



RESEARCH ARTICLE

Phylogeography at the crossroad: Pleistocene range expansion throughout the Mediterranean and back-colonization from the Canary Islands in the legume *Bituminaria bituminosa*

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Abstract

Aim: Species with widespread distributions offer excellent opportunities for investigating recent biogeographical patterns across broad scales. Here, we tested the hypothesis that, due to its intermediate geographical location, NW Africa is pivotal in explaining the phylogeographical patterns of taxa with Mediterranean–Macaronesian distributions using a legume species with short generation times.

Region: Mediterranean, with a focus on NW Africa and the Canary Islands.

Taxon: Pitch trefoil (*Bituminaria bituminosa*).

Methods: We generated genetic data and performed phylogeographical and demographic analyses at two geographical scales: Mediterranean Basin (MB), using plastid sequences (115 individuals), and Macaronesia, using plastid sequences (182 individuals) and 10 nuclear microsatellite loci (220 individuals). We also performed a literature survey focusing on phylogeographical studies of other circum-Mediterranean taxa.

Results: Northwest Africa was identified as a centre of genetic diversity (19 out of 38 haplotypes) and demographic expansion of *B. bituminosa* in the MB during the Pleistocene. Our literature review revealed two main phylogeographical patterns in widespread species: pre-Mediterranean evergreen sclerophylls versus Pleistocene facultative-deciduous (including *Bituminaria*) taxa, but on average, both functional groups show a similar, large genetic diversity (~40% of haplotypes) in NW Africa. At the Macaronesian scale, we found that Canarian *Bituminaria* is composed of two genetic sublineages that coexist and hybridize on the central islands and in the mainland Macaronesian enclave (Anti-Atlas region). Demographic analyses rejected the progression rule as the model of island colonization but strongly suggested that Anti-Atlas populations are the result of back-colonization from the easternmost islands before the Last Glacial Maximum (LGM).

Conclusions: *Bituminaria* displays a pattern of Quaternary eastward expansion in the MB that appears to be paralleled by several members of its functional plant group. Thus, our study reveals a previously undescribed dual role of NW Africa in plant biogeography, acting both as a source of species expansion to the rest of the MB and a LGM refugium of plant populations with a Macaronesian island origin.

KEYWORDS

Anti-Atlas, Approximate Bayesian Computation (ABC), Canary Islands, functional biogeography, glacial refugia, mainland back-colonization, Mediterranean phylogeography, *Psoralea bituminosa*, tедера

1 | INTRODUCTION

As a hot spot of biodiversity, the Mediterranean region, including the Macaronesian islands in the North Atlantic Ocean, has been the matter of extensive phylogeographical research in recent decades (Médail & Diadema, 2009; Nieto Feliner, 2014; Thompson, 2005). Most of these studies highlight the role of Plio-Pleistocene climatic shifts in explaining biodiversity patterns within the Mediterranean Basin (MB, hereafter; reviewed in Nieto Feliner, 2014). Thus, it is widely accepted that both the onset of the Mediterranean climate circa 3.5 Ma (Suc, 1984) and the glacial periods throughout the Pleistocene are two major events that shaped the taxonomic composition and genetic structure of extant Mediterranean lineages (Petit et al., 2005; Postigo Mijarra et al., 2009; Rodríguez-Sánchez et al., 2009). Comparative analyses across taxa have allowed biogeographers to pinpoint refuge areas where the negative impact of Quaternary climatic shifts on biodiversity may have been attenuated (Médail & Diadema, 2009). For instance, recent phylogeographical studies have stressed that NW Africa represents a key reservoir of genetic variation in the region (Bobo-Pinilla et al., 2018; García-Aloy et al., 2017; Husemann et al., 2013). However, common phylogeographical patterns across taxa are still difficult to describe in the MB, probably due to the occurrence of manifold evolutionary processes across space and time (Conord et al., 2012; Nieto Feliner, 2014).

The mosaic of genetic patterns described for Mediterranean taxa may be, at least partially, explained by the fact that Pleistocene biogeography has focused on disparate areas within the MB (e.g., see references in Vargas et al., 2018). Although each of these studies helps us understand regional processes and sometimes the underlying biogeographical links with the Macaronesian region (MAC, hereafter; e.g., García-Aloy et al., 2017; García-Verdugo et al., 2013), they hardly reflect global patterns in MB phylogeography. In this sense, species with widespread Macaronesian–Mediterranean distributions allow us to identify refuge areas and infer colonization routes in a broad spatial, and narrow temporal, context (Désamoré et al., 2011; Migliore et al., 2012; Rodríguez-Sánchez et al., 2009). Unlike taxonomically diversified lineages or species-poor lineages with narrow distributions (Sękiewicz et al., 2018; Vargas et al., 2018), widespread Mediterranean species probably attained their extant distributions quite recently, thus providing valuable insights into the processes shaping the recent (Quaternary) history of this hot spot area (Coello et al., 2020; Mousavi-Derazmahalleh et al., 2018; Rodríguez-Sánchez et al., 2009).

In addition to contrasting geographical ranges, the complex evolutionary origins of the extant MB flora (Herrera, 1992; Verdú & Pausas, 2013) impose limitations for drawing global assessments. Hence, sclerophyllous taxa, albeit conspicuous in most plant

communities, are thought to have spread across the MB under climatic conditions markedly different to those found today (i.e., colonization before the onset of the Mediterranean climate; Herrera, 1992). Because they represent iconic landscape components, this group of species has attracted a good deal of phylogeographical research (Petit et al., 2005; Rodríguez-Sánchez et al., 2009; Viruel et al., 2020; Vitelli et al., 2017). Mediterranean plant communities are, however, functionally heterogeneous; for instance, non-sclerophyllous, shrub species may be as common as pre-Mediterranean tree taxa (Margaris, 1981). Despite this fact, shrubs or short-lived perennials with widespread, circum-Mediterranean distributions are comparatively underrepresented in the phylogeographical literature (but see Coello et al., 2020; Mateu-Andrés et al., 2013). Our understanding of biogeographical patterns in the Mediterranean region would thus benefit from a more balanced representation of each functional plant group. Species with shorter generation times may indeed show stronger phylogeographical signals under scenarios of Quaternary expansion than those of long-lived species, which may improve our ability for interpretation (Désamoré et al., 2011; Rodríguez-Sánchez et al., 2009).

In the present study, we investigated the phylogeographical pattern of a short-lived species with a nearly continuous Macaronesian–Mediterranean distribution: the legume *Bituminaria bituminosa* (L.) Stirton. Laying at the crossroad between MAC and the MB, taxonomists have identified NW Africa as an area home to remarkable levels of morphological variation (Bogdanović et al., 2020; Maire, 1938), but unfortunately, genetic data in this plant lineage have never been analyzed at a broad geographical scale yet. Thus, the widespread distribution of *Bituminaria* offers the opportunity to provide insights into spatiotemporal patterns of genetic differentiation across the whole Mediterranean region. Such information is particularly needed for Macaronesian *Bituminaria* populations: Although a few, rare botanical varieties appear to occur in the Canarian archipelago (Méndez et al., 1990), many other populations (including those on the island of Madeira) tend to grow profusely in disturbed areas, which has led some investigators to cast doubt on the native status of the island populations (e.g., Acebes et al., 2010; Kunkel, 1976). Preliminary molecular studies, however, support the idea that the Canarian taxonomic varieties are genetically differentiated from mainland Mediterranean accessions (Pazos-Navarro et al., 2011), although precise information related to biogeographical patterns has not been produced thus far. Considering the widespread occurrence but suspected recent origin on the islands, Macaronesian populations of *Bituminaria* also offer an excellent arena for testing classic and emerging hypotheses on island biogeography. Assuming the simplest scenario, we could expect *Bituminaria* populations to follow the typical ‘progression rule’, that is, sequential dispersal from



older to younger islands (for a review see Shaw & Gillespie, 2016). Contrarily, if colonization has been affected by Pleistocene climatic shifts, multiple dispersal events from NW Africa, genetic admixture and/or back-colonization of mainland areas could conform the biogeographical pattern (Caujapé-Castells et al., 2017; Curto et al., 2017; García-Verdugo, Caujapé-Castells, Illera, et al., 2019).

Using a combination of genetic markers and a hierarchical sampling across the distribution of this widespread lineage, we tested three hypotheses related to its phylogeographical pattern. Based on the high levels of morphological variation described among NW African populations of *Bituminaria*, we hypothesized that this area would represent a centre of molecular diversity for this lineage within the Mediterranean region. Second, considering that *Bituminaria* displays functional traits that are markedly different from those of Mediterranean sclerophylls, we would expect a contrasting phylogeographical pattern between functional groups across the MB. Lastly, we predicted, based on the most commonly reported pattern of island colonization, that Macaronesian *Bituminaria* populations followed the progression rule, with the neighbouring mainland acting as the source of a sequential colonization process from older (easternmost) to younger (westernmost) islands. By focusing on a type of taxon seldom assessed in Mediterranean plant biogeography (i.e., non-sclerophyllous, widespread distribution and short-generation times), we aimed at contributing to balance our knowledge on the evolutionary history of the flora of this region, with a particular focus on NW Africa and the North Atlantic islands.

2 | MATERIALS AND METHODS

2.1 | Study species and population sampling

Bituminaria bituminosa s.l. is a diploid, predominantly selfing, suffruticose species with a relatively short generation time (Martínez-Fernández, 2012; Méndez et al., 1990; Pradhan et al., 2014). It typically occurs in xeric habitats, displaying several adaptations to Mediterranean drought conditions mediated by leaf physiological adjustments and facultative deciduousness (i.e., loss of aerial biomass) under prolonged water stress (Martínez-Fernández, 2012; Pang et al., 2011). *Bituminaria* populations are common on the Atlantic islands (Canary Islands and Madeira) and follow an approximately circum-Mediterranean distribution in the mainland region (Figure S1). In recent years, taxonomic studies have described several morphospecies circumscribed to very restricted geographical areas (Bogdanović et al., 2020, and references herein). However, genetic information on *B. bituminosa* is largely missing, with the exception of a few preliminary studies reporting high levels of geographically structured genetic variation among groups of Canarian and Mediterranean accessions (Juan et al., 2005; Pazos-Navarro et al., 2011).

To address the goals of the present study, we sampled *B. bituminosa* individuals at two geographical levels: the MB sensu lato (i.e., including the Canary Islands and Madeira) and, more specifically,

MAC sensu Sunding (1979; i.e., including the mainland enclave of SW Morocco). Our study was centred on the latter geographical area because recent molecular studies are showing that it is key to understand Plio-Pleistocene patterns of biodiversity in Mediterranean lineages (Durán et al., 2020; García-Aloy et al., 2017; García-Verdugo et al., 2017).

In order to obtain a representative sampling of the MB distribution, we defined six areas where *B. bituminosa* occurs, but a special emphasis was given to NW Africa, with two areas of interest considered (Anti-Atlas: ANT and Maghreb: MAG), in addition to the Iberian Peninsula (IBP), Central Mediterranean (CEN), northeast Mediterranean (NEM) and Levant (LEV; Figure S1). If phylogeographical patterns across the whole MB exist in *Bituminaria* (e.g., east vs. west or central vs. peripheral divides), we expected that a stratified sampling approach throughout the range distribution would uncover such patterns (Conord et al., 2012; De Castro et al., 2020). Thus, leaves from 18–20 individuals from each of these areas were obtained from herbarium samples or collected during field expeditions, with the exception of NEM ($N = 6$), for which attempts to obtain a larger sample were unsuccessful due to limited availability in the consulted herbaria. To provide a framework for the subsequent level of analysis, one to three samples from each Macaronesian island where *B. bituminosa* occurs were also considered. The total sample comprised 115 individuals sampled across 15 countries (see Table S1 for details on locations and voucher information).

In addition, sampling of MAC comprised 33 *B. bituminosa* populations from the Macaronesian mainland enclave (western Anti-Atlas region) and all of the main Canarian islands (typically, $N = 6$ individuals per population for plastid data; $N_{total} = 182$). Madeiran populations were not considered at this level, but our preliminary results suggested that *Bituminaria* on this island may have an introduced (or very recent) origin (see Section 3). The number of sampled populations was roughly proportional to the abundance of the species on each island/mainland area. For nuclear microsatellite analysis, one to three populations on each island/mainland area where sampled ($N = 5$ –20 individuals per population, $N_{total} = 220$), except for the island of El Hierro. For both plastid and nuclear microsatellite analyses, special care was taken to represent all of the morphological varieties described in the region (Brullo et al., 2017; Méndez et al., 1990). Voucher specimens for representative populations were deposited at the JBAG herbarium (accessions JBAG8306–JBAG8337).

2.2 | Generation of molecular data

Genomic DNA from silica-dried leaves or herbarium specimens was extracted using the DNeasy Plant Mini Kit (Qiagen, Inc.) following the manufacturer's protocol. Then, we conducted a preliminary trial using 16 samples representing the entire distribution area of *B. bituminosa*. With this subset of samples, we tested levels of polymorphism in six plastid regions using the following primers reported in Shaw et al. (2007): *psbD-trnT*, *psbE-petL*, *psbJ-petA*, *trnQ-rps16*, *trnS^(GCU)-trnG^(UCC)* and *trnV-ndhC*.

The two regions that showed the highest levels of polymorphism (i.e., *trnS*^(GCU)-*trnG*^(UCC) and *psbJ*-*petA*, totaling 2255 bp of pDNA) were sequenced for the total sample of the MB level of analysis ($N = 115$ individuals).

Next, we aimed to obtain genetic information at a deeper evolutionary scale. We therefore generated data from both nuclear and plastid markers for the samples collected at the MAC level. Thus, 182 individuals were sequenced using the previous two plastid regions and an extra region that was shown to be among the most polymorphic in our previous primer test (i.e., *psbD*-*trnT*; total length of plastid data after concatenation of the three fragments = 2547 bp). For nuclear microsatellite analysis, we initially screened a set of 14 loci selected from the set of markers characterized in Pazos-Navarro et al. (2011): Bbit-SSR004, Bbit-SSR005, Bbit-SSR010, Bbit-SSR012, Bbit-SSR013, Bbit-SSR015, Bbit-SSR034, Bbit-SSR040, Bbit-SSR055, Bbit-SSR059, Bbit-SSR060, Bbit-SSR066, Bbit-SSR070 and Bbit-SSR090. Sixteen *Bituminaria* samples from both mainland and island populations were amplified for these 14 loci following the conditions specified in Pazos-Navarro et al. (2011), with slight modifications. The PCR products were separated on a 5% polyacrylamide gel using an ABI 3130XL automated sequencer, and fragment sizes were scored using GENEMAPPER 4.0 (Applied Biosystems, Inc.). Because three loci showed low levels of allelic variation across samples in our trial (i.e., Bbit-SSR034, Bbit-SSR059 and Bbit-SSR060) and another locus (*Bbit*-SSR066) showed some ambiguities when performing allele scoring, the remaining 10 loci were used to amplify the complete sample ($N = 13$ populations and $N = 220$ individuals).

2.3 | Phylogeographical analyses in the Mediterranean region

One matrix with consensus sequences obtained from forward and reverse sequences of each individual and plastid region (i.e., *trnS*^(GCU)-*trnG*^(UCC) and *psbJ*-*petA*) were edited, aligned and trimmed with MEGA X (Kumar et al., 2018). Then, the two matrices were concatenated and haplotypes were defined based on nucleotide substitutions only (i.e., indels excluded). Nucleotide diversity (π) was determined with DNASP 5.10 (Librado & Rozas, 2009) grouping samples by geographical regions as previously defined. Our comparisons were restricted to six of these regions, because NEM was excluded due to its small sample size. In addition, to provide genetic diversity estimates for each region while controlling for differences in sample sizes, we calculated haplotype diversity (H_d) with CONTRIB 1.4 (Petit et al., 1998) after rarefaction to a size of 16 (i.e., the sample size of CAN, which was the smallest region included in our comparison). A statistical parsimony network using all the haplotypes found in the complete plastid dataset was computed with TCS (Clement et al., 2000).

Tests for past population expansion in each region were conducted using Fu's F_s (Fu, 1997) as implemented in DNASP 5.10. This statistic was chosen because it outperforms other expansion tests when using data from nonrecombining genomic regions (Ramírez-Soriano et al., 2008). Mismatch frequency graphs were also plotted

in DNASP 5.10 with the aim of exploring whether the defined regions displayed some evidence of spatial range expansion or a stationary population history (Rogers & Harpending, 1992).

Because we were interested in placing the haplotype diversification of *Bituminaria* within an explicit temporal framework, we additionally implemented a secondary calibration approach following previous studies (García-Verdugo et al., 2017, and references herein). Briefly, phylogenetic relationships among *Bituminaria* samples and outgroups of interest (e.g., *Orbexilum* spp.; Bello, 2015; Table S2) were inferred using the Bayesian approach implemented in MRBAYES (Ronquist et al., 2012). Then, the Bayesian relaxed-clock models implemented in BEAST 1.7 (Drummond & Rambaut, 2007) were used in a nested analysis, considering the phylogenetic relationships of Psoraleeae (Figure S2) and the secondary age constraints obtained from the most comprehensive analysis of the tribe Psoraleeae (Bello, 2015; see Text S1 for further details).

2.4 | Literature review

In order to examine potential associations between phylogeographical patterns of widespread Mediterranean species and plant functional groups, we searched for different combinations of the following key words in the Web of Science database (Clarivate Analytics): 'chloroplast', 'cpSSR', 'cpDNA', 'pDNA', 'plastid', 'plant', 'Medit*', 'phylogeogr*', 'haplot*', 'network', 'Pliocene', 'Pleistocene', 'Macaronesia', 'tree' and 'shrub'. Only studies considering species with circum-Mediterranean distributions and a comprehensive sampling of the species distribution were selected (see Table S3). For each selected case, we compiled information on five variables: (1) plant traits (growth form, leaf size and leaf habit) as recorded in the BROT2 database (Tavşanoğlu & Pausas, 2018), (2) putative colonization time of the MB (extracted from stem-based molecular estimates in the studies or inferred from the fossil record; e.g., Herrera, 1992), (3) estimated intraspecific divergence times (extracted from crown ages of the analyses performed in each study), (4) number of haplotypes found at both sides of the MB: west (IBP + NW Africa) versus east (Levant, including Turkey and Cyprus) and (5) number and inferred time of dispersal events to MAC, in cases where plant species were sampled in that region.

2.5 | Phylogeographical and population-level analyses in Macaronesia

Similar to the analysis at the MB level, forward and reverse sequences of each of the three plastid fragments amplified (i.e., *trnS*^(GCU)-*trnG*^(UCC), *psbJ*-*petA* and *psbD*-*trnT*) were used to create a consensus sequence for each individual, and subsequently aligned and trimmed using MEGA X (Kumar et al., 2018). The three matrices were concatenated, and levels of nucleotide diversity (π) and haplotype diversity (H_d) were calculated with DNASP 5.10 (Librado & Rozas, 2009). A parsimony-based haplotype network considering the polymorphic sites found among Canarian samples was constructed with TCS (Clement et al., 2000).



Prior to running analyses with nuclear microsatellite markers, we tested the quality of our 10 loci dataset. An exact test of linkage disequilibrium implemented in ARLEQUIN 3.5 (Excoffier & Lischer, 2010) was used to examine significant association between pairs of loci using 10,000 permutations. After sequential Bonferroni correction for multiple p tests, two loci (Bbit-SSR040 and Bbit-SSR070) displayed a significant association ($p < 0.0001$) in 12 out of 13 populations. Locus Bbit-SSR070 was discarded from all subsequent analysis because the result of our linkage disequilibrium test suggested that the two sets of primers reported in Pazos-Navarro et al. (2011) for Bbit-SSR040 and Bbit-SSR070 amplify the same locus. Furthermore, multilocus inbreeding coefficients and presence of null alleles were jointly examined with the Bayesian approach implemented in INEST 2.2 (Chybicki & Burczyk, 2009). Two models were compared: the full model ('nfb') considering null alleles, inbreeding and genotypic failures, and the random mating model ('nb'; null alleles and genotyping failures included under an assumption of $F_{IS} = 0$). The model displaying the lowest deviance information criterion was interpreted as the most likely (i.e., 'nfb'; Table S4). Analyses for each population were run with 300,000 Markov chain Monte Carlo (MCMC) cycles, with every 100th cycle sampled and a burn-in set to 30,000 cycles.

To assess the genetic structure of *Bituminaria* populations at this level of analysis, we applied the Bayesian clustering method implemented in STRUCTURE 2.3 (Falush et al., 2007; Pritchard et al., 2000) using the nuclear microsatellite dataset. We selected an admixture model with correlated allele frequencies among groups. We ran 500,000 MCMC iterations after a burn-in of 100,000 iterations for K values of 1–15, with 15 iterations for each value of K . The most probable value of K was determined using the ΔK method of Evanno et al. (2005) as implemented in STRUCTURE Harvester (Earl, 2012). Because *Bituminaria* in the Canary Islands was expected to be composed of different genetic lineages, at least roughly related to the described botanical varieties (Juan et al., 2005; Méndez et al., 1990), we examined potential introgression between genetically differentiated *Bituminaria* populations using two approaches. First, we calculated an index of admixture (HA) using a standardized version of the Shannon–Weaver diversity index for each individual from the output of our previous STRUCTURE results (see Keller & Taylor, 2010 for details). Second, we generated a matrix of pairwise genetic distances among populations using DNASP 5.10 (plastid DNA data) and ARLEQUIN 3.5 (nuclear microsatellite data) and assessed genetic relationships with Neighbor-Net for each type of marker using SPLITS TREE 4.16 (Huson & Bryant, 2006).

2.6 | Demographic analysis in Macaronesia

To investigate the demographic history of *Bituminaria* in this region and infer the most likely scenario of population divergence across the islands, we conducted Approximate Bayesian Computation (ABC) analyses using the microsatellite dataset. We generally followed the conditions adopted in previous studies (Setsuko et al., 2020, and references herein). First, patterns of selection on microsatellite loci were assessed with BAYESCAN 2.1 (Foll & Gaggiotti, 2008) to detect

any potential outlier loci affecting demographic inference. Locus Bbit-SSR090 was shown to deviate from neutral conditions (q value < 0.001 for both priors = 10 and 100). Although its effect on global differentiation was not severe (F_{ST} locus Bbit-SSR090 = 0.22 vs. F_{ST} across all loci = 0.43), it was excluded from this analysis. Then, we defined four genetically homogeneous and spatially contiguous island/mainland groups of populations (G1–G4) following STRUCTURE results, and these groups were used for testing six candidate population divergence models that encompass the most frequent scenarios of island-mainland differentiation described in this region (García-Verdugo, Caujapé-Castells, Illera, et al., 2019; Figure 1). We assumed that the pattern of sublineage hybridization detected by previous analyses conducted at the MAC scale (i.e., STRUCTURE and reticulation in the Neighbor-Net, see Section 3) was mainly originated by past genetic admixture rather than by historic events of migration (ABC Models 1–4; Figure 1). Our assumption was based on the apparent lack of specific adaptations for long-distance seed dispersal in *Bituminaria* (Martínez-Fernández, 2012; Méndez et al., 1990) and the strong spatial isolation of the four selected groups. In keeping with such an assumption, genetic differentiation among groups was markedly high ($F_{ST} = 0.34–0.46$; Figure S8). However, because population splitting can also generate admixture-like patterns (Sousa et al., 2012; Tsuda et al., 2015), we also tested two models considering lineage split without admixture (ABC Models 5–6; Figure 1). Model 1 assumed a continental origin followed by a strict progression rule across the islands, that is, from the (oldest) easternmost to the (youngest) westernmost Canarian islands (see Shaw & Gillespie, 2016). Models 2 and 3 implied a relatively recent colonization of G3 and G2 following independent colonization events from the continent. Thus, Model 2 assumed that G1 and G2 were the oldest sublineages, G3 diverged from G1 at T_2 and then G4 was formed by genetic admixture between G2 and G3 at T_1 . Model 3 assumed that G1 and G3 were the oldest sublineages, G2 diverged from G1 at T_2 , and then G4 was formed by genetic admixture between G2 and G3 at T_1 . Model 4 assumed that mainland populations are the result of back-colonization from the islands: G2 and G3 were old sublineages, and both G1 and G4 were formed by admixture between G2 and G3 at T_1 . Lastly, Models 5 and 6 assumed that the central islands were colonized from the western islands and the continent, respectively. Model 5 assumed that G1 and G4 were the oldest lineages and both G2 and G3 diverged from G4 at T_1 . Model 6 assumed that G1 and G4 were the oldest sublineages and both G2 and G3 diverged from G1 at T_1 .

Simulations were conducted with R 4.0.3 (R Core Team, 2020) and FASTSIMCOAL2 2.6.0.3 (Excoffier & Foll, 2011). Summary statistics were calculated with ARLSUMSTAT 3.5.2 (Excoffier & Lischer, 2010). Models were compared under an ABC random forest (ABC-RF) scheme implemented in the ABCRF package 1.8.1 (Pudlo et al., 2016). Posterior distributions for parameters of the best model were estimated with neural network regression implemented in the ABC package 2.1 (Blum & François, 2010; Csilléry et al., 2012). Additionally, posterior predictive check was conducted to confirm goodness of fit of the model to the observed data (Gelman et al., 2014; for further details on the ABC analysis, see Tables S5, S6 and Text S2). Generation time for *B. bituminosa* (2–5 years) was extracted from the BROT2 database (Tavşanoğlu & Pausas, 2018).

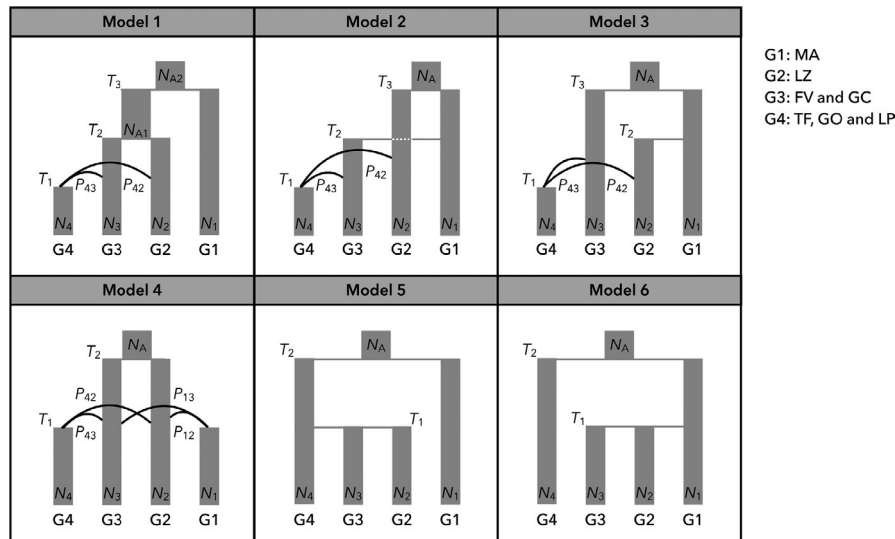


FIGURE 1 Divergence models tested for demographic inferences on Macaronesian *Bituminaria* populations using the nuclear microsatellite dataset. Genetically homogeneous and spatially contiguous island/mainland groups of populations (G1–G4) were defined following STRUCTURE results (i.e., FV = Fuerteventura; GC = Gran Canaria; GO = La Gomera; LP = La Palma; LZ = Lanzarote; MA = mainland; TF = Tenerife; see Figure 3). Current effective population sizes of each group (N_1 – N_4) and ancestral effective population sizes (N_{A1} , N_{A2} and N_A) were inferred for each scenario considering different divergence times (T_1 , T_2 and T_3) and admixture rates (P_{ij})

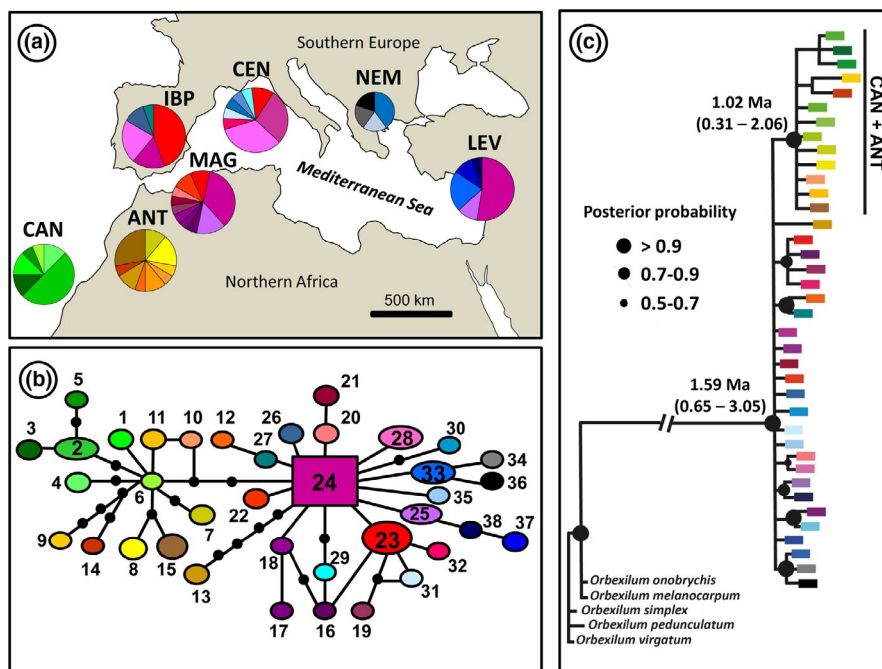


FIGURE 2 Distribution of the 38 haplotypes found across the whole distribution of *Bituminaria bituminosa* using two plastid markers (a), statistical parsimony network of haplotypes (b) and Bayesian phylogenetic reconstruction (with divergences times indicated in nodes of interest) of haplotypes (c). Each haplotype is represented by a specific colour and number. Size of pie charts (a) and haplotypes (b) are proportional to the number of individuals sampled or displaying that haplotype, respectively. Geographical areas are coded as follows: ANT = Anti-Atlas; CAN = Canary Islands; CEN = Central Mediterranean; IBP = Iberian Peninsula; LEV = Levant; MAG = Maghreb; NEM = Northeast Mediterranean

3 | RESULTS

3.1 | MB level: Patterns of haplotype distribution and diversification

Out of the 38 haplotypes obtained from the two plastid markers in *B. bituminosa*, NW Africa (ANT + MAG) harboured a significant number (19), of which 16 were private (Figure 2a). Remarkably, all seven haplotypes found in the Anti-Atlas region (ANT) were private. Our haplotype network suggested a star-like diversification of *B. bituminosa*, with a central, widespread variant (haplotype 24) found

on all areas with large sample sizes except ANT and MAC (Figure 2b). Dating analyses indicated that such a diversification (i.e., crown node of *B. bituminosa*) occurred during the Pleistocene (mean = 1.62 Ma, highest posterior densities [HPD] = 0.65–3.12, PP = 1), while further diversification occurred in Macaronesia (ANT + CAN) within the last Ma (mean = 1.02, HPD = 0.31–2.06, PP = 1; Figure 1c). Considering our two estimates (H_d and π), levels of genetic diversity across regions were particularly large in NW Africa (ANT and MAG), followed by CAN (Table 1). Neutrality tests showed certain evidence of lack of equilibrium in all regions (i.e., negative F_s values), but only results for MAG were clearly significant ($p < 0.001$; Table 1). The approximate

TABLE 1 Sample size (N), number of private haplotypes (h_p), haplotype diversity (H_d) and Fu's F_s statistic (with statistical significance) for the regions defined in the analysis of *Bituminaria bituminosa* throughout the Mediterranean basin

Region	N	h_p	$\pi (\times 10^3)$	H_d (SE) ^a	F_s (p value)
Canary Islands (CAN)	16	6	0.96	0.742 (0.104)	-0.558 (0.191)
Anti-Atlas (ANT)	18	9	1.88	0.895 (0.048)	-1.058 (0.137)
Maghreb (MAG)	20	7	0.85	0.863 (0.063)	-7.186 (0.001)
Iberian Peninsula (IBP)	18	2	0.56	0.752 (0.075)	-0.776 (0.182)
Central Med. (CEN)	18	4	0.71	0.801 (0.040)	-3.475 (0.023)
Levant (LEV)	20	2	0.42	0.668 (0.097)	-1.419 (0.128)

^aAfter rarefaction ($S = 16$).

unimodal distribution observed in mismatch distributions suggested a past signature of expansion in MAG (Figure S3).

Additionally, the haplotype detected in the single population sampled on Madeira (haplotype 23) was common in mainland regions (IBP and MAG; Table S1). Extended sequence information (i.e., sequences from the eight plastid regions used in the polymorphism test) revealed that this Madeiran haplotype was shared with one mainland Portuguese population (results not shown).

3.2 | MB level: Literature survey

After filtering records on animal taxa or plant studies without a clear phylogeographical scope, our bibliographical search retrieved 146 studies (Table S3), of which 15 cases met all the criteria chosen for a comparative framework. Our compilation showed that most species that colonized the MB before the establishment of the Mediterranean climate (i.e., Pliocene and before), are long-lived, mostly phanerophyte species with sclerophyllous, large leaves (Table 2). In contrast, species with a putative origin postdating the onset of the Mediterranean climate (i.e., Plio-Pleistocene), are typically shrubs displaying facultative deciduousness and small leaves (Table 2). Both functional groups often displayed contrasting patterns of haplotype variation at both sides of the MB: Eight of nine sclerophyllous species displayed a similar number of haplotypes, whereas five of six studies in facultative deciduous species documented a higher number of haplotypes in the west as compared to the number detected in the eastern Mediterranean area (Table 2).

Our literature review revealed that both functional groups also showed some phylogeographical similarities: (1) all Mediterranean widespread species experienced molecular diversification in the Pleistocene, (2) NW Africa harbours nearly 40% of the haplotypes found across their distribution (33% for pre-Mediterranean and 48% for Pleistocene taxa) and (3) in those species with MB-MAC distributions, time of dispersal to MAC has been always inferred within the Pleistocene, which has typically occurred through multiple, independent events in a given species (Table 2).

3.3 | Macaronesian level: Population-level analyses

Plastid sequence data revealed that Canarian *Bituminaria* populations are composed of two sublineages (CAN1 and CAN2; Figure 3b), both

showing a widespread distribution in the archipelago (Figure 3a). Although some populations of the described botanical varieties 'albomarginata' and 'crassiuscula' were represented by the same plastid sublineage (i.e., CAN1), most populations assigned to 'albomarginata' were represented by CAN2 or populations showing genetic admixture between CAN1 and CAN2; Figure 3a,b; Table S7). In keeping with the plastid data, STRUCTURE analysis with nuclear microsatellite data indicated that the most likely number of genetic clusters in the archipelago was $K = 2$ ($\Delta K = 209.71$; Figure S4). Remarkably, mainland Anti-Atlas populations did not form a separate cluster from the islands, but instead, their individuals showed a pattern of genetic admixture between the two Canarian groups (Figure 3c; Table S7). Levels of admixture were also particularly high ($HA > 0.25$) on the central islands of Tenerife and La Gomera (e.g., Pops 19 and 26; Figure 3c; Table S7). Neighbor-Net analyses reflected a pattern congruent with these latter results, with plastid data supporting reticulation between populations from CAN1 and CAN2 sublineages on the central islands (e.g., Pops 24 and 25; Figure S5a). The Neighbor-Net based on nuclear microsatellite data revealed close genetic relationships between one CAN2 population from Fuerteventura (Pop 9) and CAN1 populations, in addition to patterns of reticulation in mainland (Pops 1 and 2), La Gomera (Pop 27), Tenerife (Pop 24) and La Palma (Pop 29; Figure S5b).

The two populations sampled on the island of Lanzarote displayed the highest levels of haplotype and nucleotide diversity (Pops 3 and 4), followed by island populations displaying genetic admixture between CAN1 and CAN2 sublineages (i.e., Pops 23 and 26; Table S7). Overall, levels of within-population genetic diversity for estimates obtained with either plastid or nuclear markers correlated positively with levels of genetic admixture (e.g., Pop 24 and Pop 26 on Tenerife and La Gomera, respectively), and negatively with inbreeding values (e.g., Fuerteventura, Pops 6 and 9; Table S7).

3.4 | Macaronesian level: Demographic inference

Considering the proportion of votes and posterior probabilities, Model 4 was selected by the ABC-RF approach as the best (Table 3). However, Models 2 and 3 also showed relatively high proportions of votes (0.221 and 0.239, respectively; Table 3). Thus, we conducted paired model comparisons between these two models and Model 4.

TABLE 2 Summary information extracted from a literature review on phylogeographical studies analyzing widespread (circum-Mediterranean) plant taxa

Taxon	Traits	MB colonization/split	Hap pattern	Hap NW Africa	Dispersal to MAC	Reference/s
<i>Bituminaria bituminosa</i>	SH-FD-SL	Pleistocene/Pleistocene	W >> E	19/32	Pleistocene (1?)	This study, a
<i>Ceratonia siliqua</i>	PH-EV-LL	Miocene/Pleistocene	E ≈ W	3/6	n.a.	b
<i>Cistus monspeliensis</i>	SH-FD-SL	Pleistocene/Pleistocene	W >> E	1/4	Pleistocene (1)	c
<i>Cistus salviifolius</i>	SH-FD-SL	Pleistocene/Pleistocene	W >> E	3/7	n.a.	d
<i>Erica arborea</i>	SH-EV-SL	Plio-Pleistocene/Pleistocene	W >> E	1/8	Pleistocene (2)	e
<i>Laurus nobilis</i>	PH-EV-LL	Miocene/n.a.	E ≈ W	1/4	Pleistocene (2)	f, g
<i>Myrtus communis</i>	PH-EV-LL	Miocene/Pleistocene	W >> E	6/9	Pleistocene (2)	h
<i>Olea europaea</i>	PH-EV-LL	Pliocene/Pleistocene	E ≈ W	19/47	Pleistocene (3)	i, j
<i>Nerium oleander</i>	PH-EV-LL	Miocene/Pleistocene	E ≈ W	1/3	n.a.	k
<i>Periploca laevigata</i>	SH-FD-SL	Plio-Pleistocene/Pleistocene	W >> E	5/8	Pleistocene (3)	l
<i>Pinus pinea</i>	PH-EV-SL	Miocene/Pleistocene	E ≈ W	1/4	n.a.	m, n
<i>Quercus coccifera</i>	PH-EV-LL	Miocene/Pleistocene	E ≈ W	4/42	n.a.	o
<i>Rosmarinus officinalis</i>	SH-FD-SL	Plio-Pleistocene/Pleistocene	W >> E	4/10	n.a.	p
<i>Smilax aspera</i>	PH-EV-LL	Pliocene/Plio-Pleistocene	E ≈ W	4/13	Pleistocene (1)	q
<i>Vitis vinifera</i>	PH-FD-LL	Miocene/n.a.	E ≈ W	3/5	n.a.	r

Note: Data include inferred times for Mediterranean basin (MB) colonization (based on stem ages/fossil information) and diversification (crown ages), number of haplotypes in the western (W) versus eastern (E) Mediterranean basin and number of haplotypes found in NW Africa and estimated time (number of inferred events in parentheses) of dispersal to Macaronesia (MAC). Traits refer to the following character states: SH = shrub versus PH = tree/woody climber; FD = deciduous, semi-deciduous or facultative deciduous versus EV = evergreen sclerophylls; LL = large-leaved (typically >5 cm long or >5 cm² in size) versus SL = small leaved. (a) Bello (2015), (b) Viruel et al. (2020), (c) Coello et al. (2020), (d) Fernández-Mazuecos and Vargas (2010), (e) Désamoré et al. (2011), (f) Rodríguez-Sánchez et al. (2009), (g) Kondraskov et al. (2015), (h) Migliore et al. (2012), (i) Besnard et al. (2013), (j) García-Verdugo et al. (unpublished data), (k) Mateu-Andrés et al. (2015); (l) García-Verdugo et al. (2015, 2020), (m) Vendramin et al. (2008), (n) Saladin et al. (2017), (o) Vitelli et al. (2017), (p) Mateu-Andrés et al. (2013), (q) Chen et al. (2014) and (r) Grassi et al. (2006).

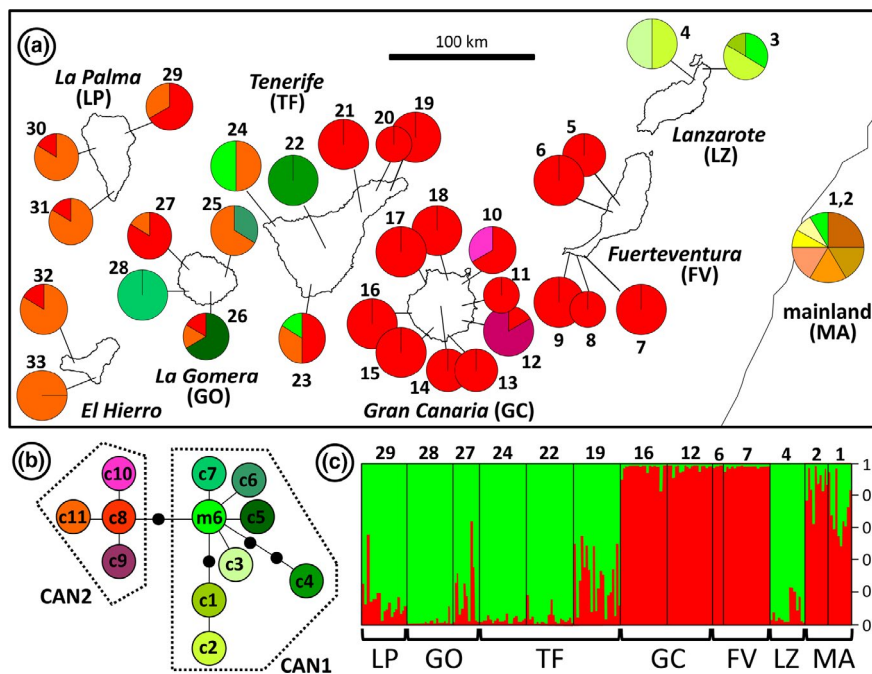


FIGURE 3 Distribution of haplotypes found in the Canary Islands and Anti-Atlas mainland populations (a), statistical parsimony network of Canarian haplotypes (b) and results of the STRUCTURE admixture analysis for $K = 2$ based on nuclear microsatellite data (c). Each population is denoted by a different number (a,c) and haplotypes by a specific code and colour (a,b). CAN1 and CAN2 represent two Canarian sublineages defined in the haplotype network (b), and the Y axis (c) represents the percentage of admixture of each individual sampled for microsatellite analysis

In both paired comparisons, Model 4 was again selected as the best with high posterior probabilities (>0.8; Table 3). For this model, all parameters except P_{13} showed a single peak (Figure S6) and most of the summary statistics predicted by posterior distributions (32 out of 34) fell near the observed values (Figure S7), which indicated

that the goodness of fit of the best model to the observed data was high. Posterior modes with 95% HPDs of current effective population sizes for G1, G2, G3 and G4 were 1892 (167–11,442), 4077 (1162–16,786), 3803 (894–16,621) and 15,692 (7750–24,510), respectively (Table S8). Posterior mode (95% HPD) of ancestral



TABLE 3 Comparison of six models of genetic divergence among Macaronesian *Bituminaria* groups following an Approximate Bayesian Computation-Random Forest (ABC-RF) approach and using data extracted from eight nuclear microsatellite loci (see Section 2 for details)

Model set	Proportion of votes for each model ^a						Posterior probability	Error rate
	1	2	3	4	5	6		
Six models	0.115	0.221	0.239	0.245	0.127	0.053	0.450	0.328
Two models	—	0.319	—	0.681	—	—	0.806	0.164
Two models	—	—	0.373	0.627	—	—	0.811	0.164

^aThe best model for each comparison is highlighted in bold.

effective population size was 19,831 (51–184,958) and larger than the extant sizes. Posterior mode (95% HPD) of foundation time for G1 and G4 (T_1) by admixture between G2 and G3 was inferred at 10,715 (1301–92,311) generations ago. Posterior mode (95% HPD) of divergence time between G2 and G3 (T_2) was 59,983 (9521–994,089) generations ago. Considering the estimated generation time of *B. bituminosa* (2–5 years; Text S2), the posterior modes of T_1 and T_2 were 24,130–53,575 (i.e., a time interval right before the Last Glacial Maximum [LGM]) and 119,966–299,915 (a time interval before the intensification of the glacial period) years ago, respectively.

4 | DISCUSSION

4.1 | Phylogeography of *Bituminaria* in the MB: Contrasting and common patterns for widespread plant functional groups

Our broad-scale analysis of *Bituminaria bituminosa* across the MB revealed a scenario of remarkably high levels of haplotype diversity in NW Africa coupled with a significant signature of expansion. In keeping with such a pattern, the star-like structure of the haplotype network for MB populations indicated an event of diversification that dating analyses roughly placed within the last 1.5 Ma. Taken together, our results pointed towards a Pleistocene expansion of *Bituminaria* from NW Africa allowing dispersal throughout the Mediterranean distribution currently occupied by this plant lineage.

Our literature review revealed that the phylogeographical pattern disclosed in *Bituminaria* is paralleled by other taxa and that distinctive patterns between functional plant groups can be identified. Thus, several widespread Mediterranean shrub species (i.e., *Bituminaria*, *Cistus*, *Periploca* and *Rosmarinus*) are characterized by high numbers of haplotypes concentrated at their western Mediterranean distribution, whereas limited haplotype diversity in the east can be interpreted as a pattern of recent range expansion (Coello et al., 2020; Migliore et al., 2012). Eastward expansion following a putative Pleistocene origin in the western MB has been inferred in several other Mediterranean plants sharing a similar functional syndrome (i.e., facultative deciduousness, small leaf size), but with different range distributions, including species in the genera *Helianthemum* (Volkova et al., 2016), *Launaea*, *Lycium*, (García-Verdugo et al., unpublished), *Lupinus* (Mousavi-Derazmahalleh et al., 2018) or *Stauracanthus* (Pardo et al., 2008). Phylogenetic evidence broadly supports the idea of syndrome-driven diversification in

non-sclerophyllous plant lineages at local scales since the onset of the Mediterranean climate (Verdú & Pausas, 2013). The results from our literature review add another layer of complexity to this observation: Pleistocene conditions may have also favoured wide geographical expansion of species with functional traits shaped by the Mediterranean climate itself (e.g., García-Verdugo et al., 2020). However, this pattern is probably restricted to species with high colonization abilities, which may overcome the deleterious effects of rapid range expansion on adaptive evolution (discussed in González-Martínez et al., 2017). Future studies focusing on comparative phylogeography and functional biogeography of co-occurring taxa would be needed to examine in deeper detail this pattern and its underlying factors.

In contrast, sclerophyllous species with long generation times appear to have expanded across the MB before the establishment of the Mediterranean climate (Table 2; see also Herrera, 1992; Vargas et al., 2018). Unlike the signature of eastward expansion detected in *Bituminaria* and other Pleistocene plant taxa, widespread sclerophylls often show a similar number of haplotypes between both sides of the MB, which suggests the occurrence of multiple Quaternary refugia (Nieto Feliner, 2014; Rosselló et al., 2007). Considering the available literature on widespread, circum-Mediterranean taxa, it is however remarkable that two common patterns between both functional groups emerge. First, NW Africa is clearly identified as a centre of haplotype diversity (nearly 40% of the haplotypes found across functional groups), and second, the role of NW Africa as a source of multiple colonizations of the North-Atlantic islands in recent (Pleistocene) times. A growing body of evidence suggests that the Macaronesian islands could have been colonized in more recent geological times than previously thought (reviewed in García-Verdugo, Caujapé-Castells & Sanmartín, 2019), and *Bituminaria* qualifies as one of such cases.

4.2 | Phylogeographical patterns of *Bituminaria* in Macaronesia: Genetic admixture and back-colonization of the mainland enclave

Haplotype sharing between Madeiran and Iberian *Bituminaria* populations suggested that this species is the result of a recent introduction on this island, although more extensive sampling is needed to confirm its wild status (Menezes de Sequeira et al., 2012). In contrast, genetic results at the species level undoubtedly support the wild condition of Canarian populations (i.e., six private

haplotypes; Figure 1b). Plastid data showed that Canarian *Bituminaria* populations are composed of two sublineages that, according to ABC results, started to diverge around 120,000–300,000 years ago. If we take this divergence time as a proxy of island colonization (García-Verdugo, Caujapé-Castells, & Sanmartín, 2019), it implies that the genetic pattern of *Bituminaria* has probably been impacted by Pleistocene climatic shifts. Our results indeed showed that a model of divergence strictly following the progression rule was rejected, which appears to be common in most Canarian lineages with temporal origins predating the Quaternary (García-Verdugo, Caujapé-Castells, Illera, et al., 2019; Juan et al., 2000).

The distribution of genetic diversity across the archipelago suggested that the north massif on the island of Lanzarote (Famara), with relatively high levels of haplotype and nuclear diversity (Table S7), may have acted as a refuge area for the CAN1 sublineage (see Villa-Machío et al., 2020 for a similar interpretation). The CAN2 sublineage, in contrast, typically displayed smaller levels of within-population genetic diversity, particularly on the island of Fuerteventura. The haplotype network (Figure 3b) suggested a recent (star-like) diversification of the main CAN2 haplotype, coming into contact with the CAN1 sublineage on the central islands. Thus, our results give strong support to the notion that genetic admixture between colonizing lineages substantially increases the level of within-population genetic variation on the central Canarian islands (Caujapé-Castells et al., 2017; Curto et al., 2017), which may have mitigated the genetic erosion caused by climatic fluctuations on the easternmost islands.

Our study on *Bituminaria* also provided new evidence of the Pleistocene biogeographical connections existing between NW Africa and the Canary Islands. Several studies have recurrently documented events of very recent dispersals from the neighbouring mainland to the Canaries (e.g., Bobo-Pinilla et al., 2018; García-Aloy et al., 2017). In agreement with this observation, we found little differentiation at nuclear microsatellite loci coupled with haplotype sharing between eastern Canarian and Anti-Atlas populations of *Bituminaria* (Figure 3; Table S7). However, STRUCTURE results suggested that Anti-Atlas populations may be the result of hybridization between the two extant Canarian sublineages, and ABC analyses pointed towards a time period close to the LGM for a presumable event of back-colonization of mainland Africa. As stated by Fernández-Palacios et al. (2016), the LGM opened an opportunity window for colonization of the continent from the Macaronesian islands, because the predominant winds favoured dispersal to the east and the islands of Fuerteventura and Lanzarote were joined in a larger land mass known as Mahan. The physical connection between both islands could have favoured genetic admixture between the two extant *Bituminaria* sublineages (García-Verdugo, Caujapé-Castells, Illera, et al., 2019; Sun et al., 2016) and subsequent dispersal to the mainland LGM refugium.

An island origin of Anti-Atlas populations of *Bituminaria* is supported by additional lines of evidence. For instance, Brullo et al. (2017) noted that specimens of *Bituminaria* from the Anti-Atlas region are much woodier than those from continental populations

elsewhere, which could be a trait derived from the insular source populations (see descriptions in Méndez et al., 1990). Furthermore, a relatively recent dispersal from the islands and in situ differentiation has been previously suggested by phylogenetic studies for other taxa endemic to this region, such as *Aeonium korneliuslemsii* (Mort et al., 2002) and *Dracaena draco* subsp. *ajgal* (Durán et al., 2020).

5 | CONCLUSIONS

In a recent review, Nieto Feliner (2014) stated that 'The main conclusion from the available evidence is that common phylogeographic patterns are scarce in the MB.' We anticipate that future studies on comparative phylogeography considering functional plant groups will help us reveal a number of such elusive patterns. Our study further suggests that merging phylogeographical information into analyses of functional biogeography is a promising venue for gaining insights into the patterns of community assembly in the Mediterranean hot-spot area. Our analysis of *Bituminaria* also illustrates the dual role of NW Africa in plant biogeography. At the crossroad of Macaronesia and the western MB, NW Africa appears to have promoted Pleistocene expansion of *Bituminaria* throughout the Mediterranean and served as LGM refugium of populations that back-colonized the continent from the islands. Our findings therefore reinforce the idea that NW Africa represents a fundamental piece for our understanding of both Macaronesian and Mediterranean plant phylogeography.

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DATA AVAILABILITY STATEMENT

All DNA sequences generated in this study are available at GenBank (accessions MW496424-MW496835), and nuclear microsatellite data and the scripts used for the ABC analysis are available at the Dryad repository (<https://doi.org/10.5061/dryad.f7m0cfxvd>).

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BIOSKETCH

Carlos García-Verdugo is a botanist with a strong interest in the evolutionary processes and mechanisms that shape plant biodiversity patterns, particularly those operating at the intersection between macroevolutionary and microevolutionary scales. To this end, his research seeks to integrate complementary sources of evidence (mostly molecular, field and common garden data, but also meta-analysis) focusing on Mediterranean and island plants.

Author contributions: C.G.-V. conceived the ideas, F.M. and C.G.-V. collected the samples, C.G.-V., M.M. and I.T. analyzed the data and C.G.-V. led the writing.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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