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A tale of two forests: ongoing aridification drives population decline and genetic diversity loss at continental scale in Afro-Macaronesian evergreen-forest archipelago endemics

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• **Background and Aims** Various studies and conservationist reports have warned about the contraction of the last subtropical Afro-Macaronesian forests. These relict vegetation zones have been restricted to a few oceanic and continental islands around the edges of Africa, due to aridification. Previous studies on relict species have generally focused on glacial effects on narrow endemics; however, little is known about the effects of aridification on the fates of previously widespread subtropical lineages.
• **Methods** Nuclear microsatellites and ecological niche modelling were used to understand observed patterns of genetic diversity in two emblematic species, widely distributed in these ecosystems: *Canarina eminii* (a palaeoendemic of the eastern Afromontane forests) and *Canarina canariensis* (a palaeoendemic of the Canarian laurel forests). The software DIYABC was used to test alternative demographic scenarios and an ensemble method was employed to model potential distributions of the selected plants from the end of the deglaciation to the present.
• **Key Results** All the populations assessed experienced a strong and recent population decline, revealing that locally widespread endemisms may also be alarmingly threatened.
• **Conclusions** The detected extinction debt, as well as the extinction spiral to which these populations are subjected, demands urgent conservation measures for the unique, biodiversity-rich ecosystems that they inhabit.

Key words: Islands, nuclear microsatellites, subtropical flora, decline, aridification, extinction, genetic conservation.

INTRODUCTION

Rare species are defined as those characterized by small population sizes, narrow geographic distributions and high habitat specificity, or any combination of these criteria (Rabinowitz, 1981). These species often display low genetic diversity, which makes them vulnerable to stochastic demographic phenomena (Kruckeberg and Rabinowitz, 1985; Hewitt, 2004). However, this relationship between rarity and genetic diversity is not an axiom: rare species may present high levels of genetic diversity (Stebbins, 1980; Gitzendanner and Soltis, 2000; Pérez de Paz and Caujapé-Castells, 2013; García-Verdugo *et al.*, 2015), whereas some widespread and common species, such as exotic invasive plants, may exhibit low levels of genetic diversity (Tsutsui *et al.*, 2000).

Relict species are rare species whose distribution ranges have been reduced to a few populations due to different processes (e.g. aridification), but with a degree of threat that gives them a great value in conservation biology (Habel and Assmann, 2009). Especially interesting are palaeoendemics, i.e. relict species that

have become rare due to widespread extinction in their original distribution range (Stebbins and Major, 1965), and which frequently act as reservoirs of phylogenetic exclusivity, as the only surviving representatives of larger clades (Cronk, 1992; Faith, 1992). Biologists often characterize these species as 'living fossils', an irreplaceable heritage in the Tree of Life (Grandcolas *et al.*, 2014). The need to conserve these rare and relict species and their habitat-restricted communities has increased in the current context of rapid human-induced climate change.

Studies on relict species have generally focused on glacial relicts, which are now constrained to some reduced ranges in mountain peaks or high latitudes (Petit *et al.*, 2003; Hewitt, 2004). By contrast, relict species that have become rare as a result of aridification have received far less attention. Genetic studies on these species, however, are of particular relevance because they may provide insights into the capacity for resilience and the foreseeable evolutionary fates of species subject to global warming.

Here we focus on one such taxon: the angiosperm plant genus *Canarina*, belonging to the bellflower family, Campanulaceae (tribe Platycodoneae). This genus comprises only three species separated by >7000 km across the Sahara desert: *C. canariensis*, associated with the endemic laurel forests of the Canary Islands, and *C. eminii* and *C. abyssinica*, which are endemic to the relict Afromontane forests of East Africa. *Canarina* is the only African representative of Platycodoneae, an early-diverging tribe within family Campanulaceae (Roquet *et al.*, 2008), while the remaining members of the tribe are distributed throughout Central and East Asia (Wang *et al.*, 2013). Thus, *Canarina* complies with the criterion of phylogenetic exclusivity in a relict rare species (Faith, 1992).

Mairal *et al.* (2015a) reconstructed the biogeographic history of *Canarina* and showed that ancestors of this genus migrated from Asia to East Africa in the Middle Miocene. They linked the disjunct sister-group relationship between *C. canariensis* and *C. eminii* (dated ~7–6 Ma) to large-scale extinction and a gradual aridification process that would have started in North Africa in the Late Miocene (Sepulchre *et al.*, 2006; Senut *et al.*, 2009). This was followed by cycles of contraction and expansion affecting both subtropical and tropical taxa (Maley, 2000; Bonnefille, 2011), in which mountainous areas in the Sahara served as refuges during arid periods (Osborne *et al.*, 2008). This Afro-Macaronesian biogeographic pattern has been observed in many other angiosperm genera, and is known as the Rand Flora pattern (Sanmartín *et al.*, 2010; Pokorny *et al.*, 2015; Mairal *et al.*, 2017a). Thus, *Canarina* also fulfils the criterion of a palaeoendemic relict – the palaeoendemic character can also be seen in the *Canarina* populations of both species, in which reservoirs of ancient genetic diversity appear in the oldest massifs and palaeo-islands (Mairal *et al.*, 2015b, 2017b).

Rare or relictual species are also generally characterized by high habitat specificity (Kruckeberg and Rabinowitz, 1985), either because the species have low environmental tolerance or a narrow niche, or because their habitats are currently under contraction. The habitats now occupied by *Canarina* (the Canarian laurel forest and the isolated patches of subtropical Afromontane forest in East African 'sky islands') have often been interpreted as the last remnants of a more humid vegetation that extended over the Mediterranean Basin and North Africa during the Early–Middle Miocene (Heald, 1951; Axelrod and Raven, 1978; White, 1983; Bramwell, 1985; McCormack *et al.*, 2009; Fernández-Palacios *et al.*, 2011); but see Kondraskov *et al.* (2015) for a different view on the relict character of the Canarian laurel forests. Plant lineages that took shelter in these enclaves have acquired a high degree of relictualism and contribute substantially to the exceptional endemicity of these areas (Fjeldså and Lovett, 1997; Juan *et al.*, 2000), which are now classified as biodiversity hotspots; i.e. biogeographic regions whose great biodiversity is threatened by habitat loss (Myers *et al.*, 2000; Mittermeier *et al.*, 2004).

Both *C. eminii* and *C. canariensis* have widespread but disjunct distribution ranges and fulfil Rabinowitz's (1981) 'large and narrow' criterion of rarity: species that are constantly sparse in a specific habitat but over a large range. *Canarina canariensis* is a terrestrial herb that propagates by seeds and shoots from its tuber (Bramwell and Bramwell, 2001); it occurs in the endemic Canarian laurel forests and (more rarely) in the adjacent thermophilous forests in the central and western islands of the Canarian Archipelago. *Canarina eminii* propagates from a long and thick root and grows as a terrestrial or (mainly) as an epiphyte of Afromontane forest trees (*Podocarpus*, *Hagenia*, *Conopharyngia*; Hedberg, 1961) in East Africa, from Ethiopia in the north to Malawi in the south (Fig. 1).

The steep topography and patchy distribution of the Eastern African forests (divided by deserts, savannahs and cultivated lands) and the deep ocean floor that lies between islands in the Canarian Archipelago have added to a high degree of geographic and ecological isolation (Fig. 1B), manifested by high genetic differentiation among populations and a marked geographic structure (Mairal *et al.*, 2015a,b, 2017a). This great interpopulational diversity is threatened in *C. eminii* by human activities, such as logging and forest clearance (fires) linked to a rapid growth of the human population and agricultural development (Hedberg, 1961; Alemayehu, 2006), which have contributed to further fragmentation and isolation among forest patches (Fahrig, 2003). In the case of *C. canariensis*, although big patches of the laurisilva are currently under legal protection, this forest type has experienced a strong contraction during the last two centuries with respect to its estimated original range, due mostly to agricultural practices (Fernández-Palacios *et al.*, 2011; but see de Nascimento *et al.*, 2015 for an alternative view of Gran Canaria's forests).

In this study, we used molecular and ecological niche modelling approaches to explore the effects of historical climate change and recent anthropogenic activity on the demographic evolution of *C. canariensis* and *C. eminii*. We developed new molecular markers from a library of nuclear microsatellites (SSRs), and used these to document levels of genetic diversity within and among populations. We also tested the alternative scenarios of population dynamics of (1) constant size, (2) bottleneck, (3) demographic expansion and (4) decline, using approximate Bayesian computation techniques (Beaumont, 2010) based on SSR data. Finally, we employed ecological niche modelling to reconstruct the climatic requirements (niche breadth) of African and Canarian populations and project them back into the past (Last Glacial Maximum, Holocene) to understand the impact of recent climate change on the geographic distribution of populations. If climate change since the end of the deglaciation or human activities have driven the retreat of these forests, we should find a genetic signal linked to a recent demographic decrease; conversely, this signal would not appear if the demographic processes were older. A demographic decline would confirm the suspicions about the serious state of the threat to these forest patches. Accordingly, our main goals are: (1) to compare levels of population genetic diversity between these two species, and with those estimated by other markers [chloroplast sequences and amplified fragment length polymorphisms (AFLPs); Mairal *et al.*, 2015b, 2017b]; (2) to search for the presence of population decline or bottlenecks that may indicate ongoing loss of genetic diversity; (3) to relate population dynamics to changes in the potential geographic distribution of *Canarina* populations over time; and (4) to provide a genetic basis to enable the design of suitable strategies for the genetic conservation of these taxa.

MATERIALS AND METHODS

Plant material, population sampling and DNA sequencing

Several sampling field campaigns were performed in East Africa and the Canary Islands (2009–14), which led to the
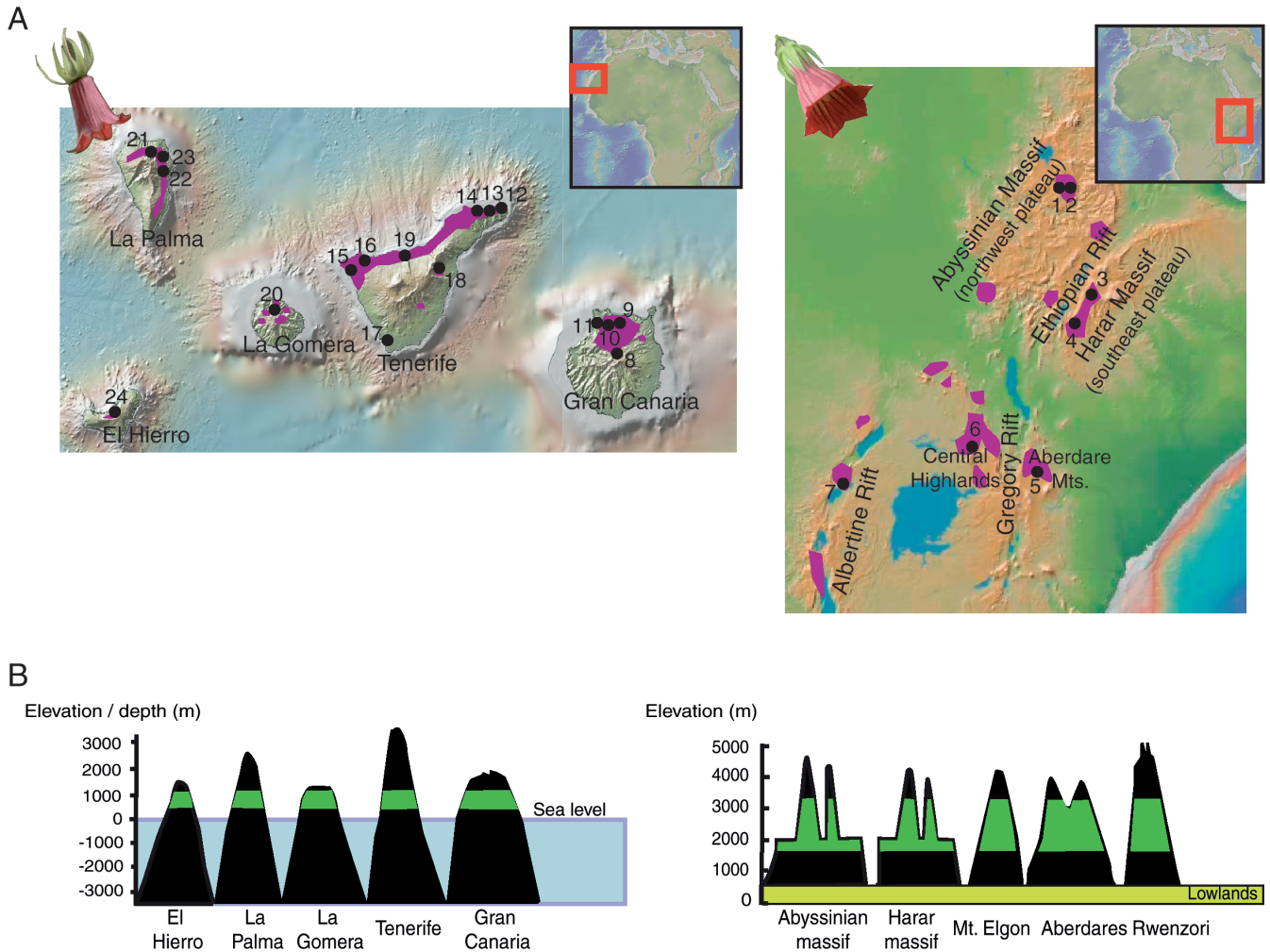
FIG. 1. Distribution and scheme of the insular systems inhabited by the studied species. (A) Geographic distribution of *C. canariensis* in the Canary Islands (left) and *C. eminii* in the Afromontane forests (right). The distribution areas are shaded in purple. Sampled populations are represented with black dots accompanied by a population code number, as specified in Table 1. (B) Schematic representations of the insular systems and the subtropical vegetation belts inhabited by the studied species: oceanic islands and laurisilva forests for *C. canariensis* (left) and sky islands and Afromontane forests for *C. eminii* (right).

collection of fresh material from 223 individuals in 24 different populations of *Canarina*: 17 of *C. canariensis* (N = 159) and seven of *C. eminii* (N = 64) (Table 1). For the largest populations, a minimum of ten samples was collected through the entire area of occupation to reduce the possible inflation in the genetic structure descriptors (Caujapé-Castells, 2010). DNA was extracted from silica-gel-dried leaves using the DNeasy Plant Mini Kit (Qiagen, CA, USA). The quality of DNA extractions was checked on 1 % agarose gels, and DNA concentration was measured using a NanoDrop spectrophotometer. All samples were diluted to 10 ng µL^{-1} prior to PCR amplification.

Development of nuclear microsatellite markers and scoring

A microsatellite library was developed for *C. canariensis* at the Savannah River Ecology Laboratory (University of Georgia). The extracted plant DNA was enriched using magnetic streptavidin beads and biotinylated oligonucleotides representing CT and GT repetitions. The library was sequenced using a Titanium Sequencing Kit (Roche Life Sciences) on the Roche 454 GS-FLX Titanium platform. We selected tri- and tetra-nucleotide repetition motifs to reduce the risk of misinterpreting as homozygotes the heterozygotes with alleles differing by a single repeat. Primer design and PCR amplifications were performed in the laboratories of Jardín Botánico Canario 'Viera y Clavijo', Unidad Asociada CSIC del Cabildo de Gran Canaria (JBCVC-CSIC).

We selected and tested 26 primer pairs for *C. canariensis*, which were also tested for cross-amplification in *C. eminii*. Fifteen loci were discarded because of amplification failure in most samples, poor electrophoretic profiles or low levels of variation. Finally, 11 SSR loci were retained for *C. canariensis* and seven for *C. eminii*; these showed good quality profiles and were used to genotype all sampled individuals from both species. DNA amplification (PCR) was performed in a final volume of 20 µL, containing 1 µL of DNA, 12.55 µL of sterilized water, 1.5 µL of 10× buffer, 0.8 µL of MgCl^{2+}, 0.3 µL of dNTP, 1 µL of BSA, 0.25 µL Taq DNA polymerase

TABLE 1. *Genetic diversity descriptors and tests used for each of the populations of* C. canariensis *and* C. eminii. *For Tenerife, names of paleo-islands are indicated in parentheses*

Population		N	A	H_e (s.e.)	N_{pa}	M
Canarina eminii, continental islands						
1. Gifta	Abyssinian massif	8	1.58	0.167 (0.220)	0.00	0.442
2. Dembecha	Abyssinian massif	8	1.80	0.244 (0.270)	0.07	0.349
3. Yirga	Harar massif	8	2.00	0.301 (0.349)	0.17	0.081
4. Harenna	Harar massif	9	2.28	0.287 (0.218)	0.33	0.299
5. Aberdare	Aberdare Mountains	8	1.83	0.382 (0.222)	0.01	0.128
6. Elgon	Mount Elgon	13	1.99	0.339 (0.295)	0.01	0.123
7. Rwenzori	Rwenzori Mountains	10	2.24	0.410 (0.272)	0.01	0.161
Canarina canariensis, oceanic islands						
8. Cueva Corcho	Gran Canaria	8	1.96	0.305 (0.34)	0.14	0.111
9. Tilos de Moya	Gran Canaria	12	2.04	0.325 (0.27)	0.06	0.158
10. El Sao	Gran Canaria	10	1.84	0.282 (0.26)	0.00	0.229
11. Andenes de Guayedra	Gran Canaria	12	1.72	0.292 (0.25)	0.00	0.224
12. Camino Chamuscadas	Tenerife (Anaga)	10	4.07	0.417 (0.29)	0.37	0.235
13. El Bailadero	Tenerife (Anaga)	11	3.89	0.378 (0.31)	0.03	0.164
14. Tope del Carnero	Tenerife (Anaga)	7	3.94	0.412 (0.29)	0.11	0.229
15. Teno Alto	Tenerife (Teno)	8	2.37	0.400 (0.26)	0.07	0.171
16. El Palmar	Tenerife (Teno)	8	2.54	0.485 (0.27)	0.25	0.198
17. Barranco del Infierno	Tenerife (Adeje)	11	2.50	0.489 (0.23)	0.00	0.184
18. Barranco de Badajoz	Tenerife	5	3.50	0.316 (0.35)	0.02	0.178
19. Barranco Ruiz	Tenerife	6	2.20	0.411 (0.22)	0.02	0.158
20. Tamargada	Gomera	13	1.67	0.235 (0.26)	0.04	0.250
21. Los Tilos	La Palma	10	2.26	0.394 (0.25)	0.14	0.202
22. Barranco del Agua	La Palma	10	2.02	0.332 (0.24)	0.01	0.188
23. Barranco La Barata	La Palma	4	1.88	0.365 (0.26)	0.00	0.258
24. El Hierro	El Hierro	12	2.24	0.415 (0.20)	0.17	0.183

A, allelic richness; N_{pa}, number of private alleles standardized to sample size; M, Garza and Williamson's M-ratio test.

(Bioline), 1 µL of forward primer (0.2 µM), 0.8 µL of reverse primer (5 µM) and 0.8 µL of reverse-tailed primer. PCR products were labelled using the fluorescent dyes FAM, VIC, NED or PET (labels according to the original kit of Applied Biosystems), and an additional 19-bp fluorescently labelled M13 primer (5′-CACGACGTTGTAAAACGAC-3′) according to the methods of Boutin-Ganache *et al.*, (2001). A tail sequence (5′-GTGTCTT-3′) was added to the 5′ end of the reverse primer to improve adenylation (addition of AMP) and to facilitate genotyping. Samples were incubated in a Verity 96 thermocycler using the following conditions: 2 min of denaturation at 94.4 °C, 30 cycles of 30 s of denaturation at 94.4 °C, 40 s at different annealing temperatures for each locus (Supplementary Data Table S1), 30 s of elongation at 72 °C and final extension for 8 min at 72 °C. Reactions were separated on 5 % polyacrylamide gels. The amplified fragments were scored using GeneMapper 4.0 software (Applied Biosystems) with the LIZ 500 size standard. We combined automatic detection of each allele with visual inspection of each sample, following Dewoody (2006) to reduce scoring errors. Finally, we identified peak profiles for each locus and allele, and we assigned a genotype to each individual.

To detect possible genotyping errors (stuttering, null alleles and allelic dropout), we analysed the genotyping matrix with the programs MICRO-CHECKER 2.2.1. (Van Oosterhout *et al.*, 2004) and INEst 1.0 (Chybicki and Burczyk, 2009). A single locus in *C. eminii* and one locus in *C. canariensis* displayed null alleles with frequencies >0.05 and high F_{IS} values, and were therefore removed from further analyses.

Other loci were excluded due to low quality of electropho-retograms, especially in *C. eminii*, where several loci were removed due to the effect of cross-amplification. The final data matrices contained nine loci for *C. canariensis* and six loci for *C. eminii*.

Genetic diversity and population structure

Deviations from Hardy–Weinberg equilibrium and linkage disequilibrium were tested using POPGENE version 4.2 (Yeh *et al.*, 1997). Standard genetic diversity statistics, such as expected heterozygosity (H_e), were calculated using the software Arlequin (Excoffier *et al.*, 2005). To account for sample size variation among populations, we estimated the allelic richness and the number of private alleles using rarefaction analysis implemented in the software HP-Rare v.1.0 (Kalinowski, 2005). To identify the signal of old or severe bottlenecks, we used Garza and Williamson's *M*-ratio test (GW test) (2001), which is better suited to the detection of these events than methods based on the deficit of rare alleles (Williamson-Natesan, 2005). The GW test detects the presence of bottlenecks by determining the ratio between the number of alleles and the allele size range, according to the statistic $M = k/r$, where k is the number of alleles and $r = S_{max} - S_{min} + 1$, where S is allele size.

To detect the genetic composition of populations, we used the Bayesian clustering method implemented in STRUCTURE v.2.3 (Pritchard *et al.*, 2000). This approach assumes that loci

are in Hardy–Weinberg equilibrium and that there is linkage equilibrium within populations. Analyses were performed separately for each species using an admixture model with correlated allele frequencies among groups. We ran 500 000 Markov chain Monte Carlo iterations after a burn-in of 100 000 iterations for *K* values of 1–10, with ten repetitions for each *K*. The most probable value of *K* was determined with the method of Evanno *et al.* (2005) implemented in STRUCTURE Harvester (Earl, 2012). The matrices were adapted to the specific format of each of the programs using Transformer-4 (Caujapé-Castells *et al.*, 2013). Microsatellite matrices are available in the DEMIURGE information system (http://www.demiurge-project.org/) with codes D-NMICR-122 (matrix of *C. canariensis*) and D-NMICR-123 (matrix of *C. eminii*). To quantify the amount of genetic variance attributable to geographic and population subdivision, a hierarchical analysis of molecular variance (AMOVA) was performed using the software ARLEQUIN v.3.0 (Excoffier *et al.*, 2005). Exploratory analyses were performed considering, alternatively, the individual populations in palaeoislands and sky islands separately, or grouped into geographic units as identified by STRUCTURE.

Demographic analysis

We used approximate Bayesian computation techniques as implemented in the software DIYABC v1.0.4.46beta (Cornuet *et al.*, 2008, 2010) to statistically evaluate alternative scenarios for the demographic history of *C. eminii* and *C. canariensis*. DIYABC uses a coalescent framework to simulate complex evolutionary scenarios without the need to estimate the underlying likelihood function (Cornuet *et al.*, 2008, 2010). Instead, simulated scenarios are summarized using variable estimates (summary statistics), which are then compared with observed values from the genetic data to estimate the posterior probability of the model parameters using Bayesian inference, and to compute measures of bias and precision for each scenario [95 % high posterior density (HPD) credibility intervals]. Due to the strong population structure observed in the two study species (Mairal *et al.*, 2015b, 2017b) and the problems that can arise by assuming panmixia, we conducted separate analyses for each population, and we grouped only those populations that were geographically (<10 km) and genetically very close according to the STRUCTURE results, i.e. Gifta and Dembecha probably form an apomictic population, and were part of the same Afromontane forest patch, nowadays fragmented because of agriculture.

We considered four competing population history scenarios: (1) a null model of constant population size; (2) a bottleneck in the past with subsequent recovery; (3) a demographic expansion; and (4) a recent population decline without recovery (Fig. 1A). We assumed different effective population sizes for each historical scenario (N_e, N_1, N_3, N_4). Table 2 shows the prior distributions of the demographic parameters for the four simulations, each simulating a change in the population size. We assumed that each locus followed a generalized stepwise mutation (GSM) model for microsatellite markers (Estoup *et al.*, 2002), using a log-uniform distribution for the geometric distribution parameter ($P = 10^{-1}–30^{-1}$) and the mutation rate ($\mu = 10^{-4}–10^{-3}$). In each of the tested scenarios, we

TABLE 2. *Prior distribution of demographic and historical parameters used as summary statistics for simulations of population size change in DIYABC (Fig. S1). Times of changes in effective population size are considered from the present (0) back in time: t_1, t_2, t_3, t_4. Mean μ is the mean mutation rate of microsatellites; Mean P is the mean of the geometric distribution parameter. All time events are expressed in numbers of generations. Conditions:* $N_1 < N_e$, $N_3 < N_e$, $N_4 > N_e$

Parameter	Prior distribution
N_e	Uniform (10–10 000)
t_1	Uniform (1–10 000)
N_1	Uniform (1–100)
t_2	Uniform (1–10 000)
t_3	Uniform (1–10 000)
N_3	Uniform (1–100)
t_4	Uniform (1–10 000)
N_4	Uniform (4000–100 000)
Mean μ	Log-uniform (1.00E–004 to 1.00E–003)
Mean P	Log-uniform (1.00E–001 to 3.00E–001)

N_e, effective population size; N_1 past effective population size in a bottleneck scenario; N_3, past effective population size in an expansion scenario; N_4 past effective population size in a decline scenario.

simulated one million datasets and assumed absence of migration between populations. First, we estimated the posterior probability of each scenario by logistic regression, using the 1 % simulated datasets with summary statistics that were closest to the observed values. Bayesian credibility ranges (95 % HPD) of each simulated scenario were compared (Cornuet *et al.*, 2008). We also estimated the posterior probability of the parameters from 1 % of the best-simulated datasets, using local linear regression and logit transformation of parameters. To make sure that the best scenario was not far off the observed data, we checked the goodness of fit by simulating 1000 data sets from the posterior predictive distribution of the parameter and compared SumStats with the observed data (Cornuet *et al.*, 2010; Budde *et al.*, 2013).

Ecological niche modelling

We modelled the current climatic niche occupied by *C. eminii* and *C. canariensis* using extant occurrence data together with climatic maps. Data points were obtained from published monographs and inventories (Hedberg, 1961), three online databases (www.jardincanario.org/flora-de-gran-canaria, www.gbif.org and www.anthos.es) and data compiled through field trips. In all, we used 108 records for *C. canariensis* and 80 records for *C. eminii* (Supplementary Data Tables S2, S3), covering the entire distributional ranges of the species. Climatic data for current conditions were obtained from WorldClim (www.worlclim.org; Hijmans *et al.*, 2005). For past climate scenarios we downscaled WorldClim data from a 30 arc-second resolution to 1 km resolution and projected current and past climate conditions for several periods after the Last Glacial Maximum, since the end of the deglaciation to the present (18, 10, 5 and 0 kiloyears; for more information see Espíndola *et al.*, 2012). Pleistocene sea-level changes [~18 kiloyears ago the sea levels were 110 m below the actual level (Rijsdijk *et al.*, 2014)] strongly altered the surface

area of the Canary Islands, and thus the extent of potentially suit-able areas for *C. canariensis*. To account for these changes, we used a bathymetry layer and constructed new rasters using the sea levels modelled by Rijsdijk *et al.* (2014) and projected cli-mate data, including sea levels (−110 m for 18 kiloyears, −46 m for 10 kiloyears, −4.7 m for 5 kiloyears). To model the distribu-tions of the two species, we combined the available occurrences with a set of six bioclimatic variables that could be estimated for all past scenarios: total annual precipitation, maximum and minimum monthly precipitation, annual mean temperature, and maximum and minimum monthly temperature. Pseudoabsences were generated by selecting 5000 random points. We used ensemble modelling (Araújo and New, 2007) to generate our predictions. Three modelling techniques – generalized additive models (GAMs), the general boosting method (GBM) and ran-dom forests (RF) – were run and summarized using R packages (R Core Team, 2014): biomod2 (Thuiller *et al.*, 2013), foreign, raster (Hijmans and van Etten, 2016), SDMTools (VanDerWal *et al.*, 2011), rms (Harrell, 2016), gbm (Ridgeway, 2015), gam (Hastie, 2016), rJava (Urbanek, 2010), dismo (Hijmans *et al.*, 2016) and randomForest (Liaw and Wiener, 2002) (references for R packages are given in the Supplementary Data; Mairal *et al.*, 2017a). We used repeated split sampling to evaluate the performance of the models, successively splitting the dataset into 70 % for calibration and 30 % for evaluation by measuring the area under the curve (AUC).

RESULTS

Genetic diversity and population structure

Based on the nine SSR loci selected for *C. canariensis* and the six loci for *C. eminii*, we detected 54 and 23 alleles, respect-ively, in the two species. The greatest number of private alleles in *C. canariensis* was detected in populations from the three palaeo-islands of Tenerife (e.g. 0.37 in Camino Chamuscadas; 0.25 in El Palmar; Table 1), which predate the age of the island in its current configuration. In *C. eminii*, the greatest number of private alleles was detected in populations from the Harar massif (0.33; Table 1). These Canarian and East African popu-lations were inferred as sources of allele migration events in previous studies based on AFLP and chloroplast sequence data (Mairal *et al.*, 2015b, 2017b). The average number of alleles per locus ranged from 1.67 to 4.07 in *C. canariensis* and from 1.58 to 2.28 in *C. eminii*. Genetic diversity values, estimated as Nei's heterozygosity (H_e), were relatively low compared with values generally obtained with microsatellite markers in endemic spe-cies (H_e = 0.42; Nybom, 2004): 0.167–0.41 in *C. eminii* and 0.235–0.489 in *C. canariensis*. Linkage disequilibrium was not significant ($P > 0.05$).

The method of Evanno *et al.* (2005) indicated that the most likely number of genetic groups was $K = 2$ for *C. canariensis* ($\Delta K = 1301$) and $K = 2$ for *C. eminii* ($\Delta K = 108$) (Supplementary Data Tables S4, S5, Fig. S2). A more complex genetic structure was revealed for *C. canariensis* by $K = 4$, delimiting two add-itional groups [$\Delta K = 60$ (Supplementary Data Table S4)]. In gen-eral, populations of *C. canariensis* displayed a clear east/west structure, with a stronger admixture within the islands of Tenerife and Gran Canaria (Fig. 2A). In *C. eminii*, the structure of $K = 2$ supported a north/south division among populations (Fig. 2B).

Hierarchical AMOVA analyses showed the largest pro-portion of genetic variation among groups, when each group was considered separately (23.22 % in *C. eminii*; 22.21 % in *C. canariensis*; Supplementary Data Table S6). Additionally, the STRUCTURE sublevels among groups ($K = 2$ for *C. eminii* and $K = 2$, $K = 4$ for *C. canariensis*) were consistent with the AMOVA analyses (Supplementary Data Table S6).

Population dynamics

All values for the GW test were M <0.68, the critical value below which populations are assumed to have suffered a recent population decline (with datasets of seven loci or more; Garza and Williamson, 2001). Though we could only reliably score six loci in *C. eminii* (see the Materials and methods section), the results of the demographic analysis (below) and the low val-ues obtained with the GW test (Table 1) support a demographic decline in all populations of *C. canariensis* and *C. eminii*.

In both species, the 'recent population decline' scenario (Supplementary Data Fig. S1) was associated with the highest posterior probabilities and the narrowest 95 % HPD intervals in the DIYABC analysis (Table 3). For some populations, we could not discriminate among alternative hypotheses, as we did not obtain robust statistics for any given scenario [e.g. Gifta, Harenna, Yirga and La Gomera (Tamargada); Table 3, Supplementary Data Table S7]. The current estimated effective population size N_e was estimated between 500 and 1300 across populations, and was about two orders of magnitude smaller than the effective popula-tion size before the decline. Population decline was estimated to have started ~2000–5000 generations ago.

Ecological niche modelling

Species distribution models indicated that the inferred poten-tial distribution for the present largely coincided with the extant distribution of both species. The AUC values were generally high (with values of 0.71 and 0.96 for *C. canariensis* and *C. eminii* respectively), suggesting that the models are consistent. Our hindcast climate niche projections showed that the areas with favourable climatic conditions for *C. eminii* and *C. canariensis* experienced a reduction from the start of the deglaciation (around 18 kiloyears ago) to the present (Fig. 3). Present projections show patches of climatic suitability (climatic refugia) that largely coin-cide with the extant distribution of the species (Fig. 3).

DISCUSSION

Low genetic diversity and strong population structure in Canarina *endemics*

Population genetic theory predicts that species subject to habitat fragmentation, e.g. due to habitat loss, are more susceptible to stochastic demographic fluctuations, such as genetic bottlenecks or declines in effective population size. This is often accompa-nied by an upward surge of inter-population genetic differentia-tion, higher inbreeding and loss of genetic variability (Stebbins, 1980; Kruckeberg and Rabinowitz, 1985; Lande, 1993), which can eventually drive a species to extinction. This 'fragmentation
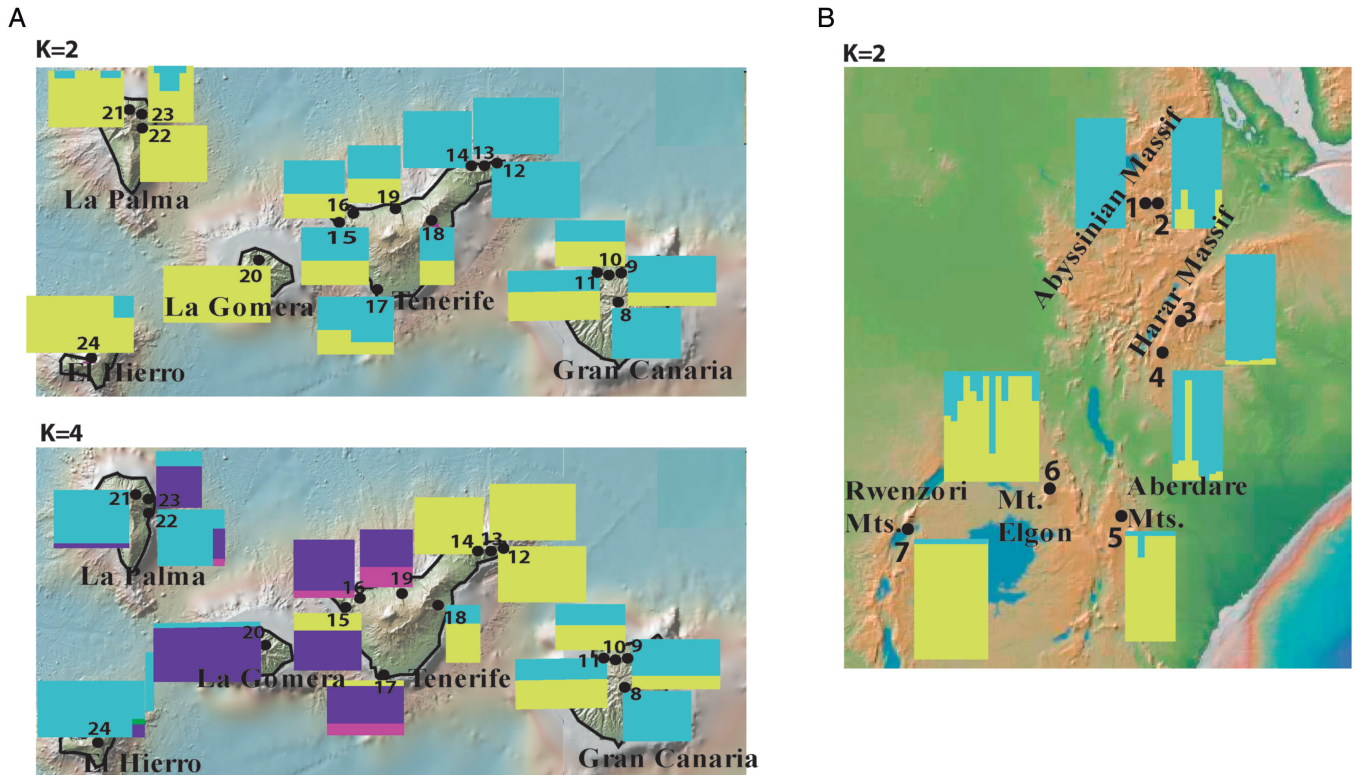
FIG. 2. Results of the genetic structure analyses for (A) *C. canariensis* and (B) *C. eminii*. Histograms show the Bayesian clustering of individuals within populations (STRUCTURE). Colours represent the individual proportion for each of the inferred Bayesian groups. All other conventions as in Fig. 1.

syndrome' (Lande, 1993) seems to be evidenced in *Canarina*. Our ecological niche models indicate that both *C. eminii* and *C. canariensis* have experienced a contraction of their climatic suitability areas since 18 kiloyears ago, which eventually led to the fragmented, patchy distribution observed today (Fig. 3). The low levels of genetic variability detected at the population level (Table 1) are in agreement with those obtained with other types of molecular marker (chloroplast DNA sequences and AFLPs; Mairal *et al.*, 2015b, 2017b). They are also generally below those obtained with microsatellites in endemic species (Nybom, 2004), and specifically below those reported in other taxa distributed in the study areas (Table 4). Note, however, that comparing genetic statistics across distantly related phylogenetic groups is problematic because their values can be influenced by taxon-specific biological differences (Gitzendanner and Soltis, 2000).

The loss of genetic diversity is also accompanied in *Canarina* by a large among-population differentiation (see AMOVA in Supplementary Data Table S6) and great genetic exclusivity in some populations, especially those that may have acted as refugia or genetic reservoirs in the past (e.g. the palaeo-islands of Tenerife or Mount Elgon in Kenya), though this diversity, detected also by AFLPs and plastid sequences, seems to be of older origin than the one reported here (Mairal *et al.*, 2015b, 2017a).

In *C. canariensis*, our SSR analyses detected a strong population structure akin to the one revealed by AFLPs and DNA sequences: an east/west genetic split, with the strongest difference found within Tenerife (Fig. 2A; Mairal *et al.*, 2015b, 2017a). The only difference is that SSRs detected higher levels of genetic admixture in the population of the paleo-island of

Adeje than the other markers (Fig. 2A; this had been predicted in Mairal *et al.*, 2015b). In *C. eminii*, SSRs detect a north/south genetic diversity structure, which was not detected by AFLPs or sequences (Mairal *et al.*, 2017b). This might reflect more recent population dynamics in agreement with similar contact patterns found in other Afromontane plants (Assefa *et al.*, 2007; Masao *et al.*, 2013). Alternatively, it could be an artefact of the lack of specificity in our SSR markers, which were designed for *C. canariensis*. Additionally, we found genetic admixture in Mount Elgon (Fig. 2B), which agrees well with its purported role as a crossroad among populations at both sides of the Rift Valley (Mairal *et al.*, 2017b), and with the descent of vegetation belts during the Last Glacial Maximum (Chala *et al.*, 2017).

Historical population declines in widespread rare species

The time lag between range reduction and the collapse of populations is known as 'extinction debt' (Tilman *et al.*, 1994). Initially, if studies are conducted shortly after habitat loss has occurred, the evolutionary cost of the extinction debt is not obvious and thus difficult to detect (Triantis *et al.*, 2010). Several generations are needed to detect the total impact of habitat loss and fragmentation (Tilman *et al.*, 1994; Tilman, 1999). This seems to be the case in *Canarina*: although this endemic genus of evergreen subtropical forests is still widely distributed in regions of East Africa and Macaronesia, it also shows a worrying population decline scenario. Indeed, demographic analyses based on SSRs (Tables 1 and 3) and ecological niche model

TABLE 3. *Results of DIYABC analyses. In each population the best supported scenario is indicated according to its posterior probability [PP; mean and 95 % Bayesian highest posterior density interval (HPD)], obtained by logistic regression from 1 % of the simulations that best approximate the observed values. NA (not available) is shown for populations for which it was not possible to discriminate a statistically robust scenario*

Population	Population	No. of individuals	Best supported scenario	PP (HPD)	N_e	N_4	t_4	N_eμmic
Canarina eminii, continental islands								
1. Gifta and Dembecha	Abyssinian massif	16	NA	–	–	–	–	–
3. Yirga	Harar massif	13	NA	–	–	–	–	–
4. Harenna	Harar massif	9	NA	–	–	–	–	–
5. Aberdare	Aberdare Mountains	8	Decline	0.7511 (0.6710, 0.8312)	5.17E+02	6.33E+E04	2.03E+03	9.73E−02
6. Elgon	Mount Elgon	8	Decline	0.6912 (0.6544, 0.7280)	5.35E+02	5.94E+E04	2.89E+03	1.39E−01
7. Rwenzori	Rwenzori Mountains	10	Decline	0.6986 (0.6631, 0.7342)	1.03E+03	5.84E+E04	3.56E+03	1.92E−01
Canarina canariensis, oceanic islands								
8. Cueva Corcho	Gran Canaria	8	Decline	0.9293 (0.9066, 0.9519)	5.50E+02	6.59E+E04	1.72E+03	9.91E−02
9. Tilos de Moya	Gran Canaria	12	Decline	0.9400 (0.7882, 1.0000)	6.60E+02	6.33E+E04	2.01E+03	1.22E−01
10. El Sao	Gran Canaria	10	Decline	0.3902 (0.3325, 0.4478)	6.83E+02	5.11E+E04	4.51E+03	1.05E−01
11. Andenes de Guayedra	Gran Canaria	12	Decline	0.6930 (0.6497, 0.7363)	5.00E+02	5.77E+E04	2.90E+03	9.07E−02
12. Camino Chamuscadas	Tenerife (Anaga)	10	Decline	0.5972 (0.5646, 0.6298)	1.25E+03	5.06E+E04	4.96E+03	4.01E−01
13. El Bailadero	Tenerife (Anaga)	11	Decline	0.9265 (0.7775, 1.0000)	1.03E+03	6.07E+E04	4.02E+03	2.10E−01
14. Tope del Carnero	Tenerife (Anaga)	7	Decline	0.6000 (0.1706, 1.0000)	1.40E+03	7.48E+E03	3.51E+03	2.78E−01
15. Teno Alto	Tenerife (Teno)	8	Decline	0.9523 (0.9360, 0.9686)	1.06E+03	6.90E+E04	2.87E+03	2.09E−01
16. El Palmar	Tenerife (Teno)	8	Decline	0.9511 (0.9354, 0.9668)	1.34E+03	6.25E+E04	3.46E+03	2.61E−01
17. Barranco del Infierno	Tenerife (Adeje)	11	Decline	0.9940 (0.9880, 1.0000)	9.31E+02	6.89E+E04	1.87E+03	1.73E−01
18. Barranco de Badajoz	Tenerife	5	Decline	0.8499 (0.8096, 0.8902)	7.41E+02	6.65E+E04	3.39E+03	1.56E−01
19. Barranco Ruiz	Tenerife	6	Decline	0, 8775 (0.8423, 0.9127)	8.70E+02	6.18E+E04	2.35E+03	1.45E−01
20. Tamargada	La Gomera	13	NA	–	–	–	–	–
21. Los Tilos	La Palma	10	Decline	0.8024 (0.7579, 0.8469)	9.00E+02	5.07E+E04	2.83E+03	1.64E−01
22. Barranco del Agua	La Palma	10	Decline	0.7253 (0.6768, 0.7738)	7.70E+02	5.71E+E04	2.37E+03	1.43E−01
24. El Hierro	El Hierro	12	Decline	0.7498 (0.6942, 0.8054)	9.18E+02	5.43E+E04	2.44E+03	1.51E−01

N_e, current effective population size; N_4, effective population size at the beginning of the decline; t_4, start time of decline, measured in number of generations; N_eμmic, current effective population size × micros mutation rate.

projections (Fig. 3) suggest that both *C. eminii* and *C. canariensis* underwent a strong and recent population decline.

When did this decline start and what were its causes? Our previous studies showed that the current disjunct distribution of genus *Canarina* across the Sahara Desert can be explained by the climatic aridification during the Late Miocene/Pliocene and the resulting contraction in the distribution ranges of subtropical forests in Africa and Macaronesia (Mairal *et al.*, 2015a, b, 2017a, b; Pokorny *et al.*, 2015). Although this hypothesis may explain the current continental disjunction, the population decline detected here is probably more recent. The DIYABC analysis detected a population decline ~2000–5000 generations ago, which could not be recovered with coalescent-based Bayesian skyline plots (Drummond *et al.*, 2005; Mairal *et al.*, 2015b). This is in agreement with observations that SSR markers detect more recent signals of genetic diversity than chloroplast DNA and AFLP markers (Chakraborty *et al.*, 2014). Since the generation time of *Canarina* is not known precisely due to its tuberous habit (it can sometimes behave as a biennial or even show longer generation times), it is difficult to assign an absolute temporal framework for this decline; also, the time scale here differed slightly depending on the population. However, the temporal range of the decline detected here was consistent within the past 10 000 years (if we consider the plants as biennial) or 50 000 years (if we consider generation times of 10 years), i.e. it probably took place during the last glacial cycle (Pleistocene–Holocene).

This decline coincides with palaeoclimate projections from our ecological niche models, which support a contraction of the distribution ranges of both species from 18 kiloyears ago to the present (Fig. 3). Caution should be exercised in the interpretation of these models due to the low-resolution climate datasets available, which could underestimate potential microrefugia (Gavin *et al.*, 2014). However, though geographic locations may be biased, the models are highly congruent with the extant distribution of the species and furnish evidence of a population decline. Furthermore, a recent study (Chala *et al.*, 2017) supports our models by projecting the descent of vegetation belts during the Last Glacial Maximum.

Interestingly, DIYABC detected a less pronounced population decline in the populations with the highest levels of genetic diversity (Table 1 and N_4 versus N_e in Table 3). These populations are located in areas that served as refugia during climatic oscillations or tectonic and volcanic events, such as Mount Elgon in Africa or the palaeo-islands of Tenerife, thereby supporting their role as reservoirs of genetic diversity against extinction, both during the Pleistocene and in more recent times (Mairal *et al.*, 2015b, 2017b; this study).
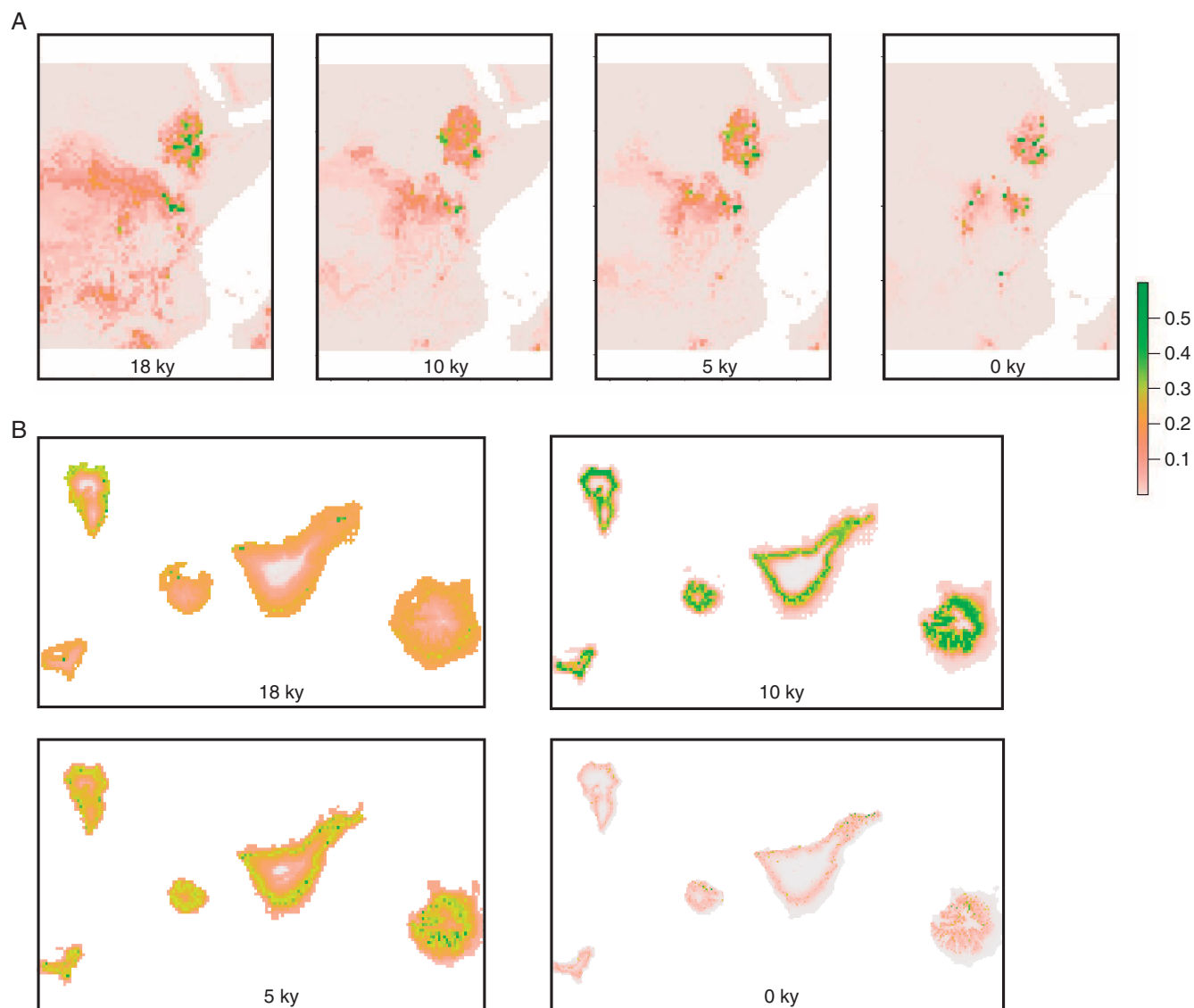
FIG. 3. Geographic projections of the climatic niche model of (A) *Canarina eminii* and (B) *Canarina canariensis* over four time periods: 18, 10, 5 and 0 kiloyears (ky) (modelling sea-level changes). Colour scale indicates climatic suitability values; brown colour indicates low values and dark green indicates high values.

By contrast, a much sharper decline is observed in the most fragmented laurel forest populations in the Canaries, i.e. Barranco de Badajoz and Barranco Ruiz in Tenerife (Table 3). In *C. eminii*, the sky islands located at lower latitudes (Rwenzori Mountains, Mount Elgon, Aberdare Mountains) harbour greater genetic diversity than populations in the massif of Abyssinia, which were probably more strongly affected by glaciations and human activities (e.g. Gifta and Dembecha in Table 1; cf. Mairal *et al.*, 2017b). Loss of ecological interactions in these areas, such as deficiency of pollinators, may also have had a direct impact on genetic diversity in rare species (Lavergne *et al.* 2004).

Conservation biology of Canarina

Our ecological niche models may render an approximation of the effect of climate change on habitat loss and the associated loss of genetic diversity during the last glacial/interglacial cycles. Nowadays, however, human activities constitute an additional threat to the maintenance of genetic diversity in *Canarina*. Afro-Macaronesian forests have been logged in the past centuries for timber, agriculture and human settlements, bringing them to the verge of extinction, especially in topographically less complex areas, which are more easily exploited.

At present, the Afromontane forests have disappeared almost entirely from the Ethiopian highlands (estimated loss of up to of 35 % of originally forested areas) (EFAP, 1994; Kebede *et al.*, 2007). This situation is aggravated by the decrease in soil quality due to changes in water resources (Kloos and Legesse, 2010), which has caused a dramatic decline in these forests, and even their disappearance in large areas (Reusing, 2000; FAO, 2001).

In the Canary Islands, the area of laurel forests has recently declined to 12.5 % of their potential distribution

TABLE 4. *Average values of microsatellite diversity statistics for several species studied in the Canary Islands and Afromontane forests*

Species	A	H_e	Reference
Canary Islands			
Canarina canariensis	2.5	0.367	–
Bencomia exstipulata	3.50	0.440	González-Pérez *et al.*, 2009a
Bencomia caudata	5.13	0.620	González-Pérez *et al.*, 2009a
Myrica rivas-martinezii	6.50	0.560	González-Pérez *et al.*, 2009b
Neochamaelea pulverulenta	7.85	0.425–0.812	Rigueiro *et al.*, 2009
Myrica faya	9.30	0.570	González-Pérez *et al.*, 2009b
Olea europaea subsp. *guanchica*	14.00	0.710	García-Verdugo *et al.*, 2010
Ilex perado subsp. *lopezlilloi*	2.13	0.480	Sosa *et al.*, 2010a
Ilex perado subsp. *platyphylla*	5.37	0.600	Sosa *et al.*, 2010a
Ilex canariensis	2.38	0.420	Sosa *et al.*, 2010a
Sambucus palmensis	6.80	0.499	Sosa *et al.*, 2010b
Silene nocteolens	15.83	0.780	Sosa *et al.*, 2010a
Pinus canariensis	22.80	0.771	López de Heredia *et al.*, 2010
Parolinia ornata	4.38	0.515	González-Pérez and Caujapé-Castells, 2014
Ruta oreojasme	7.62	0.687	Meloni *et al.*, 2015
Phoenix canariensis	4.41	0.476	Saro *et al.*, 2015
Micromeria rivas-martinezii	9.80	0.500	Puppo *et al.*, 2016
Micromeria glomerata	4.27	0.310	Puppo *et al.*, 2016
Afromontane forests			
Canarina eminii	1.93	0.300	–
Coffea arabica	2–3.20	0.050–0.242	Silvestrini *et al.*, 2007
Acacia senegal	6.90	0.667	Omondi *et al.*, 2010
Senegalia mellifera	7.00	0.620	Ruiz Guajardo *et al.*, 2010
Prunus africana	2.0 – 4.00	0.105–0.728	Kadu *et al.*, 2011

A, allelic richness.

(Fernández-Palacios *et al.*, 2011); this includes the laurel forest contractions of Gran Canaria and Tenerife to 1 % and 10 %, respectively, of their original distributions (Santos Guerra, 1990; Del Arco *et al.*, 2010; Fernández-Palacios *et al.*, 2011). The areas of adjacent suitable areas, such as thermophilous woodlands, have also declined considerably (de Nascimento *et al.*, 2015).

One of the biggest threats to rare widespread species is the sensitivity of their populations to demographic stochasticity (Kruckeberg and Rabinowitz, 1985; Barrett *et al.*, 1991). This could be a critical factor for the survival of *Canarina* in African and Macaronesian evergreen forest habitats, given the detected current decline in size of many populations (Table 3). Furthermore, the generally low levels of genetic diversity detected here make these taxa more susceptible to the effects of inbreeding (especially in small populations). Intraspecific genetic variation is the most fundamental level of biodiversity, providing the basis for evolutionary change, and is crucial for maintaining the capacity of species to adapt to new environmental conditions (Frankham *et al.*, 2002; Yannic *et al.*, 2013).

Although *C. canariensis* is locally abundant in some areas (e.g. Anaga, Teno and Tilos de Moya), greater efforts should be made to protect populations that contain exclusive genetic diversity. This is the case for populations in the three palaeo-islands of Tenerife (which also harbour pristine areas of laurisilva) and of Mount Elgon in the case of *C. eminii*. These areas act not only as reservoirs of unique genetic diversity, but also as cradles and sources of new allele migration events to nearby areas (Mairal *et al.*, 2015b, 2017b; Caujapé-Castells *et al.*, 2017). Though the results reported here refer only to two rare species (*C. eminii* and *C. canariensis*), similar patterns are expected (and have been observed) in other rare species, e.g. the Afroalpine plant *Lobelia rhynchopetalum* (Chala *et al.*, 2016).

At present, a large area covered by the endemic laurel forest (laurisilva) across the Canary Archipelago is under legal protection (Fernández-Palacios *et al.*, 2011), but this is not the case for the isolated patches of Afromontane forests in East Africa. Though some areas are preserved as National Parks (e.g. Harenna Forest, Rwenzori Mountains and Mount Elgon), conservation measures are not strictly enforced. Given the political and difficult economic situation of these countries, which makes it even more challenging to extend protection areas to preserve new populations, one crucial action in this current context would be *ex situ* conservation, via seed banks. Priority should be given to the conservation of the different geographically structured areas, covering in each of them those populations exhibiting the highest levels of ancient and endemic phylogenetic diversity. Genetic evidence gathered for *Canarina* (DNA, AFLPs and SSRs) further suggests that certain populations should be established as shrine areas in order to conserve the greater genetic variation *ex situ*: in the case of *C. eminii*, one population at each side of the Rift Valley and the population of Mount Elgon; for *C. canariensis*, one population in each palaeo-island in Tenerife (home to the best-preserved areas of laurisilva in Tenerife). *Ex situ* conservation plans should collect seeds covering these priority areas and avoid gene flow among them in order to favour potential speciation in the future. Thus, while the different areas should be managed separately (avoiding mixed reinforcements, i.e. without introducing propagules from different source areas), management within areas should stress the preservation of several populations, in order to safeguard a significant proportion of genetic variability of the species.

CONCLUSIONS

Genetic conservation studies often focus on rare species with a reduced distribution area ('narrow endemics'; Kruckeberg and Rabinowitz, 1985; Oostermeijer *et al.*, 2003; Fernández-Mazuecos *et al.*, 2014). However, geographically widespread

species can also be considered rare (relict) and subject to the same status of threat, especially if habitat loss has led to isolation of populations and small population size ('large size and narrow distribution'; Kruckeberg and Rabinowitz, 1985). Here we show that this is the case for *C. eminii* and *C. canariensis*, two endemic bellflower species associated with the last remnants of subtropical forest in Africa and Macaronesia. Although both are locally widespread (*C. canariensis* is present in most of the Canarian islands and *C. eminii* occurs over a large part of East Africa), they feature the genetic signals of recent population decline due to habitat loss. The latter seems to be an effect of both historical climate change and rapid, human-induced, forest fragmentation in recent times. Especially for the Afromontane forests, loss of habitat due to increasing aridity and the alarming rates of forecast urban growth make these threatened ecosystems areas of priority interest for the conservation community (Seto *et al.*, 2012).

SUPPLEMENTARY DATA

Supplementary data are available online at https://academic.oup.com/aob and consist of the following. Table S1: primers used for PCR amplification for each locus. Table S2: geographic coordinates used in the ecological niche modelling of *Canarina canariensis*. Table S3: geographic coordinates used in the ecological niche modelling of *Canarina eminii*. Table S4: estimated probability of the likelihood function according to the Evanno method for the STRUCTURE analyses of *C. canariensis*. Table S5: estimated probability of the likelihood function according to the Evanno method for the STRUCTURE analyses of *C. eminii*. Table S6: hierarchical analysis of molecular variance (AMOVA). Table S7: probabilities for each scenario performed with DIYABC. Confidence intervals are shown in square brackets. Figure S1: graphical representation of the four scenarios simulating changes in population size with DIYABC. Supplementary references. Figure S2: Estimated probability of the likelihood function according to the Evanno method.

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