


New molecular evidence for Canarian endemic *Ruta* (Rutaceae: Ruteae) reveals a complex evolutionary history and overlooked diversification processes

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We studied four currently recognized endemics of *Ruta* (Rutaceae) from the Canary Islands, an oceanic biodiversity hotspot, to identify genetic variation, reconstruct evolutionary relationships and clarify the taxonomic delimitation of several recently discovered populations. We analysed four plastid DNA regions for a thorough population sampling of *Ruta oreojasme* on Gran Canaria, *R. pinnata* on Tenerife and La Palma and *R. microcarpa* and the only known population of the recently described *R. nanocarpa* on La Gomera. Bayesian clock dating analysis, haplotype network and ancestral area reconstructions were carried out. Our results reveal a complex, extremely dynamic and largely overlooked pattern of colonization and evolutionary history of *Ruta* in the archipelago. Thirty haplotypes were found, but only one of them was shared across more than one island. Species divergence and all colonization events in the Canary Islands were dated from the late Pliocene to the Pleistocene, and *R. oreojasme* has an earlier origin than its Canarian congeners. Multiple secondary colonization events between and within islands account for the diversification of *R. pinnata* and *R. microcarpa*. Our data cannot discard a possible independent origin of *R. oreojasme* and do not support the currently accepted circumscription of *R. pinnata* and *R. microcarpa*. Rather, the high levels of genetic differentiation detected suggest the existence of ongoing diversification processes and of new taxa. Urgent population-level conservation efforts should be undertaken, especially focused on the single population on La Gomera that can be attributed to *R. microcarpa*, *R. nanocarpa* and genetically distinct populations from La Palma and the palaeo-islands of Tenerife.

ADDITIONAL KEYWORDS: ancestral areas – Canary Islands – conservation – divergence times – haplotype network – palaeo-islands.

INTRODUCTION

The natural discontinuation of gene flow, promoted by geographical or ecological fragmentation of oceanic islands and the interaction of population genetic diversity with the geological ontogeny and ecological diversity of the islands (Marrero, 1992; Marrero & Francisco-Ortega, 2001) underlies the swift generation of genetic differences in all the lineages that make up

their native floras, thus causing the origin of new taxa with generally high genetic diversity at a generally faster pace than in most non-oceanic enclaves (see Caujapé-Castells *et al.*, 2017, and references therein). Notably, a substantial part of the 34 biodiversity hotspots defined by Myers *et al.* (2000) are, or include, islands (updated in <http://www.biodiversityhotspots.org>).

The application of increasingly reliable molecular genetic techniques has facilitated the testing of hypotheses on the origins and evolution of oceanic island floras (e.g. Baldwin, 1992; Allan *et al.*, 2004; Harbaugh *et al.*, 2009; Dunning *et al.*, 2016) and, in the Canaries, the detection of high levels of genetic diversity (Pérez de Paz & Caujapé-Castells, 2013).

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Most molecular genetic research efforts in the Canarian flora had until recently been devoted to (1) radiating lineages with many species exclusive from different islands (Barber *et al.*, 2002; Francisco-Ortega *et al.*, 2002; Mort *et al.*, 2002, 2010; Carine *et al.*, 2004; Goodson, Santos-Guerra & Jansen, 2006; Kim, Lee & Mejías, 2007; Gruenstaeudl, Santos-Guerra & Jansen, 2013); or (2) extremely endangered taxa with populations on just one island (Batista *et al.*, 2001; Oliva-Tejera *et al.*, 2006; González-Pérez *et al.*, 2009; Suárez *et al.*, 2009; Rodríguez-Rodríguez *et al.*, 2015, 2019; Albaladejo *et al.*, 2021).

Notably, since the inception of genetic investigations devoted to Canarian native lineages not subject to radiation (Sánchez *et al.*, 2006; Jaén-Molina *et al.*, 2009), many unexpected diversification processes have been found in previously unstudied taxa.

Thus, genetic differentiation has also been recently detected in non-species-rich lineages, encompassing (1) widespread floristic elements that are thought not to be taxonomically diverse (González-Pérez *et al.*,

2014; García-Verdugo *et al.*, 2015; Puppo *et al.*, 2015; Valtueña *et al.*, 2016) and (2) taxa that are endangered but have populations on two or more islands and have thus not received so much attention as the single island endemics (SIE thereafter) (Rumeu *et al.*, 2014; Jaén-Molina *et al.*, 2015; Gramazio *et al.*, 2020). These recent and ongoing investigations provide compelling evidence that many diversification processes remain to be discovered in the Canarian flora, and some of them may result in urgent taxonomic and conservation reassessments.

The currently recognized Canarian endemic species of *Ruta* L. (Rutaceae) are known to be distributed on four of the seven major islands of the archipelago, and they occupy similar habitats of the lowland thermosclerophyllous scrub (Fig. 1). Until recently, all the populations from La Gomera were taxonomically circumscribed to *R. microcarpa* Svent., those from Gran Canaria to *R. oreojasme* Webb & Berthel. and those from Tenerife and La Palma to *R. pinnata* L.f. (Fig. 1; Bramwell & Bramwell, 1974, 2001;

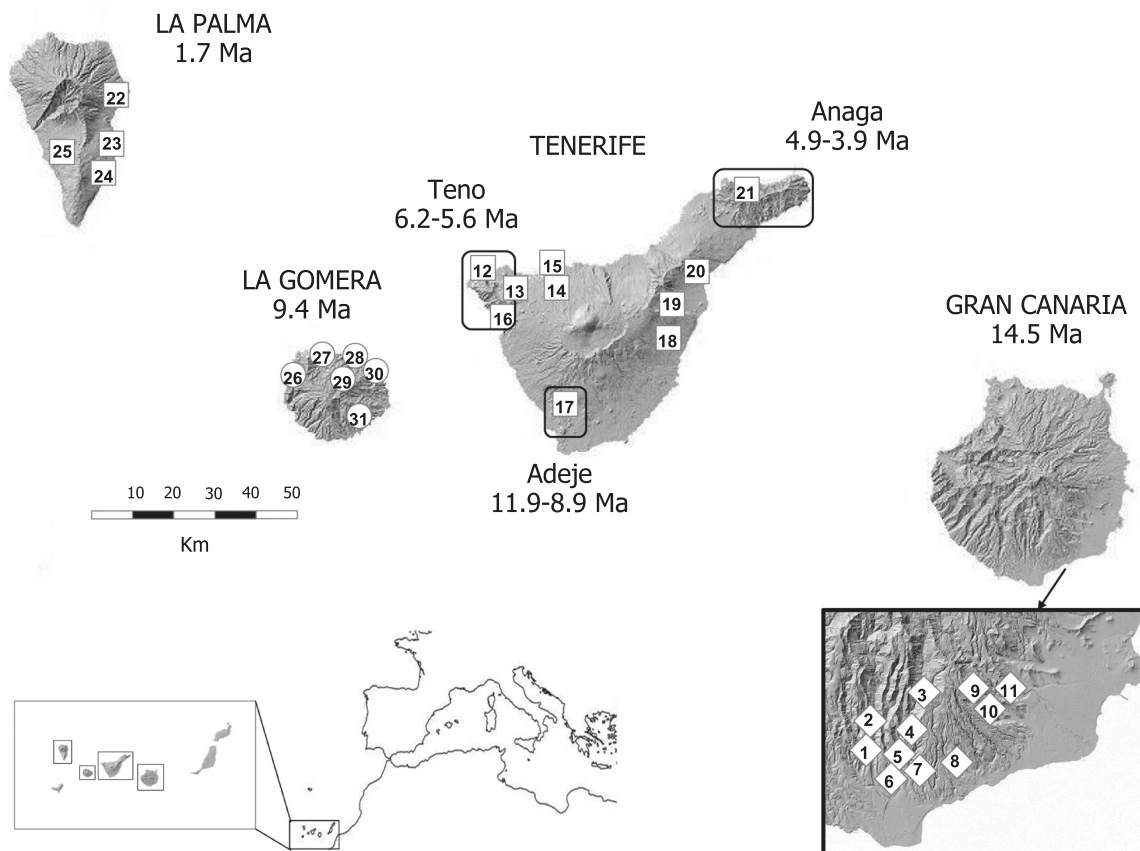


Figure 1. Sampling localities of the Canarian endemic *Ruta* on the four islands where they are distributed [with the estimated date of emergence of each island according to Carracedo, (2011)]. The palaeo-islands of Tenerife and the ages of their subaerial volcanic activities are highlighted. Population numbers correspond to Table 1. As described in Material and methods, the populations are ascribed to species on the basis of the currently accepted taxonomy.

Ceballos & Ortuño, 1976; Santos & Fernández, 1980; Acebes *et al.*, 2010). However, earlier authors recognized populations in the north of La Gomera (Hermigua) as *R. pinnata* (Burchard, 1929; Ceballos & Ortuño, 1976; Eriksson, Hansen & Sunding, 1974; Hansen & Sunding, 1985, 1993). In addition, Sventenius (1969) described new populations that he discovered in the south-west of La Gomera as *R. microcarpa*, whereas he considered other newly discovered populations in the north of that island as a possible variety or subspecies of *R. microcarpa*, if not a different species. Marrero, Jorge & Bramwell (1988) suggested that some populations in the north of La Gomera did not correspond to *R. microcarpa*, and Santos (1983) indicated that individuals of *Ruta* on La Palma were probably not *R. pinnata*, as already suspected by Sventenius.

Many other populations of these taxa have been found only in recent times, because the rugged, isolated areas of difficult access where they occur often impede thorough botanical exploration. A population found on a remote cliff on La Gomera is taxonomically quite distinct from *R. microcarpa* and has recently been described as a new species (*R. nanocarpa* Mesa, Portero-Álvarez, Martín-Carbajal & Reyes-Betancort; Mesa *et al.*, 2022). Our group also discovered several large populations of *R. oreojasme* in secluded areas in south-eastern Gran Canaria (Saturno, 2007; Olangua, 2009; Soto, 2010; Soto *et al.*, 2011). Likewise, new populations have been discovered in northern La Gomera [ascribed to *R. microcarpa* (Mesa, 1996; Mesa *et al.*, 2003; Meloni *et al.*, 2013; Conti, 2013)] or on Tenerife (TAN in Table 1, ascribed to *R. pinnata*). Increased exploration of Tenerife and La Palma would probably result in the discovery of more populations of *Ruta*; for example, a population recorded around the Barranco del Río (Barquín & Voggenreiter, 1988) has not yet been found again after several surveys in that area.

Frequent herbivore grazing by feral goats in most known populations of Canarian *Ruta* adds substantially to the natural threats posed by isolation and small population sizes. At present, *R. microcarpa* is considered critically endangered [CR B2ab(iii,v); Moreno, 2011, but see Mesa, Acevedo & Rodríguez, 2011] and *R. oreojasme* is vulnerable [VU B2b(ii) c(ii,iv); Moreno, 2011]. *Ruta pinnata* is near threatened (Moreno, 2011), although many of its populations on Tenerife are presently declining in size due to the action of introduced mammals; the conservation status of this taxon in La Palma is unknown due to the lack of thorough surveys.

Salvo *et al.* (2008, 2010) evaluated the taxonomic treatment of Ruteae *s.l.* and hypothesized that Canarian *Ruta* spp. are derived from a strongly supported single introduction event in the archipelago, and La Gomera was the inferred ancestral area within a temporal colonization interval spanning from 27.3

to 2.6 Myr (Salvo *et al.*, 2010). However, the sampling scheme of Salvo *et al.* (2008, 2010) was aimed at a basic taxonomic representation of the Canarian circumscription (just six samples encompassing the four islands of current distribution), without emphasis on the known population distribution of each taxon at the time. Similarly, fossils for molecular-clock calibration were selected from other Ruteae (fossils of *Ruta* have not been found), which in the case of the Canarian endemics might add to the temporal uncertainty surrounding the internodes of the used reconstruction (see Salvo *et al.*, 2010).

More recently, population genetic surveys with nuclear microsatellites in the endangered *R. microcarpa* (Meloni *et al.*, 2013) and in *R. oreojasme* (Meloni *et al.*, 2015) revealed considerable levels of genetic diversity. In *R. oreojasme*, this result can be interpreted as a consequence of the prolonged absence of major disturbances across south-eastern Gran Canaria (Meloni *et al.*, 2015); however, *R. microcarpa* maintains such high genetic diversity despite the high incidence of clonality in most of its small and environmentally stressed populations, mainly due to the action of introduced mammals and plants. In both taxa, Meloni *et al.* (2013, 2015) found a substantial spatial structure of genetic diversity. *Ruta nanocarpa* could not be included in Meloni *et al.* (2013), but this does not affect the conclusions with regard to *R. microcarpa* because they are taxonomically distinct (Mesa *et al.*, 2022). At present, no population genetic data have been published for *R. pinnata*. In this general context, a thorough molecular analysis of Canarian *Ruta* may have relevant implications for the estimation of their phylogeographic relationships and providing adequate conservation guidelines (Soto, 2015). In this investigation, we use the sequences of four plastid DNA regions on a comprehensive population sampling of the currently known Canarian *Ruta* spp. to infer geographical structuring of their genetic diversity in the archipelago and to estimate intra- and inter-island dispersal. As in other cases where molecular data have helped detect unsuspected genetic diversity in several Canarian lineages (see before), we will also assess whether our genetic data support the currently accepted taxonomic circumscriptions of the analysed populations. On the basis of our results, we will provide informed guidelines to help streamline the present conservation and management strategies for these taxa.

MATERIAL AND METHODS

PLANT MATERIAL AND SAMPLING

Canarian *Ruta* spp. are distinct from the remaining species of the genus by their greater size (Townsend, 1968; Bramwell & Bramwell, 2001) and their much

Table 1. Species and populations included in the datasets analysed in this study. I: Islands of distribution: C = Gran Canaria, T = Tenerife, P = La Palma and G = La Gomera; Code: population codes; *N*, number of samples analysed for haplotype network; H, haplotype codes. An asterisk (*) indicates the populations distributed in the palaeo-islands of Tenerife. All populations were included in Dataset 1. Superscripts 2 and 3 indicate populations included in Datasets 2 and 3, respectively

Species/population	I	Code	UTM	<i>N</i>	H
<i>R. oreojasme</i>					
1. Montaña La Gorra	C	GOR	28RDR 405 770	5	E
2. Cho Domingo	C	DOM	28RDR 405 785	1	I
3. Las Yeguas	C	YEG	28RDR 425 770	2	B
4. Montañeta Redonda	C	RED	28RDR 420 765	3	E, D
5. Los Culatones	C	CUL	28RDR 420 755	3	B
6. Arteara	C	ART	28RDR 440 800	9	B
7. Barranco del Cañizo ^{2,3}	C	CAN	28RDR 430 750	7	B, D, J, K
8. Barranco Taliscal del Águila	C	AGU	28RDR 455 755	9	A, C
9. El Gallego	C	GAL	28RDR 480 805	4	A, G
10. Barranco de Las Palmas	C	PAL	28RDR 495 795	4	A, C, F
11. El Sao	C	SAO	28RDR 495 825	8	H
<i>R. pinnata</i>					
12. Carretera a El Fraile	T	FRA	28 RCS 160 385	13	α, β
13. El Tanque	T	TAN	28 RCS 246 379	4	X
14. Genovés	T	GEN	28 RCS 290 390	9	Z
15. Fuente del Guincho	T	GUI	28 RCS 288 393	4	Y, Z
16. Barranco de Masca ^{*2}	T	MAS	28 RCS 195 319	4	T, U, W, β
17. Barranco del Infierno ^{*2,3}	T	INF	28 RCS 320 130	6	S, V
18. Barranco de Herques	T	HER	28 RCS 580 255	9	S
19. Barranco de Badajoz	T	BAD	28 RCS 581 315	4	P, Q
20. Barranco de Chacorche	T	CHA	28 RCS 633 403	3	P
21. Roque de Los Pinos ^{*2}	T	ANA	28 RCS 790 595	10	R
22. Lomo de Las Nieves ²	P	NIE	28 RBS 275 777	9	M, O
23. El Zumacal	P	ZUM	28 RBS 290 723	4	L
24. Tirimaga	P	TIR	28 RBS 275 647	2	N
25. Jedey	P	JED	28 RBS 194 619	1	N
<i>R. microcarpa</i>					
26. Alojera ^{2,3}	G	ALO	28 RBS 720 155	16	W
27. Roque Cano	G	CAN	28 RBS 780 195	10	γ
28. Mulagua	G	MUL	28 RBS 840 145	10	γ, δ
29. Camino del Cedro ²	G	CED	28 RBS 825 145	4	γ
30. Taguluche	G	TAG	28 RBS 875 165	10	γ
<i>R. nanocarpa</i>					
31. Roque Ipalán ^{2,3}	G	IPA	28 RBS 875 085	5	γ

larger leaves (Salvo *et al.*, 2008). Figure 2 shows the four Canarian *Ruta* spp. in their habitats.

Ruta oreojasme ($2n = 36$, Stace, Armstrong & James, 1993) is a tetraploid hermaphrodite shrub considered to be an exclusive endemic to rocky slopes, crevices and ravines in southern Gran Canaria (Fig. 1). It measures up to 0.4 m in height and has tortuous branches; it flowers from February to April and sets fruit between June and August; pinnate and thick leaves, *c.* 6 cm long and bluish-green. Flowers have yellow erect petals and are clustered in terminal inflorescences. The fruit, a small, dehiscent capsule with four light brown lobes contains small black seeds

(Webb, 1840). The populations surveyed showed a generally good recruitment and an estimated census of 33 613 individuals (Soto *et al.*, 2011), but the species is subject to pressures from introduced grazing animals, occasional fires and drought.

Ruta pinnata ($2n = 40$, Stace *et al.*, 1993) is a tetraploid hermaphrodite shrub with a conspicuous trunk, erect branches that can reach *c.* 2 m in height and up to 1.5–2.0 (4.0) m × 0.8–1.0 (1.5) m in width; it is considered to be an endemic from Tenerife and La Palma (Fig. 1). It has imparipinnate leaves, with three (or two) pairs of leaflets, yellowish flowers, cochleariform petals with irregular crenulate margins and a round, fleshy,

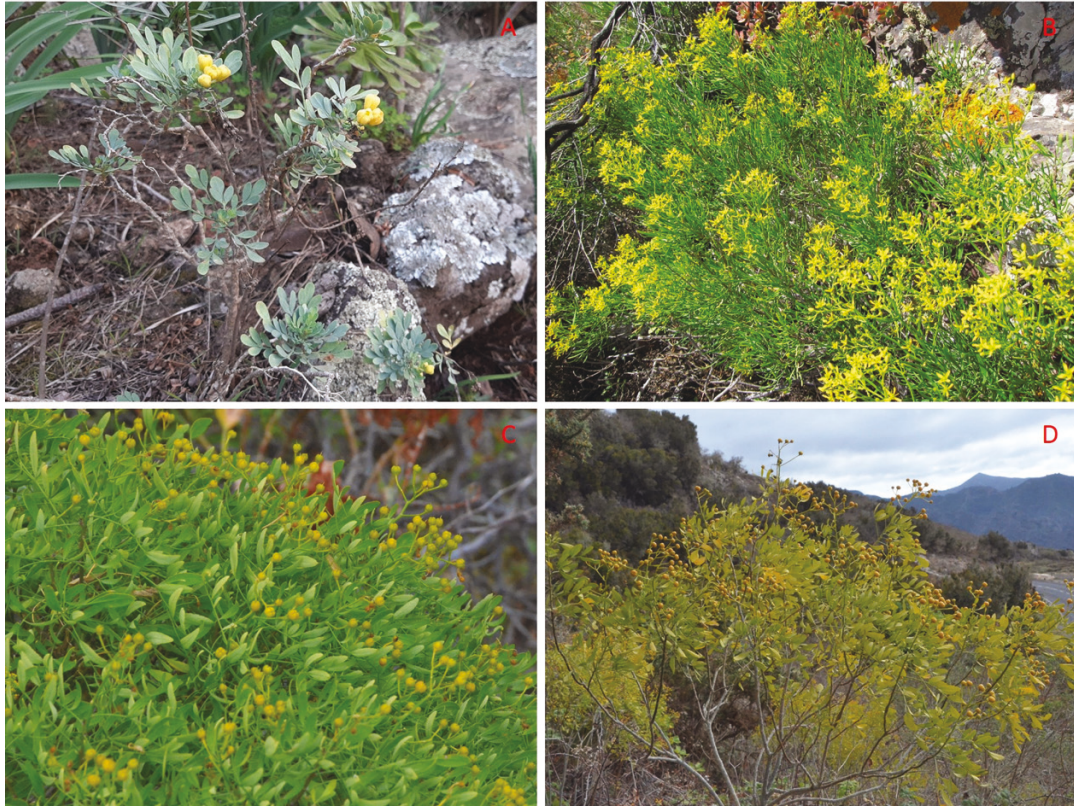


Figure 2. Pictures of selected individuals of the four Canarian *Ruta* spp. studied in their habitat. A, *R. oreojasme*, Arteara, Gran Canaria; B, *R. nanocarpa*, Roque Ipalán, La Gomera; C, *R. microcarpa*, Alojera, La Gomera and D, *R. pinnata*, Tierra del Trigo, Tenerife. Photograph credits: Á. Marrero (A, C and D) and A. Portero (B).

orange capsule (Linnaeus fil., 1782; Webb & Berthelot, 1836). It flowers from March to May and fruits in May–June; fruits ripen during the summer. Although it has not been subjected to systematic monitoring and census, field studies by our group (R. Mesa) estimate that there are < 3000 plants on Tenerife, distributed in c. 20 generally small populations ranging from < 100 to c. 500 individuals. Census data for the populations from La Palma are not available.

Ruta microcarpa is a hermaphroditic shrub with a pulvillar habit; it is similar to *R. pinnata*, but smaller, up to 0.8–1.0 (1.5) m high and 0.8–1.0 (1.5) m wide, densely branched with lax foliage. It has remotely toothed leaves with three to seven leaflets and smaller fruits and flowers (Agulló *et al.*, 1967; Sventenius, 1969). It is considered an exclusive endemic to La Gomera (Fig. 1), where it reaches up to 1.5 m in height in the northern populations (Mesa, Acevedo & Rodríguez, 2003). It flowers from March to May and fruits in May–June. It mostly occurs in rugged areas, although some populations have colonized abandoned cultivation zones. Updated censuses of this species (Mesa *et al.*, 2003) recorded 716 specimens

distributed in eight populations in the north and west of La Gomera. Three previously reported populations could not be found (Las Hoyetas, Monteforte-El Cedro and Liria). There is currently no information on the ploidy of *R. microcarpa*. Pollination of Canarian *Ruta* is favoured by Diptera and Hymenoptera (Mesa *et al.*, 2003; Soto *et al.*, 2011) and, in the case of *R. pinnata* and *R. microcarpa*, dispersal is carried out mainly by birds and lizards (Manuel Nogales, CSIC, unpubl. data).

Ruta nanocarpa is only known from one population on La Gomera (Roque Ipalán). It is similar to *R. microcarpa*, but is far more compact and leafy and smaller (40–50 cm high), and it is highly branched, with a broom habit. Leaves are green, narrowly pinnate, with narrowly oblanceolate, almost linear leaflets. Stamens are shorter than the petals, and the petals are barely longer than those of *R. microcarpa* and *R. pinnata* (Mesa *et al.*, 2022).

We sampled 1–5 g of fresh leaves from 192 individuals in 31 Canarian populations of *Ruta* (Table 1, Fig. 1; 11 of *R. oreojasme*, 14 of *R. pinnata*, five of *R. microcarpa* and the only known population of *R. nanocarpa*). These samples thoroughly represent the distribution area of

currently recognized Canarian *Ruta* spp. To represent optimally the *locus classicus* of *R. microcarpa* near Alojera (in the ‘southwestern areas of La Gomera’; Sventenius, 1969), samples from several fragmented patches in that area were collected (Teguerguenche, Andenitos Verdes and Finca la Ruda; Table 1). Our fieldwork in the surroundings of the *locus classicus* of *R. pinnata* on the north coast of Tenerife (‘in cliffs near Puerto de La Rotava’; Linnaeus *fil.*, 1782) was not successful.

Despite the morphological differences observed in the samples of some Canarian populations, we used the taxonomic ascription assigned to each population by the collectors (on the basis of the island of distribution; Fig. 1, Supporting Information, Table S1). Because the principal objectives of this investigation were restricted to inferring the phylogenetic relationships among the Canarian populations and to assess their inter-island colonization, we sampled as an outgroup for the molecular analyses a population of the mainland *R. montana* Mill., the closest congener of the Canarian taxa according to Salvo *et al.* (2010).

Sampling procedures followed the general guidelines in Caujapé-Castells *et al.* (2011). Leaves were collected and stored in silica gel until processing in the molecular facilities at the Jardín Botánico Canario ‘Viera y Clavijo’, Unidad Asociada CSIC (JBCVCSIC, hereafter). We collected at least one voucher specimen for each sampled population, and these were deposited at the LPA herbarium (Supporting Information, Table S1); silica dried leaf samples were deposited at the DNA Bank of the JBCVCSIC.

DNA EXTRACTION, AMPLIFICATION AND SEQUENCING

DNA extractions were performed using the 2 × CTAB method (Doyle & Doyle, 1987; Palmer *et al.*, 1988), with slight modifications described in Caujapé-Castells *et al.* (2011). The quality of the extracted DNA was

checked with a 1% agarose gel that was stained with SYBR Safe, Invitrogen and DNA concentration was quantified using a NanoDrop ND-2000c (Thermo Fisher Scientific, Waltham, MA, USA) spectrophotometer.

The intergenic spacers *psbA-trnH*, *rps16-trnK*, *trnT-trnL* and *atpB-rbcL* of plastid DNA (Shaw *et al.*, 2007) were selected on the basis of their general intra- and interspecific discriminatory potential detected in previous studies with several Canarian plant lineages (Jaén-Molina *et al.*, 2014, 2018; García-Verdugo *et al.*, 2015, 2017). Primers used for PCR amplifications and for sequencing are shown in Table 2. Amplification reactions followed the conditions described in Supporting Information, File S1; these were prepared for a final volume of 25 µL containing 2 µL of extracted DNA, 20 µL of ReddyMixT_M PCR Master Mix (ThermoScientific, Abgene, UK), 0.5 µL of each primer (20 µM) and 2 µL of bovine serum albumin (20 mg/mL BSA, Sigma, Las Palmas de Gran Canaria, Spain). The PCR products that provided a single band of sufficient intensity after running a 1.8% agarose gel were sent to Macrogen Inc., Korea, for bidirectional sequencing on an ABI 3730XL (Applied Biosystems, Foster City, CA, USA). Forward and reverse sequences were reviewed and edited when necessary, using the software BIOEDIT v.7.0.9 (Hall, 2007). Before further analysis, we used a fragment of each sequence as a query for a NCBI BLAST algorithm search to check that we retrieved congeneric sequences. Consensus sequences for each plastid DNA region were obtained with the cap contig assembly program implemented in BIOEDIT v.7.0.9, and for *psbA-trnH*, *rps16-trnK*, *trnT-trnL* and *atpB-rbcL* concatenated into a single matrix aligned with the algorithm ‘Muscle’ (eight iterations) using GENEIOUS PRO v.5.6.2 (Drummond *et al.*, 2011). This matrix included the 192 samples of Canarian *Ruta* plus one of their Moroccan congener *R. montana*. Identical sequences from the same population were

Table 2. Primers used in this paper and GenBank accession numbers for the submitted sequences obtained for each region

Region	Primer	Sequence (5′-3′)	References	Accession number
<i>psbA-trnH</i>	F: <i>psbA</i>	GTTATGCATGAACGTAATGCTC	Shaw <i>et al.</i> , 2005	MK156912-156962
	R: <i>trnH</i> ^{GUG}	CGCGCATGGTGGATTACAATCC		
<i>rps16-trnK</i>	F: <i>rpS16</i> × 2F2	AAAGTGGGTTTTTATGATCC	Shaw <i>et al.</i> , 2007	MK156963-157013
	R: <i>trnK</i> ^{UUU} × 1	TTAAAAGCCGAGTACTCTACC		
<i>trnT-trnL</i>	F: A2	CAAATGCGATGCTCTAACCT	Taberlet <i>et al.</i> , 1991	MK156861-156911
	R: B	TCTACCGATTTCCGCATATC		
<i>atpB-rbcL</i>	F: <i>atpB</i>	GAAGTAGTAGGATTGATTCTC	Manen <i>et al.</i> , 1994	MK156810-156860
	R: <i>rbcL</i>	TACAGTTGTCCATGTACCAG		
<i>matK</i>	F: <i>matk1</i>	ACTGTATCGCACTATGTATCA	Sang, Crawford & Stuessy, 1997	MK282767-282798
	R: <i>matk1</i>	GAAGTAGTCGGATGGAGTAG		

removed, leaving 49 sequences in the final matrix (GenBank accession numbers in [Table 2](#)).

PHYLOGENETIC ANALYSES

Maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI) analyses were conducted on the combined plastid DNA matrix. In all analyses, *R. montana* from Morocco was used as the outgroup taxon. The MP analyses were performed through heuristic search using PAUP* 4.0b10 ([Swofford, 2003](#)), with the following parameter settings: 1000 random addition replicates holding ten trees per replicate, tree-bisection–reconnection branch swapping algorithm, Multrees in effect and Steepest Descent not in effect. Gaps were treated as missing data. Strict and 50% majority-rule consensus trees were calculated for all the most parsimonious trees obtained. The robustness of clades was estimated using 1000 bootstrap replicates (BP) and the same heuristic search protocol. For ML and BI analyses, the model of sequence evolution that best fitted the sequence data was GTR (general time reversible) according to the Akaike information criterion values calculated in jMODELTEST v.0.1.1 ([Posada, 2008](#)). The ML analyses were performed using MEGA 7 ([Kumar, Stecher & Tamura, 2016](#)). The initial trees for the heuristic search were obtained automatically by applying neighbor-joining and BioNJ algorithms (default parameters). A discrete gamma distribution was used to model evolutionary rate differences among sites with four categories ([Nei and Kumar, 2000](#)). The BI analyses were carried out in MRBAYES v.3.1.2 ([Ronquist and Huelsenbeck, 2003](#)) using two identical searches with 10^6 generations each (four MCMC, chain temperature = 0.2, sample frequency = 100). Probabilities converged to the same stable value after $c. 2 \times 10^5$ generations in both runs. A 50% majority-rule consensus tree was calculated to obtain a Bayesian phylogenetic tree. The robustness of the final BI phylogenetic tree was estimated using posterior probabilities (PP) ([Alfaro, Zoller & Lutzoni, 2003](#)).

ESTIMATION OF DIVERGENCE TIMES

To date the phylogenetic tree for *Ruta* in BEAST v.1.10.3 ([Suchard *et al.*, 2018](#)), we used the concatenated matrix of the four plastid DNA markers (49 individuals, Dataset 1 from now on) and we constructed two complementary datasets at a higher taxonomic level (Datasets 2 and 3) using alternative outgroups. Dataset 2 included nine individuals from the concatenated matrix of the four plastid DNA markers, representing all Canarian taxa of *Ruta* (a sample of one population from each palaeo-island of Tenerife was also included) plus the Moroccan *R. montana*.

Dataset 3 corresponds to a matrix of *matK* sequences (1536 bp) downloaded from GenBank plus sequences of that region newly generated for this study. It comprises 11 samples representing all four Canarian endemic taxa of *Ruta* (*R. oreojasme*, *R. pinnata*, *R. microcarpa* and *R. nanocarpa*) and seven outgroup taxa (the Mediterranean *R. montana*, *R. graveolens* L., *R. chalepensis* L., *R. angustifolia* Pers. and *R. corsica* DC., plus the closely related genera *Thamnosma* Torr. & Frém. and *Boenninghausenia* Rchb. ex Meisn.). [Table 1](#) indicates the populations sampled for each dataset, and [Table S3 in the Supporting Information](#) gives details of the three datasets.

Exploratory analyses were performed to assess the reliability of our age 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 datasets was based on Bayes Factor using marginal likelihood estimation (MLE) implemented in BEAST ([Baele *et al.*, 2012](#)). The highest likelihood for the interspecific sampling (Datasets 2 and 3) corresponded to a birth–death prior with uncorrelated lognormal molecular clock and birth–death with a strict clock, respectively. For the intra-specific Dataset 1, the highest likelihood corresponds to a coalescent tree prior with a strict molecular clock ([Supporting Information, Table S2](#)). Nucleotide substitution model GTR + G was selected with jMODELTEST v.0.1.1. MCMC searches were run between 10^7 (higher taxonomic level) and 5×10^6 (population-level) generations and sampled and logged every 2000 generations. Twenty percent of the trees sampled were discarded as burn-in. We used TRACER v.1.7.1 ([Rambaut *et al.*, 2018](#)) to determine stationarity of the Markov chain and to verify that all parameters had large enough effective sampling sizes (ESS > 200). TREEANNOTATOR v.1.8.0 and FIGTREE v.1.3.1 ([Rambaut and Drummond, 2009](#)) were used to generate and visualize the resulting maximum clade credibility (MCC) chronograms, respectively. To provide additional external evidence (e.g. mutation rates and molecular-clock rate) for calibrating our phylogenetic tree more accurately and to accommodate the change in mutation from species to populations, we carried out different analyses and estimated the divergence times in the two following steps:

- (1) secondary ages estimated previously based on several Rutaceae fossil calibrations were used to analyse Dataset 3 (interspecific level). The nodes were calibrated considering the 95% highest posterior density intervals and the corresponding standard deviations provided for the origin of *Ruta* (44.56 ± 18.98 Myr) and the initial diversification of *Ruta* (19.96 ± 10.92 Myr) in [Salvo *et al.* \(2010\)](#). The ucl.d.mean was set to 10^{-6} – 10^{-1} with a uniform

distribution, and a default exponential distribution was used for the `uclid.stdev`;

- (2) the divergence times obtained in (1) for the origin of the Canarian endemics and the divergence between *R. oreojasme* and the rest of the Canarian taxa were used as priors to inform the clock rate for inter- and intra-specific relationships with *R. montana* as the outgroup. A mixed model (Ho *et al.*, 2005; Pokorny, Oliván & Shaw, 2011) was used in this second step, so that a constant-rate birth–death speciation tree prior was applied to Dataset 2, whereas a coalescent constant size model (unlinked tree prior) was applied to Dataset 1, to calibrate the haplotype divergence within populations (see Mairal *et al.*, 2015). The `uclid.mean` was set to 10^{-4} – 10^{-1} substitutions/site/Myr, the most commonly observed values for plastid markers.

ANCESTRAL AREA RECONSTRUCTIONS

The S-DIVA and S-DEC + J analyses implemented in RASP-4 (Yu, Blair & He, 2019) were used to reconstruct the possible ancestral geographical ranges of Canarian endemic *Ruta*. RASP summarized the ancestral reconstructions across the posterior distribution of the trees generated by BEAST on Dataset 1 (49 individuals). Inferences of biogeographic events at each node for S-DEC + J and S-DIVA followed Matzke (2014) and Ronquist (1997), respectively. A burn-in of 1000 (10%) BEAST trees was used to guarantee the convergence of the MCMC chain. Then, 1000 random trees from the remaining 90% distribution were chosen to account for statistical uncertainty. Range constraints of a maximum of two areas at each node were used allowing all pairwise combinations of areas. S-DEC + J was stratified according to five time slices, corresponding to the emergence of the subaerial sections of the islands: 0–1.7 Mya (La Palma), 1.7–9.4 Mya (La Gomera), 9.4–11.9 Mya (Tenerife), 11.9–14.5 Mya (Gran Canaria) and 14.5–14.9 Mya (root of the tree). One hundred percent pairwise dispersal rates were allowed between all emerged areas at each time period. According to Matzke (2014) DEC + J is most adequate to infer ‘island-hopping’ histories for island data.

HAPLOTYPE NETWORK

The relationships among the haplotypes were inferred with the concatenated matrix of the 192 samples using the statistical parsimony method implemented in TCS v.1.21 (Clement, Posada & Crandall, 2000), treating gaps as a fifth state. The maximum number of differences resulting from single substitutions among

haplotypes was calculated with 95% confidence limits. Since the number of differences (35) for the obtained haplotype of the Moroccan *R. montana* exceeded the confidence limits of the software, this sample could not be included in the analysis and therefore the resulting network was unrooted.

RESULTS

PHYLOGENETIC ANALYSIS AND DIVERGENCE TIME ESTIMATION

The combined matrix of the four markers (*atpB-rbcL*, *psbA-trnH*, *rps16-trnK*, *trnT-trnL*) had a total length of 2873 nucleotides with 2809 constant, 34 variable and 30 potentially parsimony-informative positions.

The MP, ML and BI phylogenetic analyses yielded mostly congruent topologies overall, although the BI analyses displayed better resolution and stronger branch support values. The MP analysis generated 333 trees of 79 steps each, with a consistency index (CI) of 0.81 and a retention index (RI) of 0.94. One of the most parsimonious trees is shown in Figure 3. The MP and Bayesian consensus tree (Figs 3 and 4), resulting from the analysis of the combined plastid DNA markers strongly supported *R. oreojasme* as the first-branching species with respect to the other Canarian taxa and yielded three major clades. Clade I (PP = 1, BP = 99%) comprised all 11 *R. oreojasme* populations, and clades II and III comprised populations currently considered as *R. pinnata* and *R. microcarpa* plus *R. nanocarpa* (Roque Ipalán, La Gomera). Clade II (PP = 1, BP = 99%) included *R. pinnata* from ‘Barranco de Herques’ (HER) and a few individuals from ‘Barranco del Infierno’ (INF); both populations are from Tenerife and are sister to the populations in Clade III. Clade III (PP = 1, BP = 95%) comprised three subclades: subclade IIIa (PP = 1, BP = 97%), with individuals of *R. pinnata* from Barranco del Infierno (INF) and Barranco de Masca (MAS), plus all accessions of *R. microcarpa* (*sensu* Sventenius) that represent different areas near Alojera (ALO); subclade IIIb (PP = 1, BP = 83%), with populations of *R. pinnata* from La Palma (NIE, ZUM, TIR and JED), plus samples from three adjacent populations of Tenerife: Roque Los Pinos in the northeast (Anaga, ANA) and two close areas in the east (Barranco de Badajoz, BAD and Barranco de Chacorche, CHA); and subclade IIIc (PP = 1, BP = 82%) that contains the four populations from the north of La Gomera (CAN, MUL, CED, TAG), the population of *R. sp. nova* (SOM) and populations of *R. pinnata* from north-western Tenerife (FRA, TAN, GUI, GEN), plus one individual from Barranco de Masca (MAS).

According to the parameter analysis in TRACER, the number of MCMC iterations was sufficient, with values of effective sample size (ESS) > 200 in all

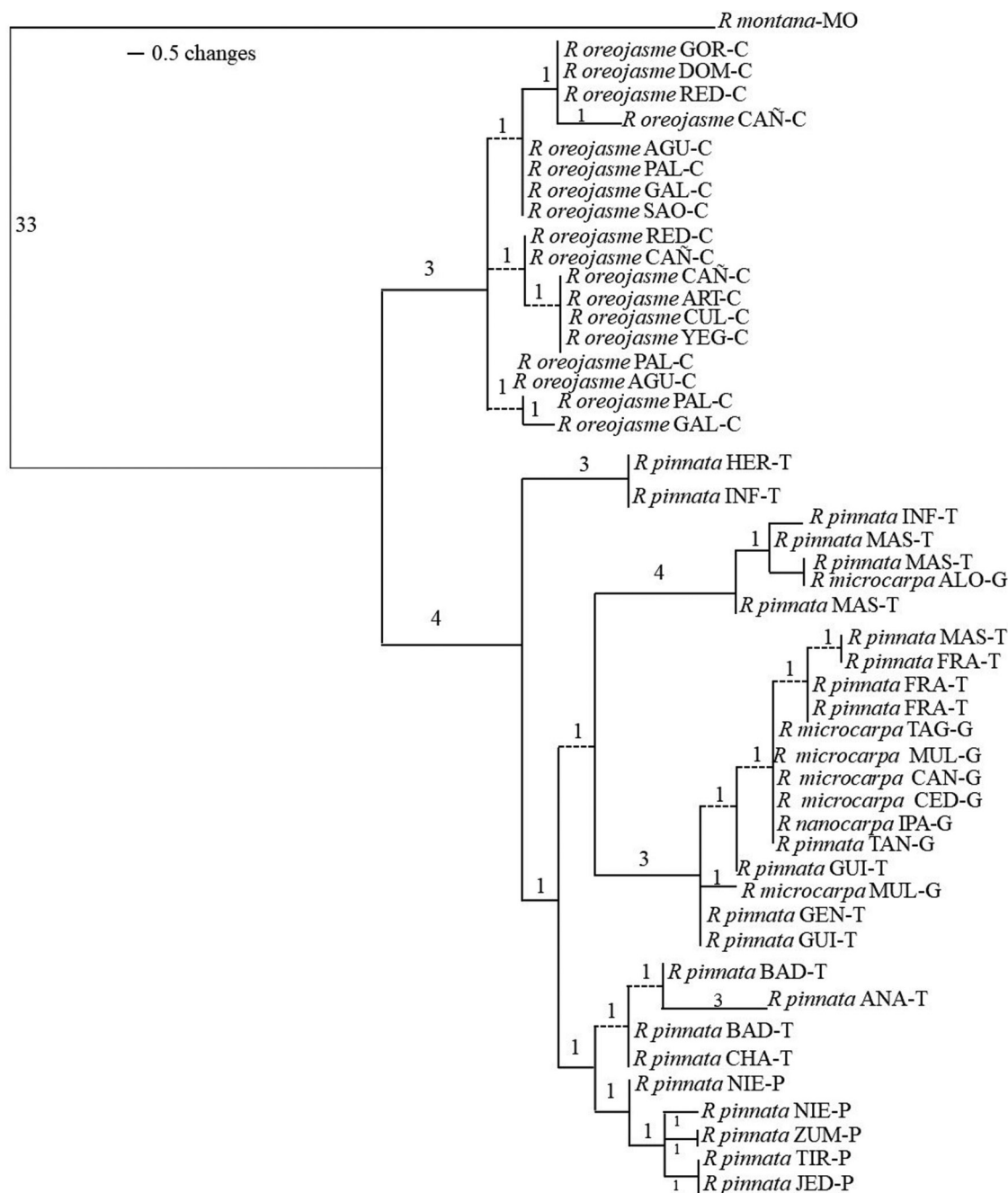


Figure 3. One of the 333 most parsimonious trees (79 steps, CI = 0.81, RI = 0.94) obtained from the analysis of the combined plastid DNA regions (*psbA-trnH*, *rps16-trnK*, *trnT-trnL* and *atpB-rbcL*). Branches that collapse in the strict consensus tree are shown as dashed lines. Numbers above the branches indicate the number of changes. Letters next to the taxon names correspond to the codes for population identification and the island of distribution in Table 1 (C: Gran Canaria, T: Tenerife, P: La Palma and G: La Gomera, see map in Fig. 1).

cases and plots showing equilibrium after discarding burn-in. The MCC tree with 95% highest posterior density intervals (HPD) for the divergence time estimates of relevant nodes (also listed in Table S2) is shown in Figure 4.

Our molecular dating analysis at the highest taxonomic level (Dataset 3, Supporting Information, Fig. S1) situates the origin of *Ruta* in the Eocene (47.87 Mya, 95% HPD = 34.35–59.06), and the initial diversification in the genus in a time window congruent

with the chronogram obtained by [Salvo *et al.*, 2010](#) (16.33 Mya, 95% HPD = 9.71–25.95). The Bayesian analyses at lower taxonomic levels (Datasets 1 and 2, four plastid DNA regions) were partially consistent with the results reported in that study; however, the inclusion of more plastid regions and more populations and taxa resulted in lower mean nodal ages and narrower intervals of confidence.

The molecular dating of Dataset 2 ([Supporting Information, Fig. S2](#)) estimates the average origin of the Canarian endemics at 11.44 Mya, and the split between *R. oreojasme* and the remaining Canarian taxa at 6.33 Mya (95% HPD = 2.13–10.38). The expanded intra-specific and inter-island composition of Dataset 1 (49 individuals, [Fig. 4](#)) resolved the relationships within and between populations better than the other datasets. The first divergence (between *R. oreojasme* and the rest of the Canarian endemics) occurred *c.* 5.03 Mya (95% HPD = 2.60–7.74). In the *R. pinnata*-*R. microcarpa* complex, the split between Clade II (HER + INF, east- and south-west Tenerife) and Clade III was dated at 3.33 Mya, 95% HPD = 1.71–5.44). Clade IIIa (with individuals from the palaeo-islands in western Tenerife and from western La Gomera), diverged *c.* 2.67 Mya from the rest of populations analysed in eastern Tenerife, La Palma and La Gomera. In Clade IIIb, the separation between Tenerife and La Palma was relatively recent (1.47 Mya, 95% HPD = 0.58–2.64). The populations from Clade IIIc diverged *c.* 0.90 Mya (95% HPD = 0.30–1.79). The three populations from north-eastern La Gomera (*R. microcarpa*) and the only population of *R. nanocarpa* diverged more recently, between 0.09 and 0.38 Mya.

ANCESTRAL AREA RECONSTRUCTIONS AND HAPLOTYPE NETWORK

The ancestral area reconstructions with the S-DEC + J and S-DIVA models gave similar results ([Fig. 5](#)) and resolved Tenerife as the ancestral area for the three species distributed in the westernmost islands, whereas Gran Canaria was the ancestral area for *R. oreojasme*. Dispersal prevailed over vicariance in the reconstructions of the two models, but the S-DEC + J model detected more events of reciprocal migration between Tenerife and La Gomera, which affected several populations of *R. pinnata* and *R. microcarpa*.

The TCS analysis identified 30 haplotypes (A– δ in [Fig. 6A](#)): 11 (A–K) in *R. oreojasme* (Gran Canaria), 17 (L–Z plus α and β) in *R. pinnata* (Tenerife and La Palma) and three (W, γ , δ) in *R. microcarpa* (La Gomera). Haplotype W was shared by two islands [western Tenerife (only Masca) and western La Gomera], and some other haplotypes were shared among populations of the same island. Southern Gran Canaria (*R. oreojasme*) was

the area with the highest genetic diversity detected (haplotypes A–K). No evident geographical structure was found among the haplotypes of this taxon. The estimated haplotype network ([Fig. 6B](#)) distinguished several groups among *R. pinnata* and *R. microcarpa* that matched the clades of the Bayesian phylogenetic tree ([Fig. 4](#)). Haplotype (S) from Herques (south-eastern Tenerife) was shared only with Barranco del Infierno (western Tenerife), which also showed an exclusive haplotype (V) that was similar to those from Barranco de Masca and Alojera (T, U, V, W). Also, Barranco de Masca and El Fraile (north-western Tenerife) shared haplotype (β). Notably, the haplotype from Alojera (W) was greatly divergent from that shared by the remaining populations of La Gomera including Roque Ipalán (γ), which was more related to north-western Tenerife (X, Y, Z, α , β). Populations from Anaga, Barranco de Chacorche and Barranco de Badajoz (all in Tenerife) showed exclusive haplotypes (P, Q and R, respectively) that were closely related to those from populations of La Palma (L, M, N, O). The network also showed a circular relationship (loop) among haplotypes in north-western Tenerife, north-eastern Tenerife and on La Palma, although haplotypes from the first area maintain a substantial distance to the others (five nucleotide substitutions at least).

DISCUSSION

A COMPLEX GEOGRAPHICAL DISTRIBUTION OF THE GENETIC DIVERSITY

Our results for the species distributed in the western islands are not fully consistent with the current taxonomic classification of *Ruta* in the Canarian archipelago ([Bramwell & Bramwell, 2001](#); [Acebes *et al.*, 2010](#)). Instead, they best agree with earlier investigations ([Santos, 1983](#); [Hansen & Sunding, 1985, 1993](#); [Marrero *et al.*, 1988](#)), highlighting the fact that the colonization of the Canarian archipelago by *Ruta* was a complex process entailing high levels of gene flow between and within different islands.

The results of our molecular analyses with a broad populational sampling differ from [Salvo *et al.* \(2010\)](#), who argued that La Gomera was the putative ancestral area of the Canarian endemic taxa of *Ruta*. In contrast, our ancestral area analysis ([Fig. 5](#)) indicates that Tenerife played a central role in the dispersal of the genus in the western part of the Canarian archipelago. Moreover, ecological shifts from a common ancestor between southern and northern Tenerife (facing and opposing the humid trade winds, respectively) may underlie the prior split between *R. microcarpa* and *R. pinnata* (2.67 Mya). Overall, our results point out that recurrent inter-island dispersal among similar habitats has been an important factor in the

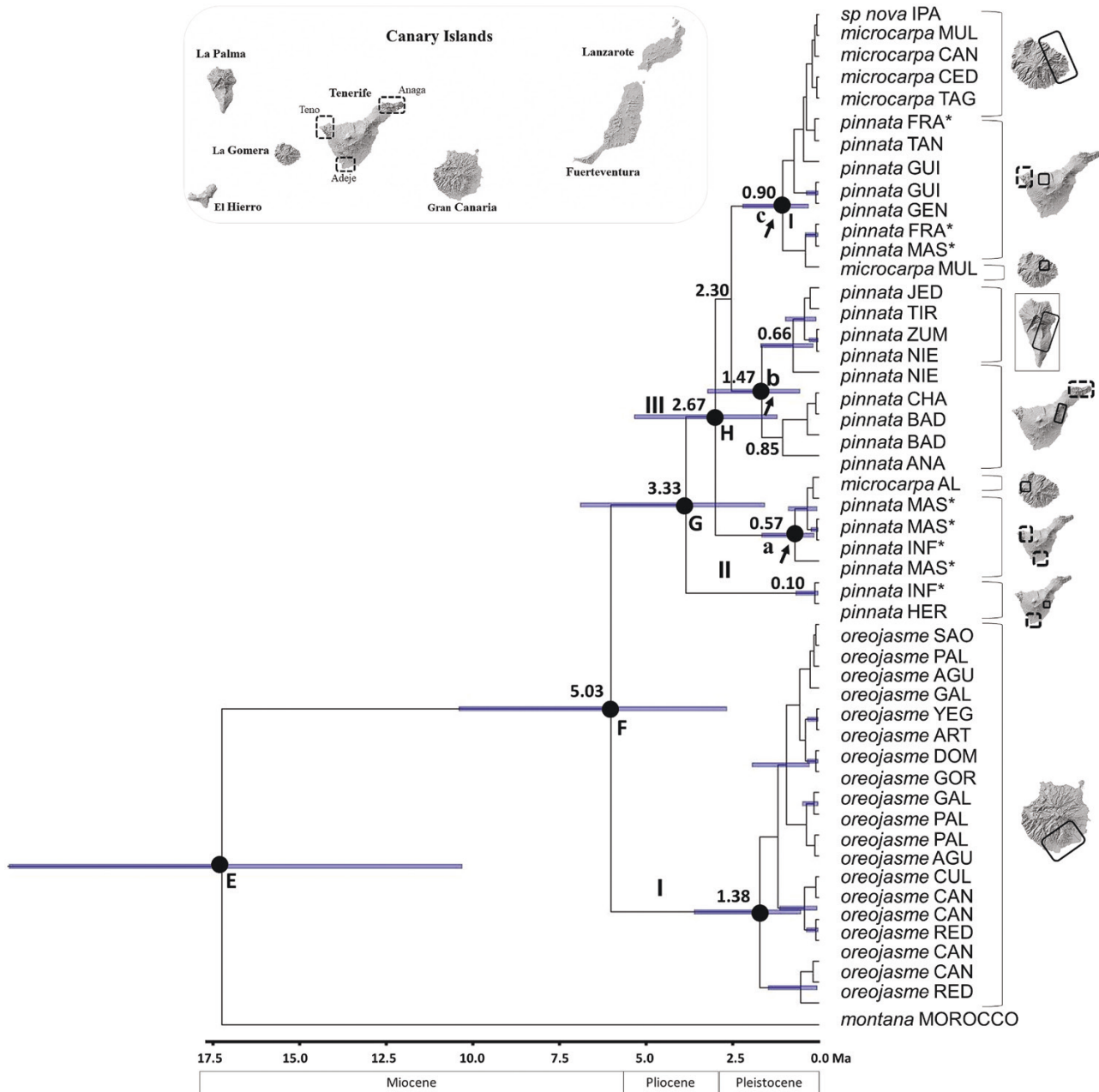


Figure 4. Bayesian chronogram obtained for 31 populations of Canary endemic *Ruta*, with *R. montana* as the outgroup (Dataset 1, 49 individuals), based on the concatenated plastid DNA regions (*psbA-trnH*, *rps16-trnK*, *trnT-trnL* and *atpB-rbcL*). The maximum clade credibility tree was inferred with BEAST under a strict clock. Mean ages of nodes of interest (Supporting Information, Table S2) are shown above the branches. Major clades are identified as I, II and III, and subclades of Clade III are labelled a, b and c. Inferred recurrent dispersal events between Tenerife and La Gomera and between Tenerife and La Palma are depicted by an arrow. Areas of distribution on the corresponding islands are highlighted with a solid line box and with a dashed line box for the palaeo-islands. Asterisks after population names indicate distribution in the palaeo-islands. Blue bars represent the 95% highest posterior density intervals for the main nodes. Major geological periods are indicated.

diversification of *Ruta* in the westernmost islands of the archipelago, where ongoing differentiation processes still appear to be occurring.

Our dating analyses suggest an earlier divergence (c. 5.03 Mya, Supporting Information, Table S2) between *R. oreojasme* and the remaining Canarian

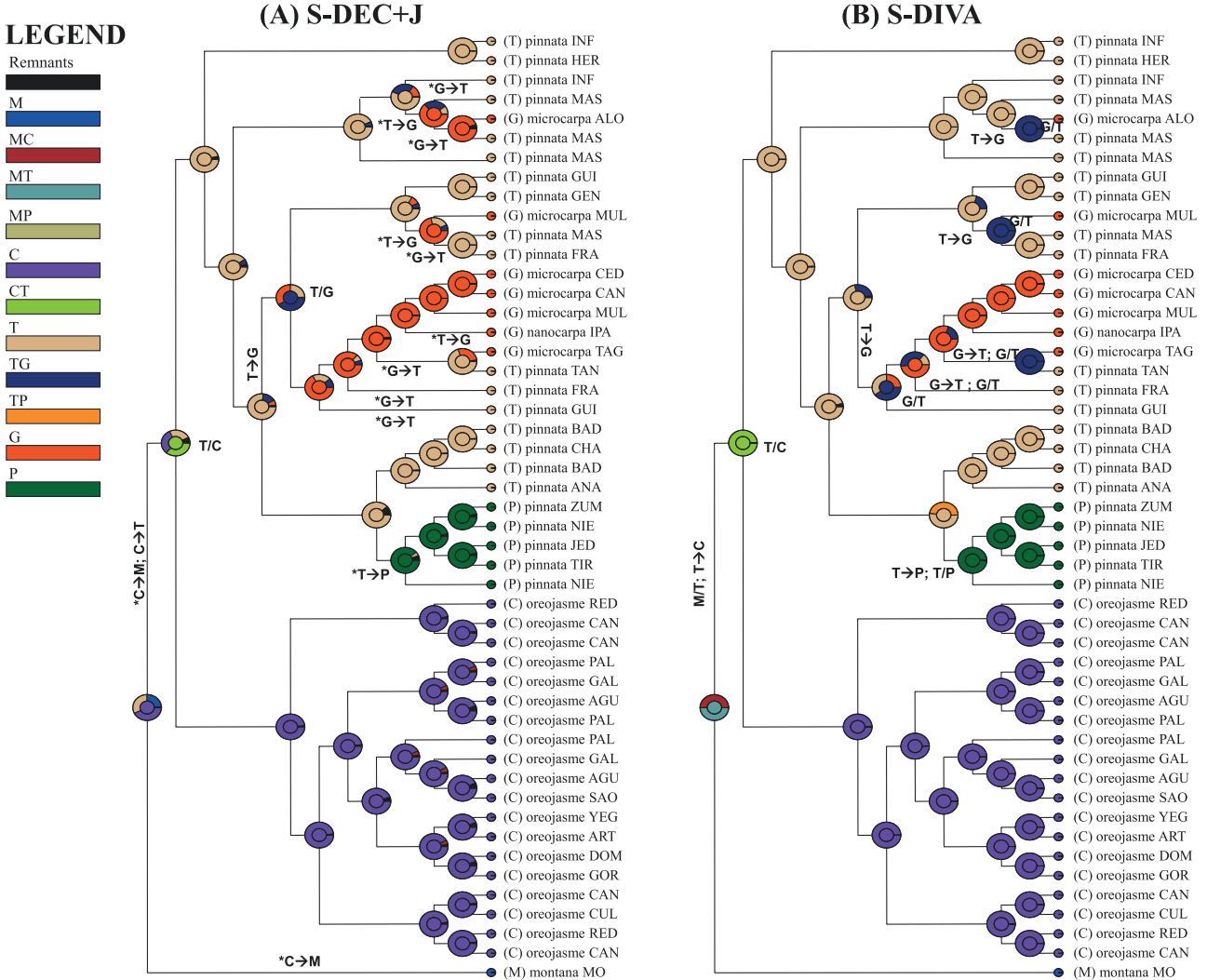


Figure 5. Ancestral geographical range reconstruction for Canary Islands *Ruta* using the posterior distribution of trees generated by BEAST on Dataset 1 (49 individuals) under models A, S-DEC + J and B, S-DIVA, following Matzke (2014) and Ronquist (1997) (see Material and methods). Colour codes of the geographical regions are given in the legend (M: Mainland, C: Gran Canaria, G: La Gomera, P: La Palma, T: Tenerife). Arrows and slanted bars indicate dispersal between islands or regions and vicariance, respectively. The central colours of the circles indicate the most probable geographical range of the corresponding node. The rings surrounding these central circles represent the relative probability of each geographical area being the ancestral area. Asterisks in A correspond to probable founder events.

endemics, which could contribute to explain the high genetic diversity detected in the former taxon (Fig. 6), together with a relatively undisturbed evolutionary history in south-eastern Gran Canaria (Meloni *et al.*, 2015).

Although the resolution of the origin of the Canary Islands *Ruta* is not among the objectives of this investigation, the 35 steps that separate the outgroup species *R. montana* from *R. oreojasme* (Fig. 3) suggest two non-exclusive possibilities. One of them is that an unsampled species could be the closest ancestor of the Canary Islands taxa. Gran Canaria is older and much closer to the continent than La Gomera or Tenerife, and it

may have been a stepping stone for the colonization of the western islands prior to the differentiation of *R. oreojasme*.

Another possibility is that the current distribution of *Ruta* in the archipelago could be due to independent introduction events giving rise to *R. oreojasme* and to the species in the westernmost islands. Although we only used one outgroup and our results do not show clear evidence of several colonizations from distantly related lineages, as in *Teline* Medik. (Percy & Cronk, 2002) or *Convolvulus* L. (Carine *et al.*, 2004), this possibility is supported by clear morphological differences in fruits, flowers and chromosome number

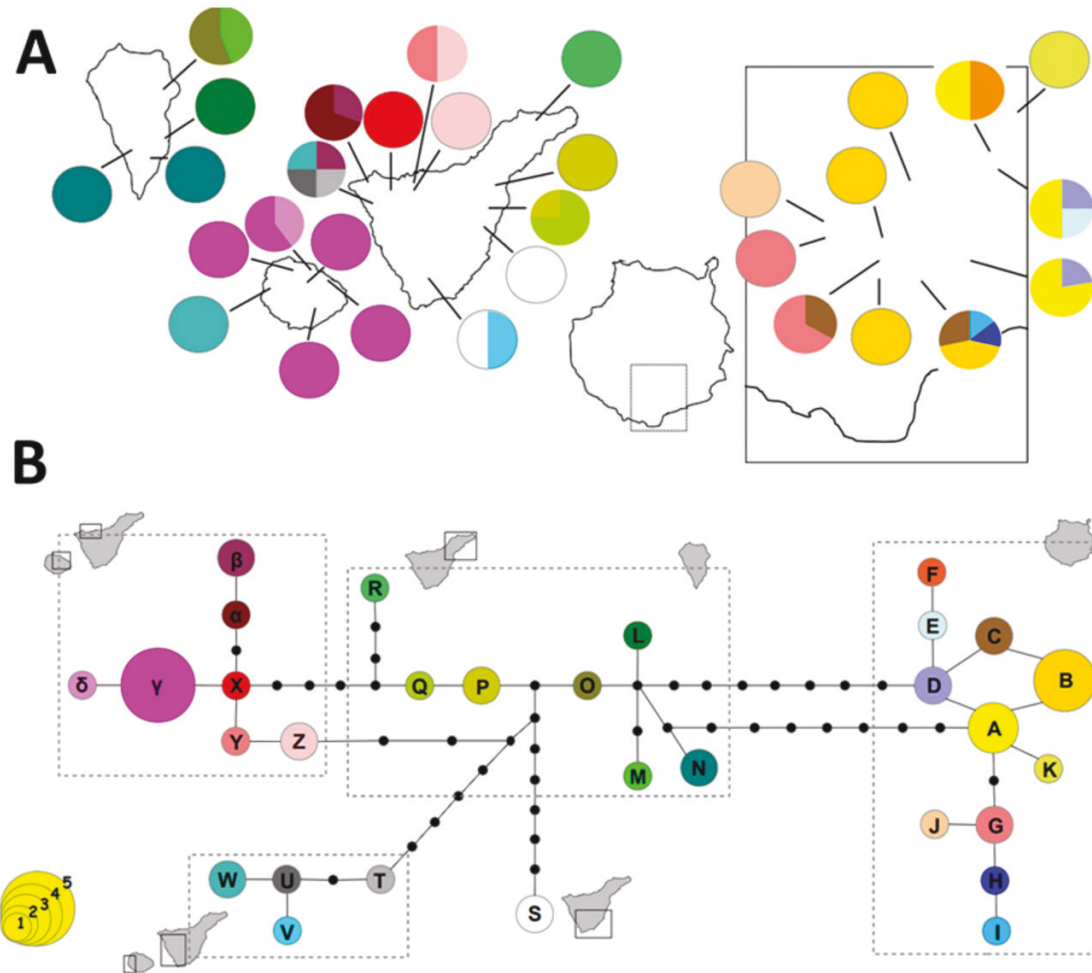


Figure 6. A, Geographical distribution of the 30 plastid DNA haplotypes detected in Canarian endemic *Ruta* (11 for Gran Canaria, 13 for Tenerife, four for La Palma and three for La Gomera). B, Haplotype TCS network based on four plastid DNA regions. Each haplotype is shown in a different colour; circle sizes are proportional to the haplotype frequencies (i.e. number of populations where each haplotype was detected). Black dots indicate missing haplotypes. Distinct groups are encased in boxes.

between *R. oreojasme* and other Canarian taxa (see Material and methods).

Our data indicate that independent secondary dispersal events have occurred relatively recently between (1) north-eastern La Gomera and north-western Tenerife (c. 0.90 Mya) and (2) western La Gomera (ALO) and the palaeo-islands of Teno and Adeje (c. 0.57 Mya) (Fig. 5, Supporting Information, Table S2). The latter divergence time estimate coincides with the latest recorded volcanic events in southern Tenerife, 0.8–0.6 Mya (Alonso-Blanco, 1989), which probably caused several population extinctions in the area.

Notably, the only haplotype detected in Alojera (W, Fig. 6) is shared with several individuals from Barranco de Masca, suggesting recent colonization from that area of Tenerife (Fig. 5). These secondary

dispersal events between Tenerife and La Gomera were probably promoted by the endozoochorous dispersal traits of these taxa (see Material and methods). Also, a relationship between the populations from the palaeo-islands of Tenerife and La Gomera is not unprecedented in the phylogeographic studies of plant lineages from the Canary Islands (Vitales *et al.*, 2014; Mairal *et al.*, 2015; Sun *et al.*, 2016).

The populations sampled in the three palaeo-islands (Table 1, Fig. 1) are placed in different clades (Figs 3 and 4), also suggesting that they may have originated from different inter-island dispersal events. Adeje is the oldest palaeo-island (with an estimated age of c. 11.9–8.9 Myr, Carracedo, 2011), and some individuals from this area (Barranco del Infierno, INF) are placed as sister to the remaining individuals in the phylogenetic tree.

In contrast, individuals of the palaeo-islands of Anaga and Teno (El Fraile) are placed in a derived position (Figs 4 and 5), like most of the palaeo-island endemics reviewed by Trusty *et al.* (2005) or Mairal *et al.* (2015). Therefore, the palaeo-islands of Tenerife probably played a relevant role as reservoirs of genetic diversity in the evolution of *Ruta* in the archipelago and as recent cradles of differentiation and dispersal (Mairal *et al.*, 2015). In *R. pinnata s.l.*, the missing haplotypes (up to ten changes between the samples from Anaga and Teno, Fig. 6B) plausibly indicate extinction in the younger area of Tenerife after the merging of the palaeo-islands (3.5 Mya, Ancochea *et al.*, 1990) and the subsequent volcanic upheavals in the central and northern areas (Ancochea *et al.*, 1999; Cantagrel *et al.*, 1999; Carracedo, 2011).

Great genetic divergence between the palaeo-islands of Teno and Anaga (north-western and north-eastern Tenerife, respectively; Fig. 1) has been reported in other widespread plant species [e.g. *Hypericum canariense* L. (Dlugosch & Parker, 2007); *Canarina canariensis* (L.) Vatke (Mairal *et al.*, 2015); *Micromeria hyssopifolia* Webb & Berthel. (Puppo, Curto & Meimberg, 2016)]. Our divergence time estimates for the colonization of the palaeo-islands by *Ruta* encompass a wide timespan (3.33–0.38 Mya, Fig. 4) probably reflecting (1) a long history of isolation among some populations in the palaeo-islands and (2) the consequences of recent geological events in central areas of Tenerife that may have had a strong impact in the divergence among the populations distributed in the palaeo-islands (in line with, e.g. Gübitz, Thorpe & Malhotra, 2000; Moya *et al.*, 2004; Puppo *et al.*, 2016). The ancestral area reconstructions and dating analyses (Figs 4 and 5) bolster the hypothesis that *R. nanocarpa* originated on La Gomera following a recent intra-island dispersal event from an ancestor related to *R. microcarpa*.

GENETIC AND TAXONOMIC SHORTFALLS IN THE CANARIAN FLORA

We contend that the discovery of such complex patterns of relationships within and among islands in the Canarian *Ruta* had remained elusive until now largely because the published phylogenetic research was based on incomplete sampling schemes. In light of the data presented here, the ongoing taxonomic evaluation of this circumscription of the genus (Marrero, in prep.) is highly needed. Both recent speciation (*R. nanocarpa* on La Gomera) and cryptic speciation (Anaga and the populations in north-western Tenerife) seem to have occurred. In other cases (e.g. in *R. pinnata* and *R. microcarpa*), our molecular data coincide with previous morphological differences highlighted by several authors, which had been systematically underestimated (see Introduction)

or discarded. Consequently, it is feasible that the genetic differences detected for other populations are associated with overlooked morphological differences that could provide a better basis for a more accurate taxonomic classification of Canarian *Ruta*.

Consistent with previous studies on both relatively widespread and narrowly distributed Canarian plant lineages (see previously), our results reveal the existence of glaring genetic and taxonomic shortfalls in this archipelago. Likewise, the current knowledge of the Canarian narrow endemic species and SIE should be revised, because it is mostly based on sampling schemes unrepresentative of the corresponding distribution ranges.

IMPLICATIONS FOR CONSERVATION

Together with other recent and ongoing molecular investigations, the present study may have important implications for the conservation of plant endemism in the Canarian archipelago. In *R. microcarpa*, our findings in the population from Alojera are relevant for conservation strategies. The morphological traits of this population, near to the *locus classicus* of *R. microcarpa* (Sventenius, 1969), fit with the original description of the species in relation to size and the linear-lanceolate, slightly thick, leaflets. The substantial genetic divergence found with respect to other populations from this island ascribed to *R. microcarpa* singles out Alojera as the only extant population of the species, with only 63 individuals at present (Mesa *et al.*, 2003). However, the category of threat for *R. microcarpa* was recently lowered from Critically Endangered to Endangered (Mesa *et al.*, 2011) due to the discovery of new populations in northern La Gomera that were ascribed by default to that species; as shown by our results, this was erroneous.

With ancillary morphological data that will be provided by the ongoing taxonomic review of the Canarian circumscription of *Ruta* (Marrero *et al.*, in prep.), our results compellingly indicate a greater relatedness of Alojera (La Gomera) to Barranco del Infierno and Barranco de Masca in Tenerife, which are currently circumscribed to *R. pinnata* (now considered ‘near threatened’, Moreno, 2011). In fact, the morphological traits of the populations in northern La Gomera fit well with the description of *R. pinnata* (Marrero *et al.*, 1988, and unpublished data).

Overall, these diverse results contribute to the extremely dynamic and largely overlooked speciation context that is being revealed in the Canaries (see Caujapé-Castells *et al.*, 2017, for a general account), thus concurring with one of the main tenets of the theory of evolution (Darwin, 1859; Dobzhansky, 1937), that is, that the population is the basic unit of natural selection and should be the minimum unit of conservation, even in the absence of genetic or other data, and especially on oceanic island biodiversity hotspots.

The concept of ‘evolution in action’ often applied to oceanic island floras, requires dynamic conservation actions that demand a more collaborative and frequent dialogue between scientists, managers, policy makers and other stakeholders. While the needed taxonomic review develops, our results for *R. pinnata* and *R. microcarpa* advise extreme caution in eventual conservation strategies, which should avoid at all costs mixed reinforcements, either on or between islands. This advice is applicable to most *in situ* conservation strategies in the Canarian flora, especially when no ancillary taxonomic, genetic or reproductive data are available to avoid errors that may lead in some cases to the rapid extinction of new, but unknown, evolutionary units.

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DATA AVAILABILITY

The data underlying this article are available in the GenBank Nucleotide Database at <https://www.ncbi.nlm.nih.gov/genbank/>, and can be accessed with the unique identifiers given in Table 2.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

File S1. PCR conditions used for the amplification of the DNA regions used in the paper. The PCR program for *psbA-trnH* included a denaturation step of 2 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 1 min at 54.3 °C (annealing) and 1 min at 72 °C, followed by an extension step of 5 min at 72 °C. Amplification of *rps16-trnK* consisted of 5 min at 95 °C, followed by 30 cycles of 1 min at 95 °C, 1 min at 50.7 °C and 1 min at 65 °C, followed by a step of 5 min at 65 °C. Amplification of *trnT-trnL* consisted of 3 min at 94 °C, followed by 30 cycles of 50 s at 94 °C, 50 s at 55 °C and 1.5 min at 72 °C, followed by a step of 10 min at 72 °C. Amplification of *atpB-rbcL* consisted of 2 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 1 min at 48 °C and 1 min at 72 °C, followed by a step of 10 min at 72 °C.

Table S1. Vouchers, year of collection and collectors corresponding to the species and populations included in this study. Collectors' codes: AM: Á. Marrero, AP: A. Portero, CS: C. Santiago, AR: A. Reid, FM: F. Medina, GH: G. Hernández, JCC: C. Caujapé-Castells, JFP: J. Fernández-Palacios, JM: J. Martín-Carbajal, MM: M. Meloni, MS: M. Soto, PR: P. Romero, RJM: R. Jaén-Molina, RM: R. Mesa

Table S2. Values obtained for the different models of substitution and molecular-clock explored using marginal likelihood estimation (MLE) implemented in BEAST. The age of the crown-node, the first divergence event in the Canarian endemics *Ruta* was constrained using a normal prior based on an age estimate from previous analyses (Salvo *et al.*, 2010, and additional analysis performed in this paper with *R. montana* as outgroup). Highlighted in bold are the results shown in Figure 4. Mean ages estimated for the nodes of the relevant clades and subclades (I, II and III a, b and c) and 95% HPD credibility interval (in brackets) are also included

Table S3. Number of taxa, populations (Pop), individuals (*N*) and the different DNA regions included in each dataset analysed in this study

Figure S1. Bayesian phylogenetic tree of Rutaceae constructed based on plastid DNA sequences *matK* (see Supporting Information, Table S2, for GenBank accessions). Mean node ages are indicated and 95% HPD confidence intervals of divergence time are represented by blue bars.

Figure S2. Bayesian phylogenetic tree obtained for the Canarian endemic taxa of *Ruta* (for *R. pinnata*, a sample from one population in each palaeo-island* of Tenerife was included) constructed based on DNA sequences of four plastid regions (see Supporting Information, Table S2 for GenBank accessions). Blue bars represent confidence intervals of divergence time, which are shown to the right of internal nodes.