



Molecular phylogenetics of *Lotus* (Leguminosae) with emphasis in the tempo and patterns of colonization in the Macaronesian region

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ABSTRACT

With a wide distribution range including Europe and Asia, *Lotus* (Leguminosae) represents the largest genus within Loteae. It is particularly diverse in the Mediterranean region and in the five archipelagos of Macaronesia (Atlantic Ocean). However, little is known about the relationships among the 14 sections currently recognized within *Lotus* and about the timing and patterns of its colonization in the Macaronesian region. In this investigation, we use four DNA regions (nuclear ribosomal ITS plus three plastid regions) in the most comprehensive sampling of *Lotus* species to date (some endemic species within the Canary Islands were poorly represented in previous phylogenetic analyses) to infer relationships within this genus and to establish patterns of colonization in Macaronesia. Divergence time estimates and habitat reconstruction analyses indicate that *Lotus* likely diverged about 7.86 Ma from its sister group, but all colonization events to Macaronesia occurred more recently (ranging from the last 0.23 to 2.70 Ma). The diversification of *Lotus* in Macaronesia involved between four and six independent colonization events from four sections currently distributed in Africa and Europe. A major aspect shaping the current distribution of taxa involved intra-island colonization of mainly new habitats and inter-island colonization of mostly similar habitats, with Gran Canaria and Tenerife as the major sources of diversification and of further colonization events. Section *Pedrosia* is the most diverse in terms of colonization events, number of species, and habitat heterogeneity, including a back-colonization event to the continent. Subsections within *Pedrosia* radiated into diverse habitat types recently (late Pleistocene, ca 0.23–0.29 Ma) and additional molecular markers and sampling would be necessary to understand the most recent dispersal events of this group within the Canary Islands and Cape Verde.

1. Introduction

Lotus represents the largest and most widely distributed genus of tribe Loteae (about 123 species divided in 14 sections, Degtjareva et al., 2006). Its main centres of species diversity are the Mediterranean and Macaronesia, the latter with ca. 52 taxa (plus three undescribed new species, Ojeda et al., 2012); both areas are considered as hotspots of biodiversity (Medail and Quezel, 1997). *Lotus* is taxonomically complex and therefore different classifications, especially at sectional and genus level, have been used through time (for example: Arambarri et al., 2005; Gillett, 1958; Kramina and Sokoloff, 1999; Degtjareva et al., 2006 or Sandral et al., 2010). The taxonomic classification of the Macaronesian taxa has also been problematic (e.g. Arambarri et al., 2005; Sokoloff et al., 2007; Brouillet, 2008; Sandral et al., 2010). In particular, there has been extensive debate about the classification of the three species

endemic to the Canary Islands that were included in the past within the genus *Dorycnium* (i.e. Tournefort, 1700; Linnaeus, 1753; Taubert, 1894; Rikli, 1901), and more recently in their own section (*Canaria*) within genus *Lotus* (i.e. Polhill, 1981; Lassen, 1986).

The Macaronesian *Lotus* species are currently classified into four sections: *Pedrosia* (including *Rhyncholotus*), *Chamaelotus*, *Lotus*, and *Canaria* that comprise about 44 taxa distributed in 29 islands of the five Macaronesian archipelagos (see Table 1).

Section *Pedrosia* is distributed in the Mediterranean region and mainland Africa, with its centre of diversification in the Macaronesian archipelagos (Azores, Madeira, Selvagens, Canary Islands and Cape Verde), and in 'the continental Macaronesian enclave', a small mainland region in Morocco in North-western Africa (Peltier, 1973; Sunding, 1979). It includes about 35 species that are mostly perennial and encompass a wide range of ecological conditions, with some rare or

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Table 1
Sections and taxa of the genus *Lotus* native in Macaronesia.

Archipelago	Section	Species	Islands of distribution
Azores	<i>Pedrosia</i>	<i>L. azoricus</i>	FL, PI, JO, SM, MA
		<i>L. creticus</i>	TE
	<i>Lotus</i>	<i>L. pedunculatus</i>	CO, FA, TE, FL, PI, GR, JO, SM, MA
Salvage Islands	<i>Pedrosia</i>	<i>L. glaucus</i>	SE
		<i>L. salvagensis</i>	SE
Madeira	<i>Pedrosia</i>	<i>L. argyroides</i>	MD, PS & D
		<i>L. glaucus</i>	MD, PS & D
		<i>L. macranthus</i>	MD, PS
		<i>L. lancerottensis</i>	MD
		<i>L. loweanus</i>	PS
Cape Verde	<i>Lotus</i>	<i>L. pedunculatus</i>	MD
	<i>Pedrosia</i>	<i>L. arborescens</i>	N
		<i>L. bollei</i>	V
		<i>L. brunneri</i>	A, V, N, S, B, M, ST, FO, Br
		<i>L. jacobaeus</i>	A, V, N, S, B, M, ST, FO
		<i>L. latifolius</i>	A
		<i>L. purpureus</i>	A, V, N, B, M, ST, FO, Br
Canary Islands	<i>Lotus</i>	<i>L. alianus</i>	A & V
	<i>Pedrosia</i>	<i>L. arinagensis</i>	C
		<i>L. berthelotii</i>	T
		<i>L. callis-viridis</i>	C, T?
		<i>L. campylocladus</i>	T, P?
		<i>L. dumetorum</i>	T
		<i>L. emeroideis</i>	G
		<i>L. eremiticus</i>	P
		<i>L. erythrochizus</i>	F
		<i>L. genistoides (nom nud.)</i>	C
		<i>L. glaucus</i>	F, GRA
		<i>L. hillebrandii</i>	P, H
		<i>L. gomerythus</i>	G
		<i>L. holosericeus</i>	C
		<i>L. kunkelii</i>	C
		<i>L. lancerottensis</i>	L, F, GRA
		<i>L. leptophyllus</i>	C
		<i>L. maculatus</i>	T
		<i>L. mascaensis</i>	T
		<i>L. pyranthus</i>	P
		<i>L. sessilifolius</i>	T, P, G
		<i>L. sessilifolius</i> var. <i>pentaphyllus</i>	T
		<i>L. sessilifolius</i> ssp. <i>villosissimus</i>	H
		<i>L. spartioides</i>	C
		<i>L. tenellus</i>	C, T
		<i>Lotus</i> sp. nov. 1	T
		<i>Lotus</i> sp. nov. 2	T
Canaria		<i>L. broussonetii</i>	C, T
		<i>L. eriophthalmus</i>	T, P, G, H
		<i>L. spectabilis</i>	T
<i>Chamaelotus</i>		<i>L. glinoides</i>	F, L, C, T, G, H, GRA

Abbreviations for the islands of each archipelago are: **Azores**: CO = Corvo, FL = Flores, FA = Faial, TE = Terceira, PI = Pico, GR = Graciosa, JO = São Jorge, SM = São Miguel, MA = Santa Maria; **Salvage Islands**: SE = Selvagens; **Madeira**: MD = Madeira, PS = Porto Santo, D = Desertas; **Cape Verde**: A = Santo Antão, V = São Vicente, N = São Nicolau, S = Sal, B = Boavista, M = Maio, ST = Santiago, FO = Fogo, Br = Brava; **Canary Islands**: C = Gran Canaria, T = Tenerife, P = La Palma, H = El Hierro, G = La Gomera, L = Lanzarote, F = Fuerteventura, GRA = La Graciosa.

endangered taxa in the Canary Islands (Bramwell and Bramwell, 2001). Within the Canarian archipelago, Gran Canaria and Tenerife harbour most of the *Lotus* species, followed by the western island of La Palma (Table 1). All these three islands are characterized by higher habitat heterogeneity (from coastal and lowland scrub to montane and in Tenerife to high montane elevations).

Although clear morphological adaptations for long distance dispersal (LDD) of diaspores are not present in *Lotus*, probably the wind, the sea and even granivorous passerine birds (endozoochorous) may have played a role in the success of this genus to colonize Macaronesia (Allan

et al., 2004; Vargas et al., 2015). Especially the *Rhyncholotus* group shows several floral traits that can favour both bird and even lizard pollination (Ojeda et al., 2013; Siverio and Rodríguez-Rodríguez, 2012), and further dispersal through endozoochory. Data from several oceanic archipelagos have proven the importance of LDD syndromes on shaping inter-island plant distribution (Vargas et al., 2015); in the Canary Islands, the relationship between the presence of LDD syndromes and more widespread distributions was statistically strongly supported (Arjona et al., 2018).

Most of the Macaronesian endemic plant groups are restricted to one archipelago (Reyes Betancort et al., 2008) and there are few examples of genera with taxa that have radiated in three of the five Macaronesian archipelagos (Williams et al., 2015). The genus *Lotus* is an exceptional case since it is distributed in all five archipelagos and in the Macaronesian continental enclave.

Therefore, this study may provide further insights into the biogeography of this hotspot of island biodiversity (Whittaker and Fernández-Palacios, 2007), and may help to address important questions on the Macaronesian endemic plant diversity to better understand six general patterns identified by molecular studies on the evolutionary history of the Macaronesian flora:

- (1) Single colonizations seem to have been common in some lineages, but also multiple independent events and subsequent secondary contact among genotypes previously confined to the mainland or to other insular regions, likely triggered evolution and radiation in many groups (see Caujapé-Castells et al., 2017),
- (2) The opportunity for Macaronesian colonization may have been during limited periods of time (“Colonisation Window Hypothesis”, Carine et al., 2004),
- (3) Rapid radiation within islands to distinct habitats and diversification between islands in similar ecological zones are prominent in the evolution of Canarian endemic plants (Francisco-Ortega et al., 1996; Kim et al., 1996; Bohle et al., 1996),
- (4) The surfing syngameon hypothesis (Caujapé-Castells et al., 2017) explains the origins of the high genetic diversity detected in the Canarian endemic flora as the dynamic result of admixture, introgression, and hybridization, and also incorporates the role of island ontogeny in the generation of the genetic diversity patterns,
- (5) Back-colonization has occurred from Macaronesia to the mainland (Allan et al., 2004; Caujapé-Castells, 2004; Jaén-Molina et al., 2009) and
- (6) Some areas (i.e. Moroccan area and palaeo-islands of Tenerife) have served as refuge of biodiversity within Macaronesia (Désamoré et al., 2011; García-Aloy et al., 2017; Mairal et al., 2015).

Since the last decade, phylogeography and dated molecular phylogenies have allowed to infer temporal colonization events and ancestral area(s) of many Macaronesian Angiosperm lineages (i.e. Curto et al., 2017; Jones et al., 2014; Mairal et al., 2015; Menezes et al., 2018; Valtueña et al., 2016). Also, divergence times that range from the Miocene and the late Miocene-Pleistocene were established for the five plant groups compared in Kim et al. (2008). In contrast, very recent time frames have been established for the origin of *Lactuca palmensis* (ca. 1.3 Ma, Dias et al., 2018), *Euphorbia lamarckii* (1.93 ± 0.98 Ma, Sun et al., 2016) or several species from *Cheirolophus* (less than 2 Ma, Vitales et al., 2014). Several studies of lineages distributed in the Canary Islands (i.e. Kondrakov et al., 2015), support the ‘relict hypothesis series’ (Cronk, 1992). Thus, palaeo and neo-endemism (taxa with different ages, i.e. paleo-endemics that arrived recently to the Canary islands, and afterwards became extinct in the continent, remaining only in the Canary Islands) coexist in the current Canarian Flora (Vargas, 2007; Fernández-Palacios et al., 2011); however, it is still necessary to conduct exhaustive sampling to obtain a more comprehensive understanding of

diversification in exceptionally species rich lineages like *Lotus*.

Here, we used various combinations of four DNA regions (ITS, *trnH-psbA*, *matK* and *CYB6*) in the most comprehensive sampling of *Lotus* species to date in order to: 1) establish the timing of the diversification of the genus *Lotus* and the sequence of colonization events in Macaronesia, 2) infer the number of colonization events and the global biogeographic patterns of *Lotus* in the five Macaronesian archipelagos, 3) understand habitat shift within section *Pedrosia*, a speciose group covering much of the ecological habitats in Macaronesia, and 4) determine the relationships among the three Canarian endemic species currently included in section *Canaria* since it has been the matter of much taxonomic debate.

2. Materials and methods

2.1. Taxon sampling and choice of DNA regions

We analysed a total of 116 taxa (species and subspecies) of *Lotus*, five genera (*Hammatolobium*, *Cytisopsis*, *Ornithopus*, *Coronilla* and *Anthyllis*) within Loteae and two species of *Sesbania* (*Sesbanieae*) as outgroups. In this study we increased the numbers of both species and populations sampled within species, and molecular markers analysed for previous studies. We selected nrITS, *trnH-psbA*, *matK* and *CYB6*, which contain DNA regions with higher representation of taxa within *Lotus* (nrITS, *trnH-psbA*, *matK*) and are very polymorphic even in closely related genera and species (Babineau et al., 2013), providing also variable sites within *Lotus* section *Pedrosia* (*trnH-psbA* and *CYB6*). Our sampling included all the species currently reported on the Macaronesian region (Table 1) except *L. gomerythus*, which it is only known from a single specimen from La Gomera (Portero et al., 2019). Also, it is important to mention that the samples of *L. pedunculatus* (sect. *Lotus*) included in this study were not collected in Macaronesia.

We first performed a comprehensive phylogenetic analysis with maximum likelihood using only nrITS (partial sequence ITS1, complete 5.8S ribosomal, and partial sequence of ITS2), including all available sequences (published and newly generated in this study) from *Lotus* and the outgroup taxa (Table S1). This analysis was based on 305 specimens (294 specimens from *Lotus* species, 94% of the species), with multiple accessions for some *Lotus* species. We selected this gene region as it is the one with the highest representation of *Lotus* taxa. This analysis allowed to identify the position of the Macaronesian species into the *Lotus* phylogeny and later concentrate further analyses including the most appropriate specimens representing each species. After this analysis (data not shown), we selected one specimen to represent each *Lotus* species for the subsequent analyses, except for the species within section *Canaria*, where we included multiple accessions (see below). Overall, our analyses covered four gene regions (Table S1) with 232 newly generated sequences and 326 sequences downloaded from GenBank.

2.2. DNA extraction, amplification and sequencing

DNA was extracted from silica gel dried material, fresh leaves and herbarium specimens according to the CTAB procedure (Doyle and Doyle, 1987). Primers and PCR conditions for the nuclear and the three plastid regions followed previous publications (Ojeda et al., 2014; Ojeda et al., 2012). The analyses in this study were performed on three datasets.

Dataset 1: ITS. This dataset contained available nrITS sequences of 135 specimens representing 116 described *Lotus* species (94% species). It also included 9 species from five closely related genera within Loteae and two species from *Sesbania* as outgroups. This dataset was used to date the phylogeny, to establish the time frame of the colonization events of *Lotus* in the five Macaronesian archipelagos, and back colonization events to the continent. In addition, this analysis was used to explore the taxonomic position of the species of the sect. *Canaria* (with multiple accessions of the three currently accepted taxa and covering all its present geographic range).

Dataset 2: ITS + *trnH-psbA*. This dataset comprised sequences of 103 specimens where both gene regions were available, representing 92 species of *Lotus* (74% species). We used the same five genera within Loteae as outgroup but excluding the two *Sesbania* species. The two gene regions were analyzed separately (to evaluate congruence) and in combination (to increase levels of resolution). This data set was used to identify with higher support the closest relatives of the lineages that colonizes Macaronesia.

Dataset 3: Four regions combined. This dataset consisted of four DNA regions (ITS, *trnH-psbA*, *matK*, and *CYB6*) from 47 specimens (38 species) and it was used to study habitat evolution within sect. *Pedrosia*.

2.3. Alignment, selection of models and phylogenetic analyses

Sequences for each of the four DNA regions were aligned using mafft (–genafpair –maxiterate 1000) (Katoh and Kuma, 2002). Each matrix was manually edited with AliView (Larsson, 2014) and summary statistics were obtained with AMAS (Borowiec, 2016). Indels were not included in the analyses. The best fitting model of sequence evolution for each gene was obtained with ModelFinder ver. 1.6.12 (Kalyaanamoorthy et al., 2017). Each matrix was analyzed with maximum likelihood (ML) with IQ-TREE (Nguyen, et al., 2015), using the best model for each region and 200 bootstrap replicates. We also performed a Bayesian analysis as implemented in MrBayes 3.2.6 (Huelsenbeck and Ronquist, 2001; Ronquist et al., 2012) using four chains, two runs of 20 million generations with the invgamma rate of variation, the GTR+Γ model of nucleotide substitution and a sample frequency of 1000. The performance of the Bayesian analysis was assessed with Tracer 1.7 (Rambaut et al., 2018) to verify whether effective samples sizes (ESS values) were higher than 200 for all parameters. ML and Bayesian analyses were performed on separate, as well as using the concatenated DNA regions (datasets 2 and 3). We used *Sesbania* as outgroup and five closely related genera (*Hammatolobium*, *Cytisopsis*, *Ornithopus*, *Coronilla* and *Anthyllis*) as part of the ingroup together with the genus *Lotus*. This outgroup and closely related genera were selected based on a previous analysis (Kramina et al., 2016). The obtained trees were visualized and formatted using FigTree (A. Rambaut, 2016) and iTOL ver. 3 (Letunic and Bork, 2016). Congruence among results from the *trnH-psbA* and nrITS was tested by comparing clade support values and level of resolution for individual clades obtained from each dataset. Details of all four DNA regions and data sets are available in Table 2.

2.4. Dating the colonization events in Macaronesia

We used the dataset 1 for dating the *Lotus* phylogeny with Beast v. 1.10.3 (Bouckaert et al., 2014; Suchard et al., 2018). An exploratory analysis was performed to assess the reliability of our date estimates with reference to the different settings (strict clock vs. uncorrelated lognormal, Yule vs. Birth-death). Choice of the best priors (clock and tree model) for our dataset was based on Bayes Factor using the Marginal Likelihood Estimation (MLE) implemented in Beast (Baele et al., 2012) that allows the comparison of Path Sampling (PS) and Stepping Stone (SS) sampling methods for alternative runs with different combinations of settings. The highest likelihood corresponded to a Birth-death tree prior with uncorrelated lognormal molecular clock (Table 3). Thus, we analyzed dataset 1 using the GTR substitution model with gamma distribution, a Birth-death speciation prior and considering the uncorrelated relaxed-clock model (UCLD, Drummond et al., 2012) with a lognormal distribution. Also, the clock model was set to accommodate the change in mutation rate from species to populations, with a uniform distribution for the ucld.mean (10^{-4} - 10^{-1}) and a default exponential distribution for the ucld.stdev. Two MCMC chains were run for 20 million generations, sampling trees and parameters every 10,000 generations and a final burn in of 10%. Tracer ver. 1.7.1 was used to assess the effective sample sizes (ESS > above 200) (Rambaut et al., 2018). Tribe Loteae, particularly *Lotus*, lacks available fossils for calibrations

Table 2

Variable and parsimony informative sites on the four data sets used on the different analyses.

DATASETS	1		2		3					
	One marker	Two markers (concatenated)	Separate analyses		Four markers (concatenated)					
	ITS	ITS+trnH-psbA	ITS	trnH-psbA	ITS	trnH-psbA	matK	CYB6	Combined	
No. specimens	135	103	103	103	47	45	45	46	47	
Alignment bp	719	1139	701	453	603	344	898	183	2028	
Missing %	16.19	18.06	14.13	26.84	0.39	5.28	2.94	0.45	5.06	
Variable sites	319 (52.7)	493 (43.3)	341 (48.6)	147 (32.5)	42 (7.0)	13 (3.8)	14 (1.6)	1 (0.5)	70 (3.5)	
Parsimony inf. sites (%)	309 (43)	323 (28.4)	246 (35.1)	79 (17.4)	16 (2.7)	7 (2.0)	10 (1.1)	1 (0.5)	34 (1.7)	

Table 3

Beast model comparison. Marginal likelihood estimates (MLE) for the dataset 1 under different clock models (Strict/Uncorrelated Lognormal = “Relaxed”) and speciation processes (Yule/Birth-Death = “Birth”) based on path sampling (PS) and stepping-stone (SS) methods implemented in BEAST v. 1.10.3.

	Strict-Yule	Strict-Birth	Relaxed_Yule	Relaxed_Birth
Path sampling	−8616.13	−8574.62	−8546.46	−8515.45
Stepping stone	−8616.98	−8575.73	−8547.32	−8515.49

(Koenen et al., 2019), and the closest group with available fossils belongs to the tribe Robinieae (*Robinia* L. wood fossil, 34 Ma) (Lavin et al., 2005). This fossil has been previously used in molecular dating analyses that included *Lotus* taxa (Farruggia et al., 2018). Here, we carried out two calibration approaches, (1) using a fossil-derived secondary age from the calibrated phylogeny included in Lavin et al. (2005), and applying a normal distribution to calibrate the root node of the clade including all Loteae taxa (mean = 14.4 Ma, SD = 1.3, 95% HPD = 11.7–18.6 Ma, node 73 in Lavin et al., 2005); (2) using again the fossil-derived secondary age estimated in Lavin et al. (2005), as calibration for the crown node of Loteae, together with an additional calibration point base on the age of La Palma (1.77 Ma, Ancochea et al., 1994), that was assigned to calibrate the node including *L. eremiticus* and *L. pyranthus*, two endemics that are distributed only in La Palma (uniform distribution with minimum age = 0 Ma and maximum age = 1.77 Ma). Therefore, the use of these two taxa as calibration points is a way to reduce the error that results from a potential time gap between the emergence and colonization of an island. The time of colonization of each lineage to Macaronesia was based on the divergence time from the sister clade.

2.5. Geographic range distribution, patterns of colonization and ancestral area reconstruction in Macaronesia

The distribution of the included species was based on the taxonomic treatments of *Lotus* in this geographic region (Sandal et al., 2006; Santos-Guerra, 2007), from records in the herbarium LPA at the Jardín Botánico Canario ‘Viera y Clavijo’-UA CSIC, and from previous studies in *Lotus* (Degtjareva et al., 2006; Kramina et al., 2016). We considered *Lotus* species as native in Macaronesia when they were recorded in the different catalogues and checklists of Macaronesian Flora (i.e. Acebes et al., 2010; Silva et al., 2010; Sequeira et al., 2011 or Sánchez-Pinto et al., 2005), and when enough evidence existed that their colonization was not aided by humans. We only excluded *L. ornithopodioides* (reported in Madeira) because available evidence suggests it most likely represents a recent human introduction. The reconstructions were made using the dispersal-extinction-cladogenesis (DEC) on the consensus tree (out of 20,000 trees) derived from the post-burnin analysis with Beast using the secondary calibration (dataset 1). In addition, to account for phylogenetic uncertainty, we also performed a statistical dispersal-extinction-cladogenesis (S-DEC) method (Beaulieu et al., 2013; Ree and Smith, 2008) using 1000 random trees obtained from the post-burnin analysis with Beast (dataset 1). We constrained the analyses to consider the geological history of the islands using the current distribution of the species and the age of the islands to account for historical

connections. We also considered the proximity of the islands and archipelagos to restrict the analyses. Both analyses (DEC and S-DEC) were inferred using RASP v.4.2 (Yu et al., 2015) and allowing only a maximum of two areas during the inference.

2.6. Habitat types shifts in section *Pedrosia*

We codified three habitat types (coastal and lowland scrub, montane, and high Canarian Mountain) within *Pedrosia* as discrete characters, following information from our own collections and previous studies (Ojeda et al., 2012; Allan et al., 2004; Sandral et al., 2006). Habitat types were mapped on the 1000 trees (to account for phylogenetic uncertainty) obtained from the ML analysis of IQ-TREE using the concatenated dataset of four gene regions (dataset 3), with maximum likelihood and the model Mk1 of trait evolution (which allows multiple characters and equal probability of change among the habitat types scored) as implemented in Mesquite ver. 3.6.1 (Maddison and Maddison, 2015).

3. Results

3.1. Phylogenetic analyses and position of section *Canaria*

Similar topologies were obtained with dataset 1 based on ML and Bayesian, with high support values for the four *Lotus* sections distributed in Macaronesia (Figure Supplementary figures 1 and 2). Overall, we recovered similar topologies with ITS and *trnH-psbA* on separate analyses of dataset 2 (Figure Supplementary figures 3–7), but with more resolved and better supported clades (>80% bootstrap) with ITS (13 sections/clades with ITS vs. only five with *trnH-psbA*). The differences on topology between ITS and *trnH-psbA* were mainly due to lack of resolution on the latter gene region, with most sections/clades poorly supported (Figure Supplementary figures 3–7). Our individual and combined analyses based on datasets 1 and 2 consistently recovered sect. *Canaria* (with three species) as monophyletic and with good support (Figure Supplementary figures 3–8). We found that this section is more closely related to the clades encompassing sects. *Chamaelotus*, *Heinekea*, *Lotea* and *Pedrosia* than to the clades comprising sects. *Bonjeanea* and *Dorycnium* (Figure 1). Previous analyses (Allan et al., 2004; Sandral et al., 2010; Kramina et al., 2016) have used only one specimen of *L. broussonetii* (cultivated in Kew Gardens) to represent sect. *Canaria*. We included the sequence of this specimen and our results indicate that this specimen is a misidentification, as it clustered with all *L. eriophthalmus* specimens analyzed (data not shown). Both the separate and the combined analyses indicate that *L. eriophthalmus* is the earliest-diverging species within sect. *Canaria* (Figure Supplementary figures 3–7).

The combined analyses of dataset 3 (four gene regions) recovered similar topologies from previous studies in *Lotus* sect. *Pedrosia* (Allan et al., 2004; Ojeda et al., 2012). With this dataset we recovered four major clades within this section, three of them exclusive to Macaronesia and the remaining to Africa (Figure 4 and Figure Supplementary figures 8 and 9). A higher number of informative sites were obtained with ITS than the plastid regions. Details and statistics of the three data sets and all gene regions are summarized in Table 3.

3.2. Tempo of the colonization events in Macaronesia

Our results suggest that *Lotus* likely diverged about 7.86 Ma (95% HPD: 5.00–11.28), with all the colonization events to Macaronesia occurring relatively recently (Pleistocene), between 2.70 Ma (95% HPD: 1.43–4.26) and 0.23 Ma (95% HPD: 0.06–0.51) (Figure 2, Table 4). Similar age estimates were obtained when using the secondary calibration of the split between *Sesbania* and *Loteae* together with the ages of the endemic species of one of the youngest islands of the Canary Islands (Figure Supplementary figure 10). Also, congruent topologies and similar ages were recovered under the birth-death or Yule model (data not shown). Our data indicate that sect. *Canaria* most likely represents the earliest colonization event of *Lotus* to the Canary Islands (ca 2.16 Ma).

Kramina et al. (2016), pointed to a very close relationship between sect. *Chamaelotus* and *L. alianus* based on morphological and molecular similarities but also because they share biogeography. Therefore, since our results are in concordance with those of Kramina, from now on we are considering *L. alianus* as another species from sect. *Chamaelotus*. Consequently, our results showed that sect. *Chamaelotus* (*L. glinoides* and *L. alianus*) seems to represent one of the most recent events (ca 0.32 Ma, Figure 2). *Lotus* sect. *Pedrosia* diverged within the last 1.28 Ma (95% HPD: 0.68–2.14) and two African lineages seem to have colonized Macaronesia region within the same time frame, the lineage of *L. eriosolen* colonized Cape Verde (ca 0.23 Ma, subclade A) and *L. jolyi*, the Canary Islands (ca 0.27 and 0.29 Ma, subclades B and C, respectively). All the diversification and colonization events of the two sect. *Pedrosia* most species-rich subclades likely occurred within the last 0.42 Ma (95% HPD: 0.22–0.69), when all islands within this archipelago were already established (Figure 2). The back-colonization event(s) to the continent from sect. *Pedrosia* occurred relatively recently (about 0.04 Ma).

3.3. Patterns of colonization and ancestral area reconstruction in Macaronesia

Our analyses based on S-DEC and DEC indicate that Macaronesia was colonized between 4 and 6 independent occasions (Figure 3) from four sections of *Lotus*: *Pedrosia* (including *Rhyncholotus*), *Chamaelotus*, *Lotus* and *Canaria*. Our ancestral reconstruction analyses suggest two likely sources for these colonization events, Africa (sects. *Pedrosia* and *Canaria*) and Europe (sects. *Chamaelotus* and *Lotus*) (Figure 3 and Supplementary figure 11). Sect. *Pedrosia* has the broadest distribution in all five Macaronesian archipelagos and our analyses suggest a minimum of two independent colonization events from two different African lineages. One lineage colonized only Cape Verde and diversified into at least six species (subclade A) (Figure 3). The second lineage most likely colonized Tenerife first, and later diversified into two main lineages: one that diversified further mainly in Tenerife and rapidly colonized Madeira and Azores (subclade C), and another that diversified in Gran Canaria (subclade B). In addition to sect. *Pedrosia*, sect. *Chamaelotus* is likely the result of two colonization events, one to Cape Verde (*L. alianus*) and the other to six of the seven major islands in the Canaries (*L. glinoides*). Sect. *Canaria* represents a single colonization event to the Canaries, and Tenerife was inferred as the most likely ancestral area for this lineage. We recovered two well supported lineages within sect. *Canaria*; one of them diversified within Tenerife and Gran Canaria (*L. broussoneti* and *L. spectabilis*), and the other (*L. eriophthalmus*) was restricted to Tenerife and then colonized La Gomera and the youngest islands of El Hierro and La Palma (Figures 2, 3, Figure Supplementary figures 10 and 11).

We recovered three species within sect. *Pedrosia* with distribution in Africa (*L. assakensis* and *L. pseudoreticus*) and in Africa/Europe (*L. creticus*) that were nested within recently diverged lineages and that most likely represent back-colonization events to the continent (Figures 1 and 3); however, our analyses could not completely determine the number of independent back-colonization events due to low level of support, even with the four gene regions combined of dataset 3

(Figure Supplementary figures 8 and 9).

3.4. Habitat shifts in section *Pedrosia*

We inferred the coastal and lowland scrub as the ancestral habitat, with at least nine independent transitions to montane habitats in only subclades B and C, and one transition to high Canary Mountain in subclade C. Our results also indicate that after the colonization of the Canarian archipelago, each of the three main lineages (subclades A-C) diversified and colonized new habitats on their respective islands (Figure 4). Later, as new habitats became available, the most recent colonization events from species of subclades B and C seems to have involved colonization events to similar habitat types to other islands.

4. Discussion

4.1. Tempo and colonization patterns of *Lotus* in Macaronesia

Lotus is considered a Mediterranean taxon with the extant diversity concentrated in Morocco, where 25% of the species are distributed, including representative species of nine sections (Degtjareva et al., 2006; Kramina et al., 2016). Our results suggest that four sections of *Lotus* colonized Macaronesia in four or six independent instances, always giving rise to monophyletic lineages that are currently distributed in Africa and in Europe (Figure 3). Previous investigations in other Macaronesian plant groups also indicate the existence of multiple colonization events, ranging from two in *Lavatera* (Fuertes-Aguilar et al., 2002), *Convolvulus* (Carine et al., 2004), *Matthiola* (Jaén-Molina et al., 2009), three events in *Festuca* (Díaz-Pérez et al., 2008), four colonizations in *Limonium* (Koutroumpa et al., 2018) and a maximum of five independent colonizations events in *Scrophularia* (Navarro-Pérez et al., 2015).

Sections *Chamaelotus* and *Pedrosia* seem to have colonized Macaronesia from diverse lineages in Africa (Saharo-Arabian and Sudano-Zambesian floristic regions) (Kramina et al., 2016). *L. alianus* is an endemic species from the northern-most islands of Cape Verde (Santo Antão and São Vicente) (Kirkbride, 2010), while *L. glinoides*, the other species from sect. *Chamaelotus* that is currently present in Macaronesia, is distributed on all islands (except La Palma, of the Canary Island archipelago), northern Africa and the Arabian Peninsula. These two taxa are recovered as sister species in previous phylogenetic analyses (Kramina et al., 2016), and our dating analyses indicate that the ancestor of these taxa colonized Macaronesia relatively recently (0.32 Ma) (Figure 2). Two possible scenarios could explain their current distribution: a) two independent colonization events, one to Cape Verde and another to the Canary Islands (Figure 3), and b) one independent colonization event, first to the Canary Islands and then from there to Cape Verde.

Denser sampling and the inclusion of faster evolving regions will be necessary to discern the most likely scenario in sect. *Chamaelotus*.

Section *Pedrosia* seems to have colonized Macaronesia in two independent instances. One involved a long-distance colonization event from West Africa to the Cape Verde archipelago at about 0.23 Ma (Subclade A). This lineage colonized all islands within this archipelago and diversified into at least six species (Sandral et al., 2006) (Figure 2). An alternative explanation is that this lineage had a wider distribution in Africa in the past, especially during the periods when the Sahara was moister than at present (Désamóré et al., 2011; Jolly et al., 1998; Mairal et al., 2015; Prentice and Jolly, 2000), and therefore the dispersion to Cape Verde probably was from a closer continental source area. The second sect. *Pedrosia* lineage colonized the Canary Island archipelago about 0.27–0.29 Ma and diversified into two main lineages (subclades B and C) (Figures 1 and 2). This lineage most likely colonized first the easternmost islands of this archipelago, Fuerteventura and Lanzarote, and then the westernmost islands. Our results suggest that the largest and most diverse islands (Gran Canaria and Tenerife) seem to have

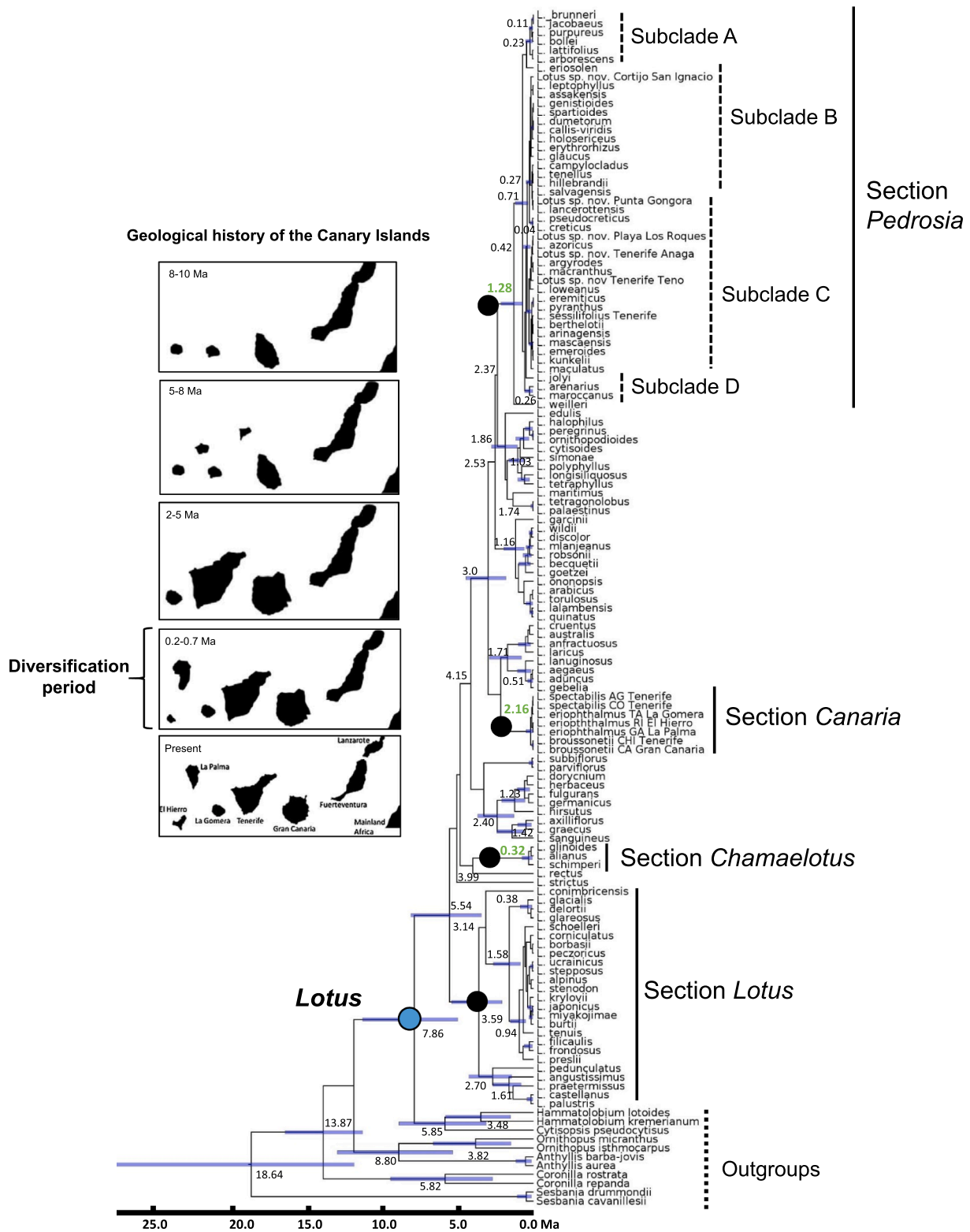


Fig. 2. Bayesian dated *Lotus* phylogeny from the relaxed molecular-clock analysis of dataset 1 (ITS, 135 accessions representing the genus *Lotus* and six genera as outgroup). The analysis was calibrated with the age estimates from [Lavin et al. \(2005\)](#) (mean 14.4 Ma, SD = 1.3) implemented on the crown node of Loteae. Time scale is in million years (Ma). Numbers near branches correspond to the average divergence time estimates, and the 95% posterior credibility intervals are represented by the blue bars. Green colour signals the ages of the independent events of colonization in Macaronesia from four sections within *Lotus* (*Chamaelotus*, *Lotus*, *Pedrosia*, and *Canaria*). Circles denote the nodes of the main divergence events. Names in red indicate species that are distributed in the continent (possible back-colonization events). On the left of the tree are included insets depict the patterns of emergence and formation of the islands up to the present time (adapted from [Caujapé-Castells et al., 2017](#)). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 4

Estimated divergence times of the major sections within *Lotus* (and subclades within *Pedrosia*) that colonized Macaronesia, based on two calibration strategies, under a Birth-death speciation process and an uncorrelated lognormal model of clock evolution.

Genus <i>Lotus</i>	Secondary age calibration		Secondary + islands age calibration	
	mean age (Ma)	CI values (95% HPD)	mean age (Ma)	CI values (95% HPD)
Sect. <i>Canaria</i>	2.16		2.27	
Sect. <i>Pedrosia</i>	1.28	0.68–2.14	1.37	0.67–2.07
subclade A	0.23	0.06–0.51	0.30	0.07–0.50
subclade B	0.27	0.13–0.47	0.35	0.14–0.47
subclade C	0.29	0.12–0.48	0.36	0.22–0.68
subclade D	0.26	0.05–0.57	0.34	0.05–0.56
Sect. <i>Chamaelotus</i> **	0.32	0.08–0.75	0.40	0.07–0.76
Sect. <i>Lotus</i> *	2.70	1.43–4.26	2.84	1.51–4.27

*The only representative of these sections that are distributed in Macaronesia is *L. pedunculatus*.

** Although when this species was described it was included in section *Lotus* (Kirkbride, 2010), due to its close relationship with *L. glinoides*, which is strongly supported by Kramina et al. (2016) and the present study, we considered this species as included in sect. *Chamaelotus*.

played a major role during the diversification of this lineage, in accordance to a previous biogeographic pattern detected for several Canarian plant lineages (i.e. *Aeonium*, *Cheirolophus* or *Micromeria*, Mort et al., 2002; Vitales et al., 2014; Puppo et al., 2015; Curto et al., 2017), which suggests that the central Canaries were the centers of diversification to both the older easternmost islands and to the younger westernmost islands (Sanmartín et al., 2008, 2010). In line with the fact that the complex geological history of the islands played an important role in generating patterns of genetic diversity (Marrero and Francisco Ortega, 2001; Caujapé-Castells et al., 2017), these two islands also host a higher variety of habitats at present, most of which must have been formed during the last 2 Ma due to volcanic activity (García-Talavera, 1999). Two sect. *Pedrosia* lineages (subclades B and C) diversified in these two islands exploiting new available habitats, and we inferred between 2 and 4 shifts from coastal scrubland and lowland to higher elevations habitat types (montane and high Canarian mountain) on each of these subclades (Figure 4). After occupying available habitats within their corresponding islands, these two lineages (subclades B and C) colonized mainly similar habitat types that became available during the most recent volcanic activity and the emergence of new islands (La Palma and El Hierro). Thus, it seems that the exploitation of new habitats within subclades B and C was achieved only early during the colonization process. For instance, the most recently diverged *L. sessilifolius* populations are distributed in the coastal and lowland scrub habitats in Tenerife, La Palma, La Gomera and El Hierro (Yang et al., 2018; Ojeda et al., 2012). Also, other dated phylogenies of Macaronesian plants (Kim et al., 2008; Guzmán and Vargas, 2009; Curto et al., 2017; Menezes et al., 2018) reveal different evolutionary processes underlying lineage diversification. Habitat shifts from a generalist habitat to laurel forest and subsequently to sclerophyllous zones, and geographic isolation have been reported as important on the diversification of *Pericallis* in the Canary Islands (Jones et al., 2014). In *Cheirolophus*, ecological adaptation has allowed the diversification into different habitats within the same island and into similar habitats in different islands (Vitales et al., 2014).

The other source area of colonization events to Macaronesia is related to lineages currently distributed in mainland Europe. *Lotus pedunculatus* is widely distributed in Europe and it has been introduced to other temperate regions in the world. The current distribution of this species restricted to Madeira and Azores (while absent in other islands within Macaronesia), might indicate that it colonized these two

archipelagos without human aid. However, because in this study we did not include samples from these islands (only samples collected in the mainland of Spain and Canada), we can neither determine the time frame of its colonization, nor completely rule out human intervention on its current distribution in Macaronesia.

4.2. Phylogenetic position and colonization patterns of section *Canaria*

All our analyses support this Canarian endemic lineage as monophyletic (Figure 1, Figure Supplementary figures 3–7), and support the previously reported relationships of sect. *Canaria* to the CHZ (*Canaria*, *Heinekenia*, *Zygocalix*) clade within *Lotus* clade based on a single specimen (Kramina et al., 2016). Thus, sect. *Canaria* is more closely related to sects. *Heinekenia*, *Lotea*, and *Pedrosia* than to sects. *Dorycnium* or *Bonjeana* (Kramina et al., 2016). This section seems to represent the oldest colonization event of *Lotus*, which our estimates suggest at about 2.16 Ma (Figure 2 and Table 4); however, this group seems to have diversified in the Canary Islands ca 0.19 Ma, more recently than the diversification in sect. *Pedrosia* subclades B and C (ca 0.27 and 0.29 Ma, respectively, Figure 2). Our extensive sampling indicates that the most recent colonization within sect. *Canaria* occurred by *L. broussonetii* that in a period less than 0.20 Ma ago (Figure 2), diversified from *L. spectabilis* (only present in the most recently formed areas of Tenerife) and *L. eriophthalmus* (distributed in the youngest islands, La Palma and El Hierro) But unlike sect. *Pedrosia*, sect. *Canaria* either experienced higher extinction rates (there is a large gap between the stem age and the crown age of the current endemics of this section) and/or had lower diversification rates or lacked the adaptive traits to exploit the availability of new habitats.

4.3. Back-colonization events to the continent

The geographical proximity between the Canary Islands and the continent has facilitated the interchange of biodiversity, especially in the past when the sea level was lower (García-Talavera, 1999; Rijdsdijk et al., 2014). Back-colonization from the Canaries to the continent (“boomerang effect”, Caujapé-Castells, 2004) has been suggested for several taxa distributed in both Macaronesia and mainland areas (i.e. *Aeonium*, *Androcymbium*, *Convolvulus* or *Matthiola*, see Jaén-Molina et al., 2009). More recently, Sun et al. (2016) and Gruenstaedl et al. (2017) concluded that *Euphorbia* and *Tolpis* are examples of Macaronesian plant lineages that have experienced back-dispersal events to the continent (North Africa and Mediterranean Europe). Our results suggest that three species within subclade B (*L. creticus*, *L. pseudocreticus* and *L. assakensis*) are nested within recent diverged lineages, indicating back colonization events from this group to the continent. Although we have support for a back-colonization event, we do have enough support to determine the number of independent events (Figure Supplementary figures 8 and 9). *Lotus creticus* is widely distributed in mainland Europe, Africa and the Mediterranean region, while *L. pseudocreticus* and *L. assakensis* are restricted to Africa (Sandral et al., 2006). Considering the recent age of this event (within the last 0.04 Ma), and that all three species are grouped within the same lineage, the most plausible scenario is a single colonization event to Africa, and further dispersion of *L. creticus*. A denser sampling within *L. creticus* and faster evolving regions will be necessary to fully determine the number of back-colonization events in *Lotus*. In the absence of evidence for dispersal vectors in *Lotus*, palaeowinds (the ‘Westerlies’, Rognon and Coude-Gaussen, 1996) are the more likely option to explain this pattern (see Caujapé-Castells, 2011 for a more general context), that has been also observed for *L. lancerottensis* (endemic from Fuerteventura and Lanzarote), possibly involved in the “boomerang” between the Canaries and mainland north Africa that gave rise to the Moroccan *L. assakensis* (Allan et al., 2004).

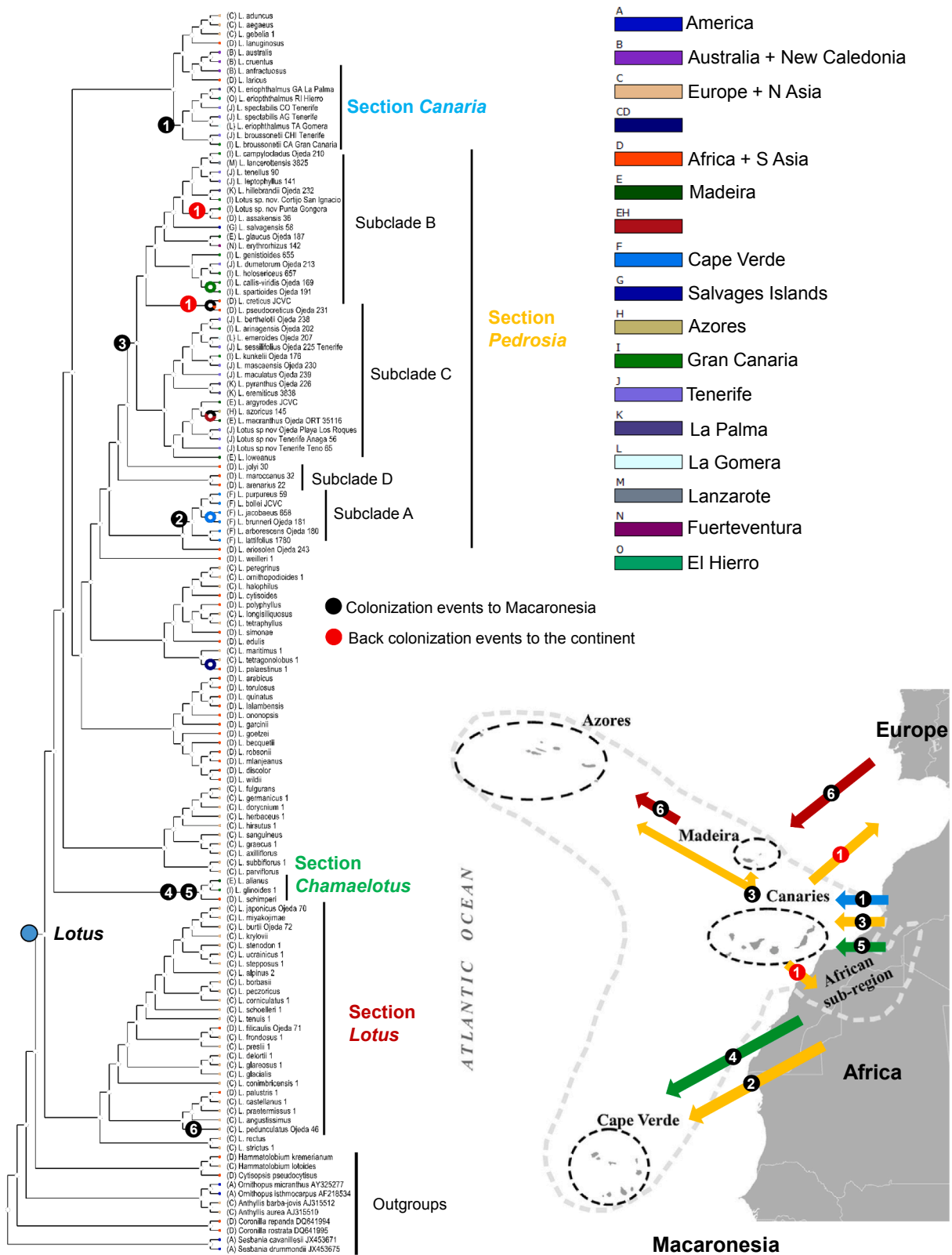


Fig. 3. Inference of the colonization events of *Lotus* sections (*Pedrosia*, *Canaria*, *Chamaelotus*, and *Lotus*) in the Macaronesia region using the S-DEC as implemented in RASP. Colonization events to Macaronesia are indicated with black circles where the number indicates the likely chronological order (see Figure 2). Red circles indicate the likely number of back-colonization events from Macaronesia to the continent (Africa and Europe). Colors indicate species distribution in the major areas (see legend). Color of the arrows corresponds to the four *Lotus* sections and the hypothesized routes of colonization. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

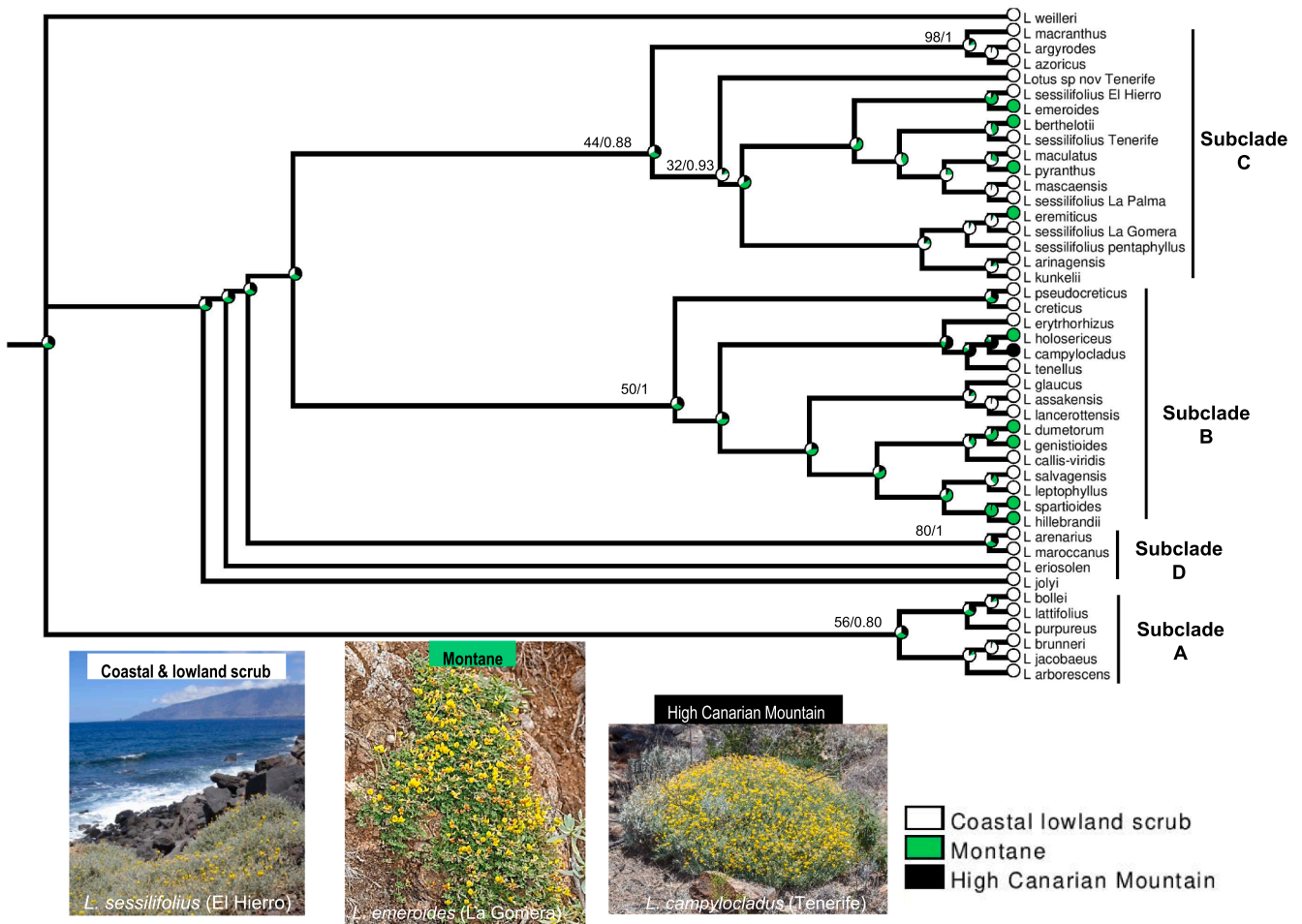


Fig. 4. Reconstruction of habitat types in section *Pedrosia* using the 1000 maximum likelihood (ML) trees obtained from the four genes combined (dataset 3, ITS+trnH-psbA+matK+CYB6). Color circle at the tip of the phylogenetic tree indicates current habitat types. Pie charts on the nodes represent the probability of the ancestral state for each node. Values next to the branches indicate bootstrap/Bayes support. Photographs of the three habitat types are depicted for selected species. Photo credits: D. I. Ojeda and G. García Casanova.

5. Conclusions

Our analyses highlight *Lotus* as a genus with a very complex and dynamic evolution in Macaronesia. At least four *Lotus* lineages (sections) seem to have colonized Macaronesia in between four to six independent occasions since the Pleistocene. Both Africa and Europe seem to have been important sources of these colonization events. We recovered distinctive patterns of colonization, with some lineages colonizing only one archipelago (sect. *Canaria*), while other lineages were able to colonize more than one archipelago (sect. *Pedrosia* and sect. *Chamaelotus*), although we cannot rule out that extinction processes could have taken place (Marrero, 2004) and that in the past, e. g. sect. *Canaria*, was also present in other archipelagos. Section *Pedrosia* represents the most successful of the lineages distributed in Macaronesia, comprising the larger number of species, habitat diversity and distribution range. Our results highlight the importance of Tenerife and Canaria in the diversification of sects. *Pedrosia* and *Canaria* and further colonization of adjacent archipelagos (except Cape Verde). Faster evolving regions and a denser sampling will be necessary to further determine the relationships and colonization patterns within the most recently diversified lineages of sects. *Lotus*, *Chamaelotus* and *Pedrosia*, including the number of independent back-colonization to the Africa and Europe in the latter section.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ympev.2020.106970>.

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