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Southern isolation and northern long-distance dispersal shaped the phylogeography of the widespread, but highly disjunct, European high mountain plant *Artemisia eriantha* (Asteraceae)

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We investigated the range dynamics of *Artemisia eriantha*, a widespread, but rare, mountain plant with a highly disjunct distribution in the European Alpine System. We focused on testing the roles of vicariance and long-distance dispersal in shaping the current distribution of the species. To this end, we collected AFLP and plastid DNA sequence data for 17 populations covering the entire distributional range of the species. Strong phylogeographical structure was found in both datasets. AFLP data suggested that almost all populations were genetically strongly differentiated, with 58% of the overall genetic variation partitioned among populations. Bayesian clustering identified five groups of populations: Balkans, Pyrenees, Central Apennines, one southwestern Alpine population and a Widespread cluster (eastern Pyrenees, Alps, Carpathians). Major groups were supported by neighbor-joining and NeighbourNet analyses. Fourteen plastid haplotypes were found constituting five strongly distinct lineages: Alps plus Pyrenees, Apennines, Balkans, southern Carpathians, and a Widespread group (eastern Pyrenees, northern Carpathians, Mt. Olympus). Plastid DNA data suggested that *A. eriantha* colonized the European Alpine System in a westward direction. Although, in southern Europe, vicariant differentiation among the Iberian, Italian and Balkan Peninsulas predominated, thus highlighting their importance as glacial refugia for alpine species, in temperate mountain ranges, long-distance dispersal prevailed. This study emphasizes that currently highly disjunct distributions can be shaped by both vicariance and long-distance dispersal, although their relative importance may be geographically structured along, for instance, latitude, as in *A. eriantha*. *Botanical Journal of the Linnean Society*, 2014, **174**, 214–226.

ADDITIONAL KEYWORDS: AFLP – chloroplast DNA – European Alpine System – glacial refugia – vicariance.

INTRODUCTION

Disjunctions are defined as cases in which populations, closely related species or higher level taxa occur in widely separated regions, but are absent from intervening areas. Generally reflecting past events, disjunctions may be traced back to dispersal across geographical barriers or may result from the fragmentation of a formerly continuous distribution area (i.e. vicariance) (Lomolino, Riddle & Brown, 2006). In biogeography, disjunctions are one of the longest studied and best illustrated phenomena; the distribution patterns of the southern beeches (*Nothofagus* Blume) and the flightless ratite birds strongly shaped

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the ideas of, for instance, Charles Darwin and Joseph Dalton Hooker (Hooker, 1844–1859; Darwin, 1859).

Events that cause disjunctions have been the subject of a long-lasting debate. Darwin (1859) regarded long-distance dispersal (LDD) as a potential driving force generating disjunctions, whereas other researchers of his time invoked the disruption of pre-existing land connections to explain disjointed distribution patterns (e.g. Hooker, 1844–1859). Although vicariance can potentially explain several events simultaneously, as a single geographical barrier may affect the distribution areas of multiple taxa (e.g. the break-up of Gondwanaland), LDD patterns are often idiosyncratic, and thus their predictability is limited. Nevertheless, the idea that rare and stochastic LDD events, mostly caused by non-standard vectors, may strongly shape distribution patterns is gaining increasing support (Nathan *et al*., 2008). This renewed interest in LDD is, in part, a result of the widespread application of genetic markers that often allow recent LDD events and vicariance to be distinguished. In the case of vicariance, the disruption of formerly continuous distribution areas should lead to reciprocally monophyletic lineages, whereas descendants of a recent LDD event are expected to be closely related to their source populations and thus exhibit a certain degree of nestedness. However, this genetic signal may erode over time, rendering the distinction between vicariance and ancient LDD difficult (e.g. De Queiroz, 2005; Kropf, Comes & Kadereit, 2006; Schneeweiss & Schönswetter, 2010).

A geographical system suitable for elucidating the influence of recent LDD and old vicariance on the range dynamics of extant biota is provided by the European Alpine System. It comprises several disjoint high mountain ranges (Sierra Nevada, Cordillera Cantábrica, Pyrenees, Alps, Apennines, Carpathians, mountain ranges of the Balkan Peninsula). These central and southern European ranges have experienced a complex history of geotectonic events and climatic fluctuation, including Neogene orogeny (Plaziat, 1981) and successive Quaternary glacial advances and retreats characteristic of the Northern Hemisphere (Bennett, 1997).

Reflecting the geographical complexity of the European Alpine System, many species of the European mountain flora exhibit disjunctions on a variety of geographical scales (Favarger, 1975; Meusel & Jäger, 1992). Several studies have explored the relationships between neighbouring mountain ranges, such as the Alps and the Pyrenees (e.g. Küpfer, 1974; Schönswetter *et al*., 2002; Kropf *et al*., 2006) or the Alps and the Carpathians (Mráz *et al*., 2007; Paun *et al*., 2008; Ronikier, Cieślak & Korbecka, 2008), but studies of endemic plants distributed throughout the entire or almost entire European Alpine System are more scarce (Kropf, Kadereit & Comes, 2003; Csergö *et al*., 2009; Schneeweiss & Schönswetter, 2010). These studies show that range formation in widely but disjunctly distributed alpine species is often complex, involving both vicariance and dispersal events, but that their relative importance remains inadequately understood.

A suitable model for studying the impact of vicariance and LDD on range formation in the European Alpine System is *Artemisia eriantha* Ten. [*A. petrosa* (Baumg.) Jan. ex DC]. This rare, herbaceous perennial mountain plant is found in small and isolated populations in a highly disjunct distributional area in the southern European mountain ranges from the Pyrenees across the south-western Alps and the Apennines to the Carpathians and the Balkan Peninsula (Fig. 1). Here, it grows on siliceous or, more rarely, limestone rock ledges and in crevices at elevations between 1500 and 3400 m (Piękoś-Mirkowa & Miechówka, 1998; Villar, Sesé & Ferrández, 2003). *Artemisia eriantha* is mainly wind pollinated, but is also visited by insects (Vallès & McArthur, 2001). Nothing is known about its breeding system, but, for the closely related *A. granatensis* Boiss, self-incompatibility has been determined experimentally (Hernández-Bermejo *et al*., 1999). As a result of its rarity, the species is included in Annex V of the EC Habitats Directive (European Community, 1992) and the Red Data lists of the Polish Carpathians and Slovakia (Mirek & Piękoś-Mirkowa, 1992; Maglocký & Feráková, 1993). It has been suggested that populations from the Carpathians and the central Apennines, respectively, belong to distinct subspecies (Futák, 1975; Pignatti, 1982). *Artemisia eriantha* is diploid ($2n = 18$; Vallès & Oliva, 1990) and is thought to be patroendemic (Favarger & Siljak-Yakovlev, 1986) within the dysploid–polyploid alpine members of *Artemisia* L. group *Leucophorae* (Gutermann, 1979). Favarger (1975) considered this species to be a member of the central and southern Tertiary European orophilous flora, the morphology of which has not changed since the separation of the mountain chains. In contrast, Küpfer (1974) pointed out that its cytological and morphological homogeneity can also be explained by its recent arrival in the Pyrenees and the Alps during Pleistocene glaciations.

Here, we explore the range dynamics of *A. eriantha*. Specifically, we test whether the currently highly disjunct distribution is solely the result of vicariance, as suggested by Favarger (1975), or whether LDD also contributed to range formation, as proposed by Küpfer (1974). To this end, we apply highly polymorphic, mostly nuclear-derived (Meudt & Clarke, 2007) AFLP markers with maternally inherited (in Asteraceae: Corriveau & Coleman, 1988) plastid DNA sequences.

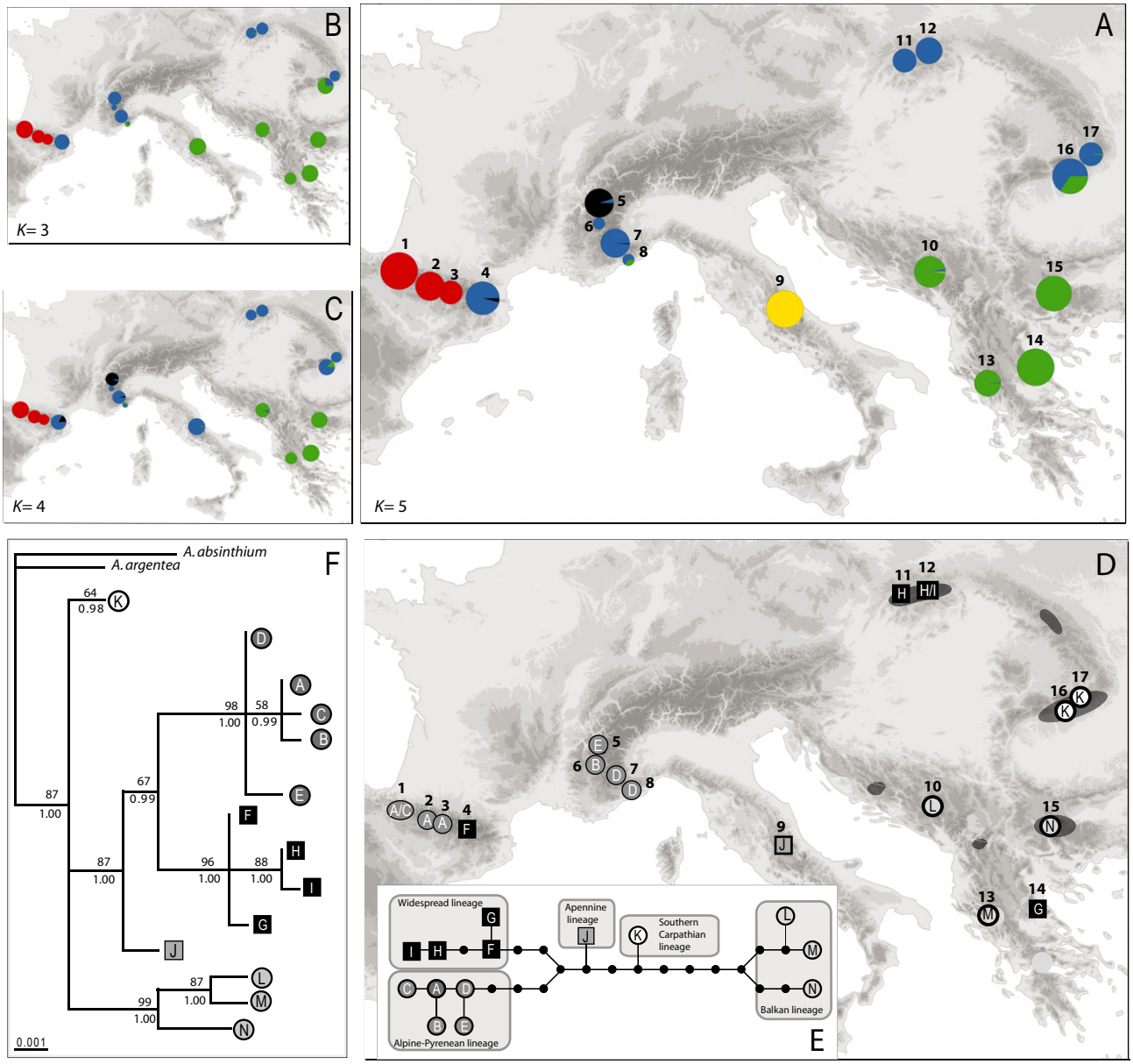

Figure 1. Geographical distribution, plastid DNA haplotype diversity and AFLP clusters of *Artemisia eriantha*. A–C, Results of Bayesian clustering analysis of AFLP data with the program STRUCTURE with *K*, the number of gene pools, set to 3–5. B and C are suboptimal clustering solutions, whereas A received the highest likelihood scores (see Fig. S2). The size of the pie charts corresponds to the number of investigated individuals per population and the colours represent different gene pools (in the optimal grouping: red, Pyrenean cluster; blue, Widespread cluster; green, Balkan cluster; black, Galibier cluster; yellow, Apennine cluster). D, Distributional area (covered by the symbols or shaded in grey), sampled populations (numbered, for details see Table 1) and plastid DNA haplotypes designated with capital letters. E, Statistical parsimony network of plastid DNA haplotypes. Designation of sampled haplotypes as in (D) and (F), unsampled mutational steps are illustrated as black dots. F, Phylogenetic relationships among plastid DNA haplotypes based on Bayesian inference. Bayesian posterior probability and bootstrap support values derived from a maximum parsimony analysis are given below and above the branches, respectively.

MATERIAL AND METHODS

Sampling

One hundred individuals from 17 populations covering the entire distributional range of *A. eriantha* were sampled in 2004 and 2005 (see Supporting Information Table S1; Fig. 1; Table 1). Whenever possible, leaf material of five to ten plants per population was collected and stored in silica gel; exceptions were populations 6-Laurichard and 8-Ladres, where only a single individual was found in each case. Voucher specimens were deposited at the herbaria of the Centre de Documentació de Biodiversitat Vegetal de la Universitat de Barcelona (BCN), the Botanical Institute of Barcelona (BC), the Polish Academy of Science in Kraków (KRAM) or the University of Belgrade (BEOU).

DNA extraction, AFLP fingerprinting and DNA sequencing

Total genomic DNA was extracted from 10 mg of silica gel-dried leaf material following Schönswetter *et al*. (2002). The AFLP procedure followed Gaudeul, Taberlet & Till-Bottraud (2000), but reaction volumes in the polymerase chain reactions (PCRs) were halved. The following three selective primer combinations were selected from the 12 tested (fluorescent dye in parentheses): *Eco*RI ACA (6-FAM)–*Mse*I CAC; *Eco*RI ACC (NED)–*Mse*I CAG; *Eco*RI AGG (VIC)–*Mse*I CTG. For each individual, 1.2 μL 6-FAM-, 2 μL VIC- and 3 μL NED-labelled selective PCR products were combined and precipitated with sodium acetate, washed with 70% ethanol, dried, resuspended in 0.2 μL GeneScan ROX 500 (Applied Biosystems, Foster City, CA, USA) and 9.8 μL formamide (Applied Biosystems), and run on a capillary sequencer ABI 3100 (Applied Biosystems). Negative controls, within and between run replicates, were routinely included to test for contamination and reproducibility. Raw data were collected and aligned with the internal size standard using GeneScan 3.7 (Applied Biosystems). The GeneScan files were imported into GENOGRAPHER 1.6 (formerly available at http://hordeum.oscs.montana.edu/genographer) for scoring. Fragments in the size range 75–500 bp were scored. The data were exported as a presence/absence matrix. Duplicated individuals were used to test reproducibility and to calculate the error rate (Bonin *et al*., 2004). Markers which were non-reproducible and markers scored as present for fewer individuals than the error rate were removed from the dataset.

Two to four individuals per population were screened for plastid DNA sequence variation, except for the populations 6-Laurichard and 8-Ladres. Three regions were amplified and sequenced using the following primers: SP43122F and SP44097R (Hershkovitz, 2006) for the intergenic spacer *ycf3-trnS*; 3′-trnG^{UUC} and 5′-trnG2G (Shaw *et al*., 2005) for the *trnG* intron; rpl16F71 (Jordan, Courtney & Neigel, 1996) and Rex2 (5′-GATATTCCCTTCATTCTTCCT-3′; R. T. Kimball, University of Florida, Gainesville, FL, USA, pers. comm.) or rpl16Fc (5′-CAGTCAAGATATGATATATTGTTC-3′) and RexC (5′-AGAGTTTCTTCTCATCCAGCTC-3′) for the *rpl16* intron. The PCR profile was the same for all three regions and included a pre-heat step (94 °C for 4 min) and 35 cycles of amplification under the following conditions: 95 °C for 1 min, 52 °C for 1.5 min and 72 °C for 2 min, followed by an additional extension step of 10 min at 72 °C. The reactions were performed in a total reaction volume of 25 μL, consisting of 2.5 μL Taq buffer (Applied Biosystems), 2.5 μL $MgCl_2$ (25 mM), 2.5 μL deoxynucleoside triphosphates (dNTPs) solution (1 mM), 12.3 μL milliQ-H_2O, 0.2 μL AmpliTaq (Applied Biosystems), 1.0 μL of each primer (10 μM) and 3.0 μL total genomic DNA of unknown concentration. PCR products were purified with a Qiaquick PCR purification kit (Qiagen Inc., Valencia, CA, USA) or DNA Clean & Concentrator-5 (Zymo Research, Orange, CA, USA). Direct sequencing of the amplified DNA segments was performed using BigDye Terminator Cycle Sequencing v.3.1 chemistry (Applied Biosystems) following the manufacturer's protocol, and electrophoresis was performed on an ABI PRISM 3100 DNA Analyzer (Applied Biosystems).

Analysis of AFLP data

We performed a neighbor-joining (NJ) analysis based on Nei & Li genetic distances with TREECON 1.3b (Van de Peer & De Wachter, 1997). Support for the branches was estimated with 1000 bootstrap replicates using the same program. The tree was rooted with *Artemisia genipi* Stechm. based on previous analyses (M. Sanz, unpubl. data). To aid the visualization of putatively present reticulate patterns in the AFLP data, a NeighbourNet diagram based on the same distance matrix was produced with SPLITSTREE4 4.8 (Huson & Bryant, 2006).

To study the underlying genetic structure in more detail, we used two different approaches to identify genetically homogeneous groups by genetic mixture analysis: the Bayesian multilocus assignment method implemented in BAPS 2 (Bayesian Analysis of Population Structure; Corander *et al*., 2003) and model-based clustering based on a Bayesian Markov chain Monte Carlo (MCMC) approach implemented in STRUCTURE 2.2 (Pritchard, Stephens & Donnelly, 2000; Falush, Stephens & Pritchard, 2007). In contrast with STRUCTURE, BAPS uses stochastic optimization (Corander, Marttinen & Mäntyniemi, 2006) instead of MCMC, treats *K* (the appropriate number

Table 1. Geographical origin, summary of AFLP variation and plastid DNA haplotypes detected (number in parentheses) among the investigated populations of *Artemisia eriantha*. *n*, number of individuals investigated with AFLP; f_{tot}, total number of AFLP fragments per population; f_{poly}, number and percentage of polymorphic AFLP fragments; f_{pp}, number of polymorphic private AFLP fragments; f_{fp}, number of fixed private AFLP fragments

Population	Sampling locality	Longitude/latitude	*n*	f_{tot}	f_{poly}	f_{pp}	f_{fp}	Average gene diversity ± SD	Haplotypes
Pyrenees									
1-Infierno	Spain: Collado del Infierno	–0°13′/42°48′	10	88	47 (53.41)	1	0	0.0788 ± 0.0432	A(1)/C(1)
2-Urdiceto	Spain: Ibón de Urdiceto	0°15′/42°40′	6	97	38 (39.17)	1	0	0.0744 ± 0.0447	A(2)
3-Posets	Spain: Posets	0°25′/42°38′	4	98	19 (19.39)	0	0	0.0434 ± 0.0301	A(4)
4-Casamanya	Andorra: Pic de Casamanya	1°33′/42°34′	8	107	74 (69.16)	2	0	0.1319 ± 0.0736	F(4)
			28	157	116 (73.41)	11	0	0.1627 ± 0.0812	
Alps									
5-Galibier	France, Hautes-Alpes: Col du Galibier	6°24′/45°03′	6	106	63 (59.43)	5	5	0.1211 ± 0.0716	E(2)
6-Laurichard	France, Hautes-Alpes: Col de Laurichard	6°24′/45°06′	1	114	0	0	0	0	B(1)
7-Ischiator	Italy, Alpi Maritime, Cuneo upper lake Ischiator	7°02′/44°18′	6	111	65 (58.55)	0	0	0.1339 ± 0.0790	D(2)
8-Ladres	France, Alpes-Maritimes: Pas des Ladres	7°24′/44°05′	1	102	0	0	0	0	D(1)
			14	164	118 (71.51)	11	0	0.2021 ± 0.1047	
Central Apennines									
9-Corvo	Italy: Monte Corvo	13°30′/42°28′	10	94	53 (56.38)	2	0	0.0904 ± 0.0494	J(2)
Western Carpathians, High Tatras									
11-Liliowe Turnie	Poland: western Tatra Mountains, Liliowe Turnie	19°54′/49°13′	4	97	30 (30.92)	1	0	0.0724 ± 0.049	H(2)
12-Kondratowa	Poland: Kondratowa	19°57′/49°14′	5	91	38 (41.76)	1	0	0.0765 ± 0.0480	H(1)/I(1)
			9	122	67 (54.92)	2	0	0.1248 ± 0.0685	
Balkan Peninsula									
10-Durmitor	Montenegro: Durmitor, Sedlo	19°02′/43°08′	7	98	62 (63.26)	3	0	0.1101 ± 0.0632	L(2)
13-Ploskos	Greece: Ploskos	20°46′/39°58′	5	93	35 (37.63)	0	0	0.0739 ± 0.0465	M(2)
14-Olympus	Greece: Olympus	22°22′/40°05′	10	99	42 (42.42)	0	1	0.0674 ± 0.0372	G(2)
15-Rila	Bulgaria: Josifica Mountain	23°30′/42°00′	9	99	60 (60.60)	3	0	0.0992 ± 0.0548	N(2)
			31	149	104 (70.00)	13	0	0.1365 ± 0.0681	
Southern Carpathians									
16-Bucegi	Romania: Bucegi	25°27′/45°26′	4	95	37 (38.95)	1	0	0.0869 ± 0.0585	K(2)
17-Făgăraş	Romania: Făgăraş	25°39′/45°35′	4	107	38 (35.51)	2	6	0.0898 ± 0.0604	K(2)
			8	140	74 (52.85)	9	0	0.1375 ± 0.0767	

of groups) as a variable to estimate and gives a list of the best partitioning and their likelihood scores. In two sets of BAPS runs, both individuals and populations were used as clustering units, and values of K (the maximum number of clusters) in the range 2–17 (the number of geographical samples) were explored using three replicates for each value of K.

For the STRUCTURE analysis, we used a model with admixture, uncorrelated allele frequencies and recessive alleles. We performed ten replicate runs for each K value in the range 1–9 and 10^6 MCMC replicates (10^5 additional replicates as burn-in). The similarity between the results of different runs for the same K value was calculated according to Nordborg *et al*. (2005) using the R-script STRUCTURE-SUM (Ehrich, 2006). We identified the optimal number of main groups as the value of K at which the increase in likelihood started to flatten out, the results of replicate runs were identical and no empty groups were encountered. The replicate runs of the best K value were merged with CLUMPP 1.1.1 (Jakobsson & Rosenberg, 2007) using the full-search algorithm. The relative 'cluster membership coefficients' of all individuals were then averaged for each population.

Several statistics were calculated for each population and geographical group (Table 1), including the total number of AFLP fragments, the number and percentage of polymorphic loci, and polymorphic private (f_{pp}) and fixed private (f_{fp}) fragments. Polymorphic private fragments are confined to a single population or geographical area, whereas fixed private fragments are found in all investigated individuals of a single population or geographical area. Pairwise F_{ST} distances among geographical regions, analyses of molecular variance (AMOVAs; Excoffier, Smouse & Quattro, 1992), average gene diversity over loci (in the following termed 'genetic diversity') corrected for small sample size and its standard deviation for both sampling and stochastic processes were computed for populations and geographical regions using ARLEQUIN 3.0 (Excoffier, Laval & Schneider, 2005; available at http://cmpg.unibe.ch/software/arlequin3).

Plastid DNA analysis

The plastid DNA sequences were manually aligned using BIOEDIT 5.0.9 (Hall, 1999). To visualize the genetic relationships between the observed haplotypes, we constructed a statistical parsimony network using TCS 1.21 (Clement, Posada & Crandall, 2000). Indels longer than 1 bp were shortened to single base pair gaps and treated as a fifth character state.

In order to determine support for clades, phylogenetic trees were constructed using MRBAYES 3.1.1 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). MRMODELTEST 2.2 (Nylander, 2004) was used to determine the best-fit nucleotide substitution model. Bayesian analyses were initiated with random starting trees and four Markov chains were run simultaneously for 10^6 generations and sampled every 100 generations. For all analyses, the variance of split sequences was 0.01, indicating convergence of chains (Huelsenbeck & Ronquist, 2001). The first 25% of the runs were discarded as 'burn-in'. The 50% majority rule consensus phylogeny and posterior probabilities (PPs) of the nodes were calculated from the remaining sample. Nodes with PPs ≥ 0.95 were considered to be statistically supported. In addition, maximum parsimony analysis was performed in PAUP 4.0b10 (Swofford, 2002) using tree bisection–reconnection (TBR) branch swapping with character states (including gaps) specified as unordered and unweighted. We conducted a heuristic search with 1000 replicates with random taxon addition. Bootstrap support (BS) values were generated from 1000 replicates using the heuristic search option. Trees were rooted with *Artemisia absinthium* L. and *A. argentea* L'Hér. based on previous phylogenetic studies (Sanz *et al*., 2008). We tested the hypothesis of allopatric differentiation between European mountain ranges (H_1, vicariance hypothesis) using the approximate Bayes factor ($2 \times \ln BF$; Kass & Raftery, 1995), defined as the ratio of the estimated marginal likelihoods of the two compared models (i.e. unconstrained vs. vicariance model) computed using MRBAYES, where $2 \times \ln BF < -10$ and $2 \times \ln BF > 10$ indicate significant support for and against the vicariance hypothesis, respectively.

RESULTS

AFLP

Using three primer combinations, 229 AFLP fragments were scored, 185 (80.4%) of which were polymorphic. The error rate was 1.3%. Genetic diversity ranged from 0.0434 (population 3-Posets) to 0.1339 (population 7-Ischiator) (Table 1). There were no significant differences in genetic diversity among mountain ranges. Populations 5-Galibier and 17-Făgăraş harboured the highest number of fixed private fragments (five and six, respectively). The Pyrenees, Alps and Balkans were the mountain ranges with the highest number of polymorphic private fragments (11, 11 and 13, respectively; Table 1).

The NeighbourNet diagram (Fig. 2) was largely congruent with the NJ tree (see Supporting Information Fig. S1). Supported major groups were formed by four populations from the Balkans (10-Durmitor, 13-Ploskos, 14-Olympus, 15-Rila) and three populations from the western Pyrenees (1-Infierno, 2-Urdiceto, 3-Posets). In addition, a relatively

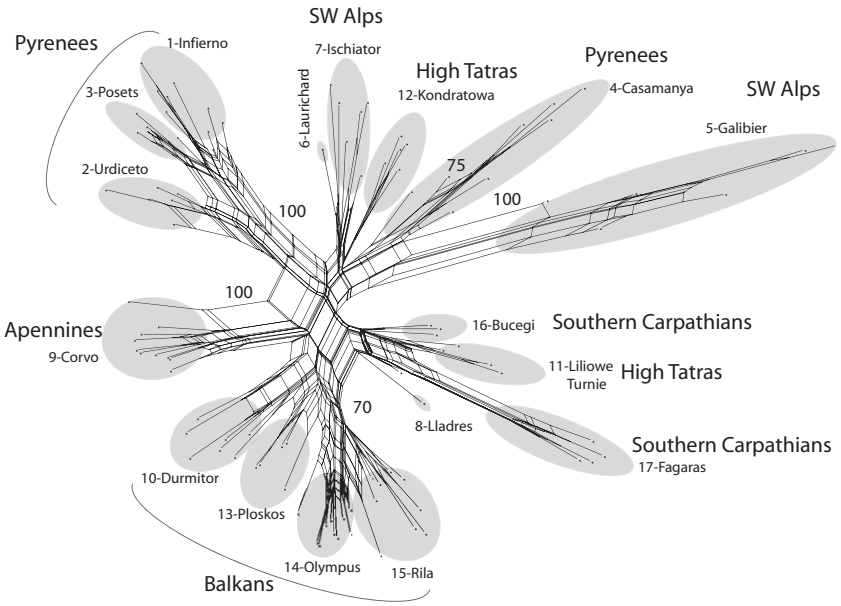

Figure 2. NeighbourNet of AFLP phenotypes of *Artemisia eriantha* constructed with SPLITSTREE4 from a Nei & Li distance matrix. Numbers are bootstrap values > 50% from a neighbor-joining analysis of the same matrix given for major groups only.

strongly weighted split separated the populations from the Balkans and population 9-Corvo from the remaining populations.

STRUCTURE inferred $K = 5$ as the optimal number of groups (for details, see Supporting Information Fig. S2), resulting in the following main clusters (Fig. 1A): (1) the Pyrenean cluster (populations 1-Infierno, 2-Urdiceto, 3-Posets); (2) the Balkan cluster (populations 10-Durmitor, 13-Ploskos, 14-Olympus, 15-Rila); (3) the Galibier cluster (population 5-Galibier); (4) the Widespread cluster (populations 4-Casamanya, 6-Laurichard, 7-Ischiator, 8-Ladres, 11-Liliowe Turnie, 12-Kondratowa, 16-Bucegi, 17-Făgăraş); and (5) the Apennine cluster (population 9-Corvo). Only populations 8-Ladres and 16-Bucegi showed admixture above a cut-off level of 64%. All of the main genetic groups were geographically coherent, with the exception of the Widespread cluster, which included populations from the Pyrenees, Alps, Tatras and southern Carpathians. To illustrate the hierarchical splitting of gene pools, the STRUCTURE clusters derived at $K = 3$ and $K = 4$ are also shown (Fig. 1B, C).

In separate BAPS analyses, using both individuals and populations as clustering units, the partition with the highest log marginal likelihood (–6499.40) consisted of seven clusters (PP = 1) in which populations 4-Casamanya and 17-Făgăraş were separated as independent clusters (see Fig. S1). The partition at $K = 5$ was identical to the STRUCTURE result (data not shown).

A non-hierarchical AMOVA attributed 58% of the overall genetic variation to the among-population component. In the hierarchical analysis, 20.4% of the variation was found among mountain ranges, whereas separate analyses revealed strong differentiation among populations within mountain ranges (42.7–54.7%; Table 2).

Plastid DNA sequences

DNA sequences of each haplotype per population were submitted to GenBank (Table S1). Fourteen variable characters were found in the 679-bp-long intergenic spacer *ycf3-trnS*, 11 in the intergenic spacer *trnG* (598 bp) and 14 in the *rpl16* intron (483 bp), resulting in a total of 39 variable characters, including indels, in 1760 bp (2.21% variability). Concatenating the three plastid sequences, assuming that the plastid genome forms a single linkage group, gave a total of 14 haplotypes in the 36 individuals analysed. Most populations did not show intrapopulational variation. Exceptions were 1-Infierno and 12-Kondratowa, which each harboured two different haplotypes separated by a single mutational step.

The statistical parsimony network allowed the recognition of five distinct lineages (Fig. 1D, E): (I) the Alpine-Pyrenean lineage, which includes haplotypes A–E from the south-western Alps (5-Galibier to 8-Ladres) and the western Pyrenees (1-Infierno to 3-Posets); (II) the Widespread lineage, which comprises haplotypes F–I from the Tatra mountains

Table 2. Analyses of molecular variance (AMOVAs) for AFLP phenotypes in *Artemisia eriantha*. *All *P* values were < 0.001

Source of variation	d.f.	Sum of squares	Variance components	% Total variance	F_{ST}*
Among all populations	16	1511.25	14.45	57.74	0.58
Within all populations	83	878.28	10.58	42.26	
Among STRUCTURE groups	4	824.89	6.96	26.33	0.60
Among populations	10	686.36	8.89	33.65	
Within populations	83	878.28	10.58	40.02	
Among mountain regions	5	791.74	5.27	20.39	0.59
Among populations	11	719.51	10.01	38.71	
Within populations	83	878.28	10.58	40.90	
Among Alpine populations	3	109.00	15.72	51.74	0.52
Within populations	12	146.33	14.93	48.26	
Among Pyrenean populations	3	259.56	11.28	52.43	0.52
Within populations	24	245.68	10.23	47.57	
Among Tatra populations	1	54.68	10.37	54.67	0.55
Within populations	7	60.20	8.60	45.33	
Among Carpathian populations	1	49.70	9.89	49.33	0.49
Within populations	6	61.00	10.16	50.67	
Among Balkan populations	3	199.96	7.45	42.62	0.43
Within populations	27	271.13	10.04	57.38	

(11-Liliowe Turnie, 12-Kondratowa), the southern Balkan Peninsula (14-Olympus) and the eastern Pyrenees (4-Casamanya); (III) the Apennine lineage with haplotype J from 9-Corvo; (IV) the southern Carpathian lineage (16-Bucegi, 17-Făgăraş) with haplotype K; and (V) the Balkan lineage, which includes haplotypes L–N from the Balkan Peninsula (10-Durmitor, 13-Ploskos, 15-Rila). To exclude the possibility of DNA template contamination and/or mix-up, leaf material of individuals from populations 4-Casamanya and 14-Olympus was re-extracted and re-sequenced. This confirmed the position of these two populations.

Genealogical relationships inferred using maximum parsimony (consistency index, 0.97; retention index, 0.99) and Bayesian analyses (based on the F81 model of sequence evolution) were congruent with the plastid DNA haplotype network (Fig. 1F). In both analyses, the lineages found in the haplotype network were confirmed with high BS and PP. The hypothesis of vicariance among mountain ranges was strongly rejected, as indicated by $2 \times \ln BF = 399.94$.

DISCUSSION

The historical range dynamics of the rare and disjunctly distributed *A. eriantha* in the European mountain system have been shaped by both vicariance and extensive LDD. The relative importance of these processes can, however, differ within different parts of the distributional area, as detailed below. This agrees with the historical biogeographies of other widely, but disjunctly distributed, European mountain plants (Kropf *et al*., 2006; Dixon *et al*., 2009; Schneeweiss & Schönswetter, 2010), underlining that conceptual restriction to one of these processes (for example, in vicariance biogeography: Wiley, 1988) is obsolete.

The earliest phase of range formation in *A. eriantha*, as indicated by the plastid DNA data (Fig. 1E, F), was westward migration from the Balkan Peninsula and the southern Carpathians via the Apennines to the Alps and Pyrenees. Colonization of European mountain ranges probably started in western Asian mountain ranges, such as the Caucasus or the Anatolian mountains, which harbour close relatives of *A. eriantha* (e.g. *A. aschurbajewi* C.Winkl. and *A. splendens* Willd; Sanz *et al*., 2008). The trend of westward colonization seen in *A. eriantha* agrees with general patterns for the colonization of Europe by *Artemisia* as a whole. Molecular phylogenetic reconstructions of the genus (Sanz *et al*., 2008; Tkach *et al*., 2008) and the oldest (Oligocene) records of *Artemisia* pollen from western China (Miao *et al*., 2011) support the hypothesis of Ling (1992) that the genus originated in the steppe areas of Central Asia from where it spread westward into Europe.

Subsequent isolation and diversification in different refugia in southern European peninsulas (Iberian, Apennine and Balkan Peninsulas) are suggested by both datasets (Figs 1, 2). Specifically, each peninsula harbours a specific haplotype lineage (Alpine-Pyrenean, Apennine and Balkan lineages; Fig. 1E, F) and a specific AFLP gene pool (Pyrenean, Apennine and Balkan clusters; Fig. 1B, C). This suggests that

the three 'classic' southern European refugia (Hewitt, 2004) also played an important role in the Pleistocene history of *A. eriantha*. Southern European refugia have been identified for numerous animal (e.g. Bilton *et al.*, 1998; Benke *et al.*, 2009) and temperate plant (e.g. Petit *et al.*, 2003; Arrigo *et al.*, 2011) species, but more rarely for alpine plants (Kropf *et al.*, 2003, 2006; Csergö *et al.*, 2009), which probably experienced range expansions during periods of climatic cooling (Birks & Willis, 2008). As in southern latitudes, suitable habitats probably remained isolated and alpine species mostly responded by elevational range shifts (Surina, Schönswetter & Schneeweiss, 2011); this isolation resulted in genetic differentiation. Considering that the divergence seen in slowly mutating, maternally inherited plastid sequences (Charlesworth, Charlesworth & Barton, 2003) and in mostly nuclear-derived AFLP markers (Meudt & Clarke, 2007) probably arose at different time horizons, this congruent differentiation in different refugia either happened over a long time or on a number of occasions owing to Pleistocene climatic oscillations. Extended isolation is also supported by private markers (Table 1) and hierarchical AMOVAs of AFLP data, in which the among-groups component explained 58% of the overall genetic variation (Table 2). Overall, for a wind-pollinated species, high genetic differentiation also supports the importance of the isolation effect in *A. eriantha* populations (Nybom, 2004).

Despite the evident role of vicariance in the range formation of *A. eriantha*, several discrepancies lend support to the outright rejection of the hypothesis of strict allopatric differentiation based on a Bayes factor test. First, ties between different mountain ranges lead to genetic heterogeneity within them. For instance, the Alpine haplotype B is derived from the Pyrenean haplotype A, and haplotype K from the southern Carpathians and haplotypes H and I from the Tatra Mountains in the north-western Carpathians fall into different clades, precluding simple north-westward colonization in the Carpathians (Fig. 1E, F). Furthermore, and perhaps most significantly, the Widespread lineage (Fig. 1D) comprising haplotypes F–I is highly disjunctly distributed in the eastern Pyrenees, on Mt. Olympus in northern Greece and in the Tatra Mountains. In contrast, the Widespread cluster based on AFLP data (Fig. 1A) is disjunctly distributed from the Pyrenees across the Alps to the Tatra Mountains and the southern Carpathians, where it merges with the Balkan cluster. Strong AFLP differentiation within mountain ranges is also illustrated by the high proportion (43–55%) of overall variation attributed to the among-population component by non-hierarchical AMOVAs (Table 2). Connections between the temperate mountain ranges (Pyrenees–Alps–Carpathians) have also been observed for other alpine plants (Mráz *et al.*, 2007; Paun *et al.*, 2008; Schneeweiss & Schönswetter, 2010). Topological relationships of the haplotypes of the Widespread lineage (Fig. 1D, E) and the position of a genetically admixed population (16-Bucegi) at the south-eastern edge of the distributional range of the Widespread cluster suggest a secondary eastward (re)colonization direction, contrasting with the general direction inferred for the southern European peninsulas. Discrepancies between the circumscription of the Widespread lineage and the Widespread cluster, most prominently in the western Alps and on Mt. Olympus, are probably a result of differences in the evolutionary dynamics of the employed markers. Whereas plastid haplotypes may have been readily fixed in isolated and often small populations of the Widespread lineage (Piękoś-Mirkowa & Miechówka, 1998; R. Vilatersana, pers. obs.), and are generally less prone to be replaced by immigrant genotypes (Currat *et al.*, 2008), gene flow with local and/or immigrant genotypes may lead to AFLP homogenization via genetic swamping (Avise, 2004).

Several LDD events must be invoked to explain the current distribution of different plastid DNA lineages (Fig. 1D). The cypselas of *Artemisia* are devoid of a pappus (with the exception of a few species that have a crown of scales: Hobbs & Baldwin, 2013), and are therefore not well adapted for LDDs, although hydrochory, anemochory and zoochory have been suggested for *Artemisia* (Vallès, 1988). However, fruits of alpine *Artemisia* spp. are able to persist for extended periods in the soil (Schwienbacher, Marcante & Erschbamer, 2010), which may enhance the establishment of the species following LDD events. Furthermore, migration possibilities might have been greater in the open, steppe landscapes during cold periods of the Pleistocene (Nathan *et al.*, 2008), including occurrences in northern refugia located in the lowlands or in unglaciated mountainous regions north of the southern European refugia (Stewart & Lister, 2001; Birks & Willis, 2008; Schneeweiss & Schönswetter, 2010). However, in the absence of fossil evidence, these hypotheses remain difficult to test.

Some regional AFLP patterns may be explained in terms of glacial history or hybridization with closely related taxa. For instance, differences in the extent of the Quaternary glaciation of the western and eastern Pyrenees (Calvet, 2004) may have resulted in the genetic break observed in both datasets (Fig. 1A, D). Population 4-Casamanya exhibits ties to the northernmost Alpine population 5-Galibier (Figs 1A–C, 2), which otherwise forms a separate AFLP cluster. Morphological features and genome size data (Garcia, 2007), its phylogenetic position as sister to all other populations of *A. eriantha* in the NJ tree rooted with *A. genipi* (see Fig. S1) and the high number of polymorphic and fixed private fragments (Table 1)

render introgression by the sympatric and abundant *A. genipi* a probable explanation for the observed divergence of population 5-Galibier.

CONCLUSIONS

The phylogeographical history of *A. eriantha* is much more complex than expected from its current distribution pattern, as are the results obtained for other species with small and scattered populations (e.g. *Cardamine resedifolia* L.: Lihová *et al*., 2009; *Androsace vitaliana* Lapeyr. and *A. lactea* L.: Dixon *et al*., 2009; Schneeweiss & Schönswetter, 2010). Instead of simple allopatric differentiation in multiple refugia, the range dynamics in *A. eriantha* are characterized by a combination of vicariant differentiation in southern European refugia and LDD events mainly involving temperate mountain ranges, partly leading to admixture between formerly isolated lineages. As vicariance and LDD probably operated on a number of occasions and at different times to differing degrees in different regions, the interpretation of contemporary phylogeographical patterns is hampered (Stewart & Lister, 2001) and requires evidence from several independent markers (Eidesen *et al*., 2007; Humphries & Winker, 2011), as demonstrated here for *A. eriantha*. The genetic uniqueness of most populations of *A. eriantha* reinforces its inclusion in Annex V of the EC Habitats Directive, as the use and exploitation of this species should be subject to management measures to avoid over-exploitation.

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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:

Figure S1. Neighbor-joining analysis of AFLP phenotypes of *Artemisia eriantha* based on Nei and Li's (1979) genetic distances. Numbers above the major branches are bootstrap values > 50%. The optimal grouping achieved by Bayesian model clustering using BAPS 2 (Bayesian Analysis of Population Structure) is plotted to the right of the geographical area names with symbols. *Artemisia genipi* was used as outgroup.

Figure S2. Summary of analyses of the AFLP dataset for *Artemisia eriantha* using the program STRUCTURE 2.2. (A) Likelihood of each number of clusters (*K*) for each of ten runs plotted against *K* values. (B) Average similarity between runs for each *K* value. Circles represent the mean of all pairwise comparisons between the ten runs, whereas triangles indicate the standard deviation. Similarity between runs was calculated according to Rosenberg *et al*. (2002) with the R-script STRUCTURE-sum (Ehrich, 2006). According to Rosenberg *et al*. (2002), a similarity value > 0.85 corresponds to a generally similar population structure.

Table S1. Localities and voucher information for the populations studied, and GenBank accession numbers for the three plastid DNA regions sequenced.