PALRIO). The average values between species ranged from 0.484 (between *B. hermosae* and *B. rupicola*) to 0.601 (between *B. palmensis* and *B. hermosae*), with the intermediate value of 0.513 (between *B. palmensis* and *B. rupicola*). It is interesting to compare these high F_{ST} values found between species with the averages among populations within species, these being 0.136 (*B. hermosae*), 0.174 (*B. rupicola*) and 0.199 (*B. palmensis*). The highest F_{ST} values among populations of the same species were detected in *B. palmensis*, between the two populations of La Palma and the one in Tenerife, these being of 0.276 (PALAND – PALRIO) and 0.266 (PALVIZ – PALRIO).

Consistent with the AMOVA results, the first two axes of the PCoA accounted for a high proportion of the total variance (53.26%) with 34.80% explained by the first axis and 18.45% by the second (see Fig. 2). This revealed three clearly differentiated groups of individuals of each species.

In addition, the UPGMA tree confirmed the differentiation between the three species, supported by high values of bootstraps, in agreement with the topology found by Pelser et al. (2007) (Fig. 3).

Table 2 Basic genetic diversity indices for *B. hermosae. B. palmensis* and *B. rupicola* populations

Species	Population	*N*	*NA*	*Ar*	*PA*	*PAr*	H_o	H_e	F_{IS}	Bottleneck test		
										L	H_d/H_e	*P*
B. hermosae	HERCAN	30.8	16	1.57	0	0.01	0.237	0.252	0.059^{ns}	5	0/5	0.016^{**}
	HERGAR	48.9	19	1.57	0	0.01	0.208	0.212	0.017^{ns}	7	3/4	0.234^{ns}
	HERTEJ	40.9	27	2.00	3	0.18	0.335	0.365	0.083^{ns}	9	2/7	0.018^{**}
Total		120.6	27	2.14	4	0.34	0.258	0.302				
B. rupicola	RUPAGA	4.0	19	1.90	0	0.02	0.400	0.321	-0.297^{ns}	6	2/4	0.078^{ns}
	RUPLAJ	10.9	22	1.86	1	0.10	0.336	0.309	-0.093^{ns}	7	2/5	0.234^{ns}
	RUPOJI	4.9	23	2.15	0	0.11	0.240	0.356	0.351^{ns}	7	5/2	0.765^{ns}
	RUPCAR	59.0	30	2.03	2	0.07	0.288	0.359	0.199^{***}	7	3/5	0.156^{ns}
Total		78.8	33	2.66	6	0.52	0.297	0.382				
B. palmensis	PALAND	19.9	31	2.34	0	0.11	0.391	0.420	0.072^{ns}	10	3/7	0.278^{ns}
	PALVIZ	22.0	25	2.03	0	0.06	0.414	0.379	-0.093^{ns}	10	4/6	0.161^{ns}
	PALRIO	33.9	28	2.00	2	0.24	0.359	0.358	-0.003^{ns}	10	5/5	0.161^{ns}
Total		75.8	38	2.99	10	1.18	0.383	0.454				

N mean sample size over loci, *NA* number of alleles, *Ar* rarefied allelic richness, *PA number of private alleles, PAr* rarefied private allelic richness, H_o observed heterozygosity; H_e unbiased expected heterozygosity, F_{IS} inbreeding coefficient, *L* number of polymorphic loci used in the bottleneck tests, H_d/H_e number of loci with heterozygote deficiency and heterozygote excess (respectively) according to the TPM model, *P* probability of the Wilcoxon test for heterozygote excess

Not significant (ns); $^{***}P < 0.001$; $^{**}P < 0.05$

Table 3 AMOVA analysis for *B. hermosae. B. palmensis* and *B. rupicola* at the interspecific and intraspecific hierarchical levels

Source of variation	Degrees of Freedom	Sum of squares	Variance components	Percentage of variation (%)	*F*-statistics
B. hermosae vs *B. rupicola* vs *B. palmensis*					
Among species	2	619.3	1.567	44.2	$F_{CT} = 0.442^{***}$
Among populations within species	7	125.8	0.355	10.0	$F_{SC} = 0.179^{***}$
Within populations	542	878.6	1.621	45.8	
Total	551	1,623.7	3.543		$F_{ST} = 0.542^{***}$
B. hermosae					
Among populations	2	37.1	0.217	13.7	
Within populations	239	325.6	1.362	86.3	
Total	241	362.7	1.579		$F_{ST} = 0.137^{***}$
B. rupicola					
Among populations	4	30.1	0.378	17.7	
Within populations	154	269.9	1.753	82.3	
Total	157	300.0	2.131		$F_{ST} = 0.177^{***}$
B. palmensis					
Among populations	2	58.5	0.557	22.7	
Within populations	149	283.1	1.900	77.3	
Total	151	341.6	2.457		$F_{ST} = 0.226^{***}$

$^{***}P < 0.001$

Including the whole set of samples, the Bayesian structure analysis identified three genetic clusters based on the highest ΔK (K = 3, Online Resource 3). This is congruent with the other results in the species differentiation, since all individuals were aggregated according to their taxonomic origin with more than 80% of assignment. However, in the analysis for each species individually, we detected that each of the three species presented one (*B. rupicola*) or two genetic clusters (K = 2), assuming the admixture model with correlated frequencies (Fig. 4). In accordance with the AMOVA and

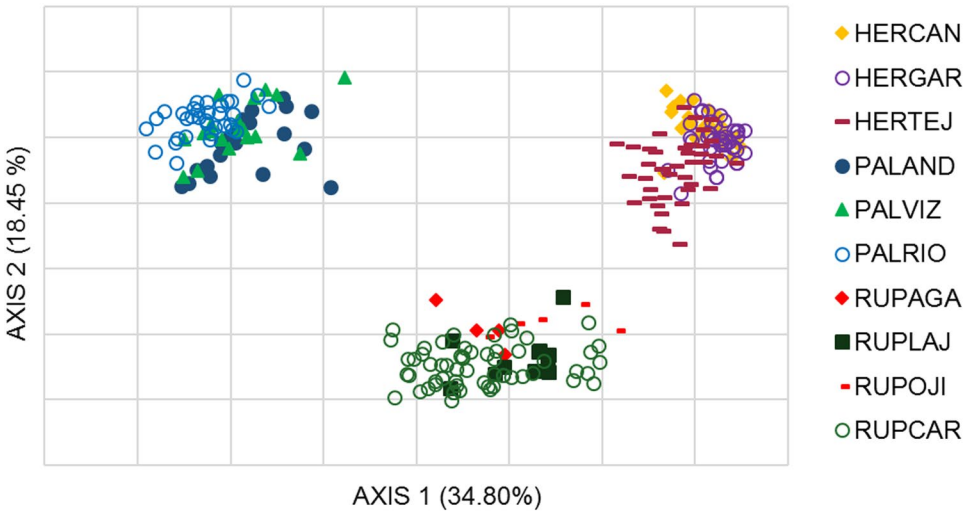

Fig. 2 Principal coordinates analysis (PCoA) based on the genetic distance among individuals. The first two axes explained 53.26% of the total variation. Percentage of variation for each axis are indicated within brackets. Population codes are detailed in Table 1

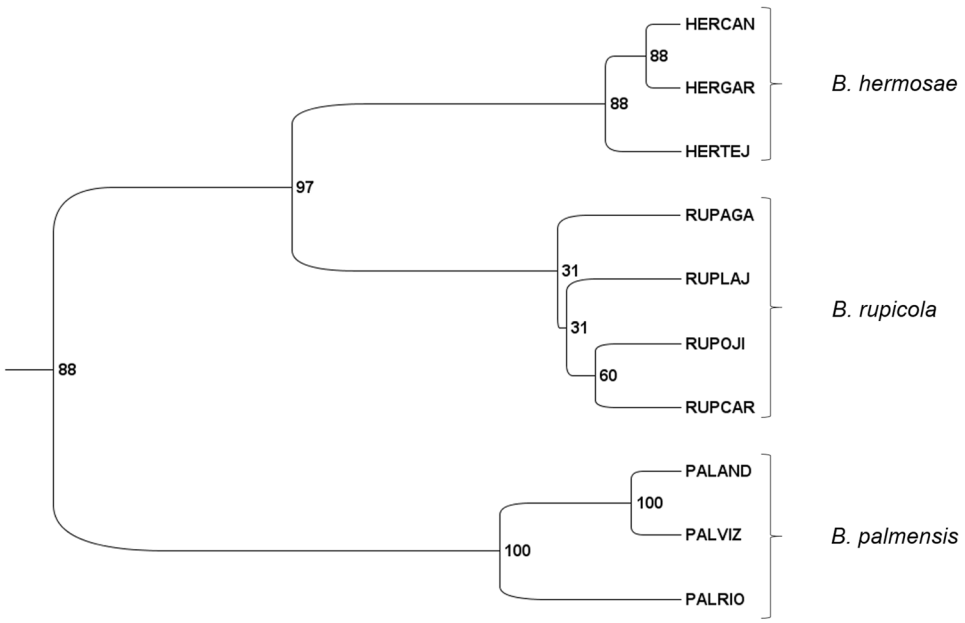

Fig. 3 UPGMA based on Nei (1983) genetic distance among the eleven localities of *Bethencourtia hermosae, B. rupicola* and *B. palmensis*. Bootstraps values are indicated on the right of the nodes. Population codes are detailed in Table 1

F_{ST}, the strongest genetic structure was found in *B. palmensis*, separating the Tenerife population from the ones in La Palma. On the other hand, we did not find a clear structure in *B. rupicola*, while *B. hermosae* presented more assignment of the HERTEJ population to one of the clusters.

Discussion

One of the main objectives of this article was to determine the level of differentiation among populations within *Bethencourtia*, in order to resolve taxonomic uncertainties for conservation purposes, an important issue in conservation biology (Frankham et al. 2002). The results of the analysis for genetic differentiation and structure in the endemic genus *Bethencourtia*, such as the PCoA and UPGMA, strongly reveal that the three species conform to different entities to a high degree, in concordance with their morphological differences (Nordenstam 2006a). The Bayesian analysis implemented in STRUCTURE for the whole set of samples also indicates a clear grouping of the three taxa, along with the results at the species-hierarchical level in AMOVA (44.2% of variation between species). Consistently, pairwise F_{ST} values between species were clearly higher than those between populations within species. Although *B. hermosae* and *B. rupicola* share the same island, the differentiation levels between these two species are similar to the differentiation between each of them and *B. palmensis*. The high F_{ST} value found between species ($F_{ST} = 0.542$) reveals the lack of interspecific gene flow.

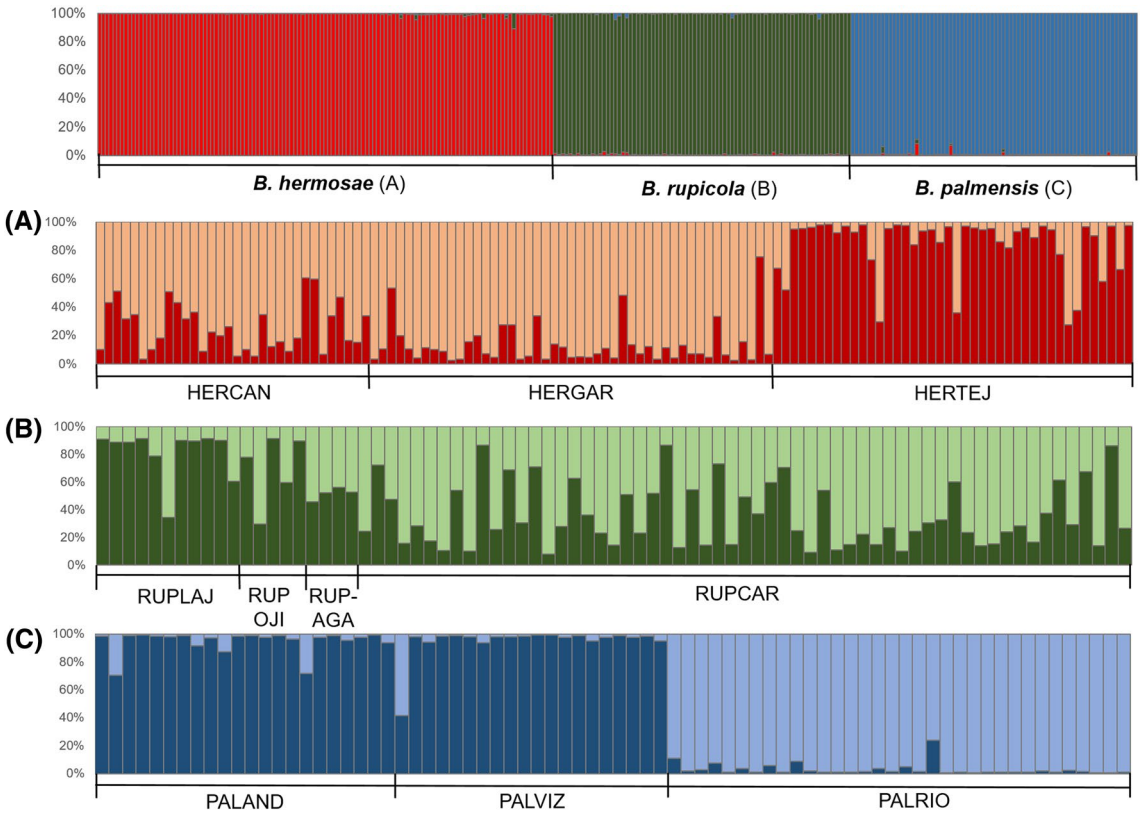

Fig. 4 Bar plots for the proportion of coancestry inferred from Bayesian cluster analysis implemented on STRUCTURE and CLUMPP. The first plot includes the whole set of *Bethencourtia* samples (K = 3), (A) *B. hermosae*, (B) *B. rupicola* and (C) *B. palmensis* with (K = 2) each. Population codes are detailed in Table 1

Similar percentages of differentiation were detected between the different lineages of *Ilex* in Macaronesia, with 36.38% between species (Sosa et al. 2013). Within the *Cheirolophus* genus (Asteraceae), a percentage of explanation among taxonomical groups of 38.63% was also found by Vitales et al. (2014). In addition, the results obtained for the differentiation between *B. hermosae* and *B. rupicola* in La Gomera are higher than other related species inhabiting the same island, such as *Crambe tamadabensis* and *Crambe pritzelii* (8.84% of the total variation between species) (Soto et al. 2016).

The genetic structure at the intraspecific level showed different degrees of homogenization. On the one hand, *B. rupicola* and *B. hermosae* presented a high degree of gene flow among the populations studied. In *B. rupicola*, this is supported by the lack of high assignment of any of its individuals to the two clusters in the STRUCTURE analysis. In *B. hermosae*, HERTEJ presented a higher assignment to one cluster and more private alleles. As occurred in *B. rupicola*, the pairwise F_{ST} values and intraspecific variation were low. The anemochorous dispersal syndrome of this group and the reduced geographical distance between localities could be promoting gene flow within species.

On the other hand, in *B. palmensis*, the genetic structure is different: two defined clusters (K = 2) to which the Tenerife (PALRIO) and La Palma populations (PALAND and PALVIZ) were assigned. The AMOVA results at the intraspecific level were higher than in *B. hermosae* and *B. rupicola*, with 22.7% of the variation found between populations. This was much as expected due to the differences between the islands of Tenerife and La Palma with the ocean acting as a natural barrier. The relatively high F_{ST} values detected between the two islands and the high presence of private alleles in PALRIO also indicate lower levels of gene flow between islands. Although there may be speciation processes taking place in this species due to isolation, we did not find enough evidence to treat the two island populations as different units. Although we only used the PALRIO locality for our research, to test this differentiation, further studies at the morphological and/or molecular level could include more localities from Tenerife. Indeed, due to their habitat complexity high population differentiation is common in the Canary Islands (Mairal et al. 2015), as well as within the same species between islands (González-Pérez et al. 2009a), leading to adaptive radiation in plant genera (Pérez de Paz and Caujapé-Castells 2013). Moreover, dry fruits like those

of *Bethencourtia* usually lead to species-rich lineages with divergence between islands (García-Verdugo et al. 2014).

According to the selfing rate results from this study, the species in *Bethencourtia* would be partially or totally self-incompatible. This conclusion agrees with Ortega and González (1986), who mentioned low seed viability in in-vitro cultivated individuals, possibly caused by self-incompatibility. It has also been pointed out that total or partial self-incompatibility is a common trait in island colonizers in the Asteraceae family. When multiple colonization events have been possible, as in the Canaries due to their proximity to the continent (Francisco-Ortega et al. 2000; Pérez de Paz and Caujapé-Castells 2013), outcrossing or pseudo-self-compatible ancestors could have provided a higher genetic diversity than selfing ones (Crawford et al. 2009). The high homogenization in *B. rupicola* and *B. hermosae* can also be supported by this reproductive system. Furthermore, the HW equilibrium found in almost all populations shows a lack of inbreeding that usually occurs in selfing species (Jarne and Charlesworth 1993).

However, the overall genetic diversity values obtained are low for an outcrossing group. Indeed, the genetic diversity values detected in *Bethencourtia*, specially *B. hermosae* and *B. rupicola* are much lower than in other oceanic endemics in Asteraceae, such as the self-incompatible *Tolpis azorica* ($H_e = 0.716$) (Silva et al. 2016), and *Leontodon filii* ($H_e = 0.530$) (Dias et al. 2014). The values found in *Bethencourtia* were also lower than in other Canarian endemics analyzed with microsatellite markers, such as the insular endemics *Ruta oreojasme* ($H_e = 0.687$) (Meloni et al. 2015), *Parolinia ornata* ($H_e = 0.515$) (González-Pérez and Caujapé-Castells 2014) and *Silene nocteolens* ($H_e = 0.780$) (Sosa et al. 2011), the critically endangered *Sambucus palmensis* ($H_e = 0.500$) (Sosa et al. 2010) and the related species in the Canary Islands *Senecio chrysanthemifolius* ($H_e = 0.700$) (Brennan et al. 2012).

In addition, significant analysis in BOTTLENECK for HERCAN and HERTEJ indicate that the populations from *B. hermosae* could have suffered a recent reduction of their effective size. The possible causes of this reduction are difficult to infer, although land use and competition with invasive species may have diminished all populations in La Gomera. In fact, low numbers of seedlings and juveniles have been detected in both species (Gobierno de Canarias 2009).

Outcrossing species with most of their genetic variability within populations are bound to suffer a greater diversity loss due to habitat fragmentation than selfing species (Aguilar et al. 2008). Naturally rare species that are inherently associated with specific habitats and endemics that form small populations usually have reduced genetic diversity due to bottlenecks, genetic drift and inbreeding. Moreover, populations with low effective sizes are less capable of confronting external disturbances (Barrett and Kohn 1991; Ellstrand and Elam 1993; Frankham 1998). These rare endemic species are commonly expected to have lower variance than widespread ones (Cole 2003) and insular endemics with less variation than continental species (Frankham 1997; Sosa et al. 2011). *Bethencourtia* is a typical case of rare insular endemics with habitat specificity, so low genetic diversity values are to be expected, in consonance with those hypotheses.

Accordingly, further studies of the conservation status of this genus should be considered, with demographic and reproductive studies that would help understand the causes of the bottleneck events and the low genetic diversity found. In this vein, the conservationists of the Garajonay National Park have started to monitor *B. hermosae* and *B. rupicola*, with annual population censuses (Fernández-López and Velázquez-Barrera, unpublished report). In extreme cases, if the germination *in-situ* continues to be unsuccessful, in-vitro propagation could be provided for the maintenance of the populations (Ortega and González 1986). Indeed, to maintain and enhance genetic diversity within each species on La Gomera, propagules from all localities should be taken to construct a reservoir in case of stochastic events or decline of the populations in the near future.

In conclusion, reduced population sizes due to habitat specificity, coupled with bottleneck events and the difficulties in finding available mates may be the most important factors affecting genetic diversity in *Bethencourtia*. Consequently, these aspects should be considered when conservation programs are designed.

Implications for conservation

We genetically characterized the endemic genus *Bethencourtia* in the Canary Islands. The delimitation of the three species has been successfully clarified through microsatellite markers, especially needed between *B. hermosae* and *B. rupicola*. Consequently, the inclusion of *B. rupicola* in the official checklists should be of primary importance to its conservation. Certainly, the facts of its reduced geographical distribution and its presence in only five localities with a low number of individuals in Agando and Ojila, are sufficient reasons to catalog this taxon as "Endangered" or "Vulnerable" by the IUCN.

Considering that *Bethencourtia* species are possibly self-incompatible, we do not expect "inbreeding depression" (Ellstrand and Elam 1993), therefore, efforts should be focused on reinforcing the natural populations with individuals belonging to the same area, and preserving the genetic structure which has been found. The population genetic structure within species was low, apart from the divergence in *B. palmensis* between islands. Therefore, conservation strategies ought to be focused on avoiding anthropological translocations among the distinct populations found in this research. First, the transfer of propagules from *B. rupicola* to

B. hermosae localities should be avoided. In Vallehermoso, the HERTEJ population was found to be distinct with higher genetic diversity, which should be considered for management purposes. Secondly, further attention must also be paid to *B. palmensis* populations, trying to avoid the introduction of individuals from Tenerife to La Palma or vice versa. Homogenization of already differentiated localities could lead to the loss of genetic diversity and interrupt speciation processes.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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