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Bayesian hypothesis testing supports long-distance Pleistocene migrations in a European high mountain plant (*Androsace vitaliana*, Primulaceae)

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ABSTRACT

Colonization of the south-western European mountain ranges is suggested to have predominantly progressed from the Iberian Peninsula eastwards, but this hypothesis has never been tested in a statistical framework. Here, we test this hypothesis using *Androsace vitaliana*, a high elevation species with eight mostly allopatric subspecies, which is widely but disjunctly distributed across all major south-western European mountain ranges. To this end, we use plastid and nuclear sequence data as well as fingerprint (amplified fragment length polymorphisms) data and employ Bayesian methods, which allow co-estimation of genealogy and divergence times using explicit demographic models, as well as hypothesis testing via Bayes factors. Irrespective of the ambiguity concerning where *A. vitaliana* started to diversify – both the Alps and the mountain ranges of the Iberian Peninsula outside the Pyrenees were possible – colonization routes were not simply unidirectional, but involved Pleistocene connections between the Alps and mountain ranges of the Iberian Peninsula bypassing the interjacent Pyrenees via long-distance dispersal. In contrast, the species' post-glacial history is shaped by regional gene pool homogenization resulting in the genetic pattern showing good congruence with geographical proximity in agreement with a vicariance model, but only partly supporting current taxonomy.

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1. Introduction

In recent years, phylogeography has undergone a shift from employing *ad hoc* explanations for observed patterns of genetic variation to using statistical tests of historical scenarios and demographic parameters within explicit models (Knowles and Maddison, 2002; Knowles, 2004). While the complexity of biologically plausible models can be high, for instance due to temporal phases of migration, isolation, admixture or bottlenecks, the complexity of actually testable models is mainly determined by the discriminatory power of the available data (Rosenberg and Nordborg, 2002; Knowles, 2004). Unfortunately, the well resolving AFLP (amplified fragment length polymorphisms) markers are not yet amenable to model-based approaches (but see Luo et al., 2007), rendering sequence data from the plastid genome an indispensable alternative (Petit and Vendramin, 2007). Plastid sequences have the disadvantages that the plastid genome behaves as a single locus and that the encountered variation is low (Schaal et al., 1998). These drawbacks are compensated by the applicability of

increasingly sophisticated analytical methods for sequence data (Knowles, 2004; Anderson et al., 2005; Drummond et al., 2006; Excoffier and Heckel, 2006; Minin et al., 2008). Among these, Bayesian methods are particularly attractive as they can handle “nuisance” parameters (i.e. parameters, which need to be estimated as part of the model, but are not of primary interest) such as effective population sizes (Dixon et al., 2007) or genealogies (Drummond et al., 2005) by marginalizing over them (Shoemaker et al., 1999).

Here, we use Bayesian methods in a phylogeographical study of the morphologically and karyologically well investigated European high mountain plant *Androsace vitaliana* (Primulaceae; Chiarugi, 1930; Favarger, 1958; Schwarz, 1963; Kress, 1997a,b, 1999), currently divided into eight mostly allopatric subspecies (see Section 2.1). As this species is found in a highly disjunct range in most high mountain ranges from the Sierra Nevada in southern Spain to the eastern Alps and the Apennines in Italy (Fig. 1), it provides the opportunity to investigate phylogeographical relationships across the major south-western European mountain ranges. Although our knowledge of these phylogeographical relationships is still limited due to the restricted distribution of the investigated species (Diadema et al., 2005) or the inclusion of a subset of mountain ranges only (Schönswetter and Tribsch, 2005; Gaudeul, 2006; Kropf et al., 2006; Schmitt et al., 2006; Valcárcel et al., 2006; but

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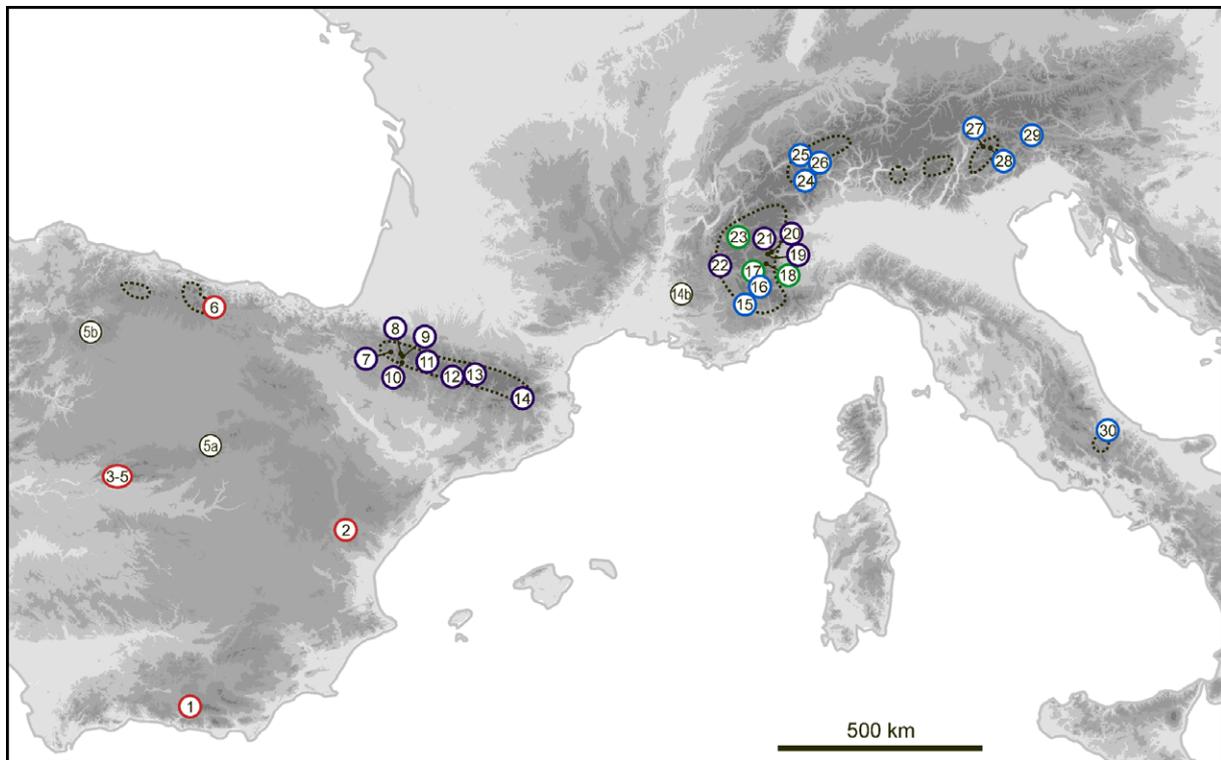


Fig. 1. Map of south-western Europe showing the distribution of *Androsace vitaliana* (dotted line). Locations of sampled populations (numbered according to Table 1) are indicated by circles (the locations of populations 14a and 24a are congruent with those of populations 14 and 24 and are not shown). Distribution of major haplotype lineages is indicated by colors (black outline indicates populations, where only ITS data are available), matching those used in Fig. 5.

see Kropf et al., 2002, 2003), previous studies do indicate that colonization of these mountain ranges predominantly progressed from the Iberian Peninsula eastwards (Comes and Kadereit, 2003; Vargas, 2003). An origin on the Iberian Peninsula and, implicitly, an eastward migration has also been suggested for *A. vitaliana* (Schwarz, 1963), but this hypothesis has not been tested in a statistical framework yet. Many authors considered elements of the high mountain flora with Mediterranean or Asian affinities to be of late Tertiary origin (summarized in Comes and Kadereit, 2003). This is also the case for *A. vitaliana*, whose highly disjunct distribution was taken as indication for a Tertiary origin (Lüdi, 1927; Chiarugi, 1930; Schwarz, 1963), a notion challenged by molecular dating results suggesting a Pleistocene differentiation (Vargas, 2003).

We will use *A. vitaliana* as a study system to address the above hypotheses on the phylogeographical relationships between the south-western European mountain ranges. To this end, we first used AFLPs, highly resolving markers derived almost exclusively from the nuclear genome, which are expected to reflect more recent events than plastid sequence data and to be rapidly homogenized by gene flow (Bussell et al., 2005; Meudt and Clarke, 2007). Secondly, we also used nuclear and maternally inherited (Harris and Ingram, 1991) plastid DNA sequences. Specifically, we wanted to infer (i) the colonization patterns in the south-western European mountain ranges, for instance, whether colonization progressed westwards, as suggested by the distribution of *A. vitaliana*'s sister species (*A. wulfeniana* and *A. brevis*) in the Alps (Schneeweiss et al., 2004), or vice versa, and (ii) whether race differentiation within *A. vitaliana* dates to the Tertiary, as suggested by early authors (Lüdi, 1927; Chiarugi, 1930; Schwarz, 1963) or whether differentiation is of Pleistocene origin (Vargas, 2003). Additionally, we tested whether currently recognized subspecies correspond to genetically defined lineages.

2. Materials and methods

2.1. Study species

Androsace vitaliana is a perennial caespitose to cushion-forming herbaceous plant found on both siliceous and calcareous bedrock in usually open vegetation at altitudes between 1500 and 3300 m (Kress, 1997a). As the density of the indumentum is quite variable and the geographical distribution is fairly patchy, eight mostly allopatric subspecies have been recognized: Sierra Nevada (subsp. *nevadensis*), Sistema Central (subsp. *aurelii*), Sierra de Javalambre (subsp. *assoana*), Montes de León and Cordillera Cantábrica (subsp. *flosjugorum*; from here on we will refer to these five mountain ranges collectively as Iberian mountain ranges), south-eastern Alps (subsp. *sesleri*), Apennines (subsp. *praetutiana*), and both western Alps and Pyrenees (subsp. *vitaliana* and subsp. *cinerea*). The species has yellow distylous flowers, which are chiefly pollinated by butterflies (Hess, 2001), but whose style polymorphism differs from the typical heterostyly found in *Primula* in the lack of differentiation in pollen grain size, stigmatic surfaces and stamen length (Kress, 1967). The seeds are relatively small (around 1 mm) and have no apparent adaptations for long-distance dispersal. The species is paleopolyploid, as the entire *Aretia* clade (Schneeweiss et al., 2004), with a chromosome number of $2n = 40$ with occasional polyploidy (Kress, 1984), which does not show any correlation with taxonomy or geography (Favarger, 1965).

2.2. Laboratory techniques

Plants were collected from 30 populations across the natural range of *A. vitaliana* (Table 1; Fig. 1) and stored in silica gel. Voucher specimens were deposited at the herbarium of the Institute

Table 1
 Sampling locations, voucher information and GenBank accession numbers of *Androsace vitaliana* (populations 1–30) and outgroup taxa as well as number of analyzed individuals and cpDNA haplotypes of *A. vitaliana* only.

Number/ species	Location	Longitude	Latitude	Voucher specimen	Number of individuals ^a	Haplotype (haplotype lineage)	GenBank accession numbers ^b
1	Cauchiles, Sierra Nevada	3.350°W	37.050°N	WU 10882	5/7	I (1)	EF512034, FJ606080–FJ606085; EF512062, FJ605951–FJ605956; EF512094, FJ692483–FJ692488; EU109186
2	Sierra de Javalambre	1.000°W	40.067°N	304PV04	5/6	I, II (1)	EF512035, FJ606086–FJ606090; EF512063, FJ605957–FJ605961; EF512095, FJ692489–FJ692493; FJ654497
3	Nava del Barco, Sistema Central	5.594°W	40.222°N	160PV01	5/3	II (1)	EF512036, FJ606091–FJ606092; EF512064, FJ605962–FJ605963; EF512096, FJ692494–FJ692495; EU109184
4	Nava del Barco, Sistema Central	5.594°W	40.222°N	161PV01	5/3	II (1)	EF512037, FJ606093–FJ606094; EF512065, FJ605964–FJ605965; EF512097, FJ692496–FJ692497; FJ654489
5	Nava del Barco, Sistema Central	5.594°W	40.222°N	162PV01	5/3	II (1)	EF512038, FJ606095–FJ606096; EF512066, FJ605966–FJ605967; EF512098, FJ692498–FJ692499; FJ654490
5a	Sierra de Ayllón			No voucher	–/–		–; –; –; EU109185
5b	Montes de Leon			No voucher	–/–		–; –; –; EU109182
6	Peña Labra, Cordillera Cantábrica	4.384°W	43.024°N	WU 8888	5/9	III (1)	EF512039, FJ606072–FJ606079; EF512067, FJ605943–FJ605950; EF512099, FJ692475–FJ692482; EU109183
7	Puerto d'Acher, Pyrenees	0.654°W	42.807°N	WU 8886	4/6	XI (3)	EF512040, FJ606059–FJ606063; EF512068, FJ605930–FJ605934; EF512100, FJ692462–FJ692466; FJ654496
8	El Portalet, Pyrenees	0.441°W	42.796°N	WU 8879	4/5	XI (3)	EF512041, EF587867, FJ606056–FJ606058; EF512069, EF587883, FJ605927–FJ605929; EF512101, EF587899, FJ692459–FJ692461; FJ654495
9	Formigal, Pyrenees	0.400°W	42.767°N	149PV01	5/5	XI (3)	EF512042, FJ606068–FJ606071; EF512070, FJ605939–FJ605942; EF512102, FJ692471–FJ692474; EU109188
10	Peñas de Aso, Pyrenees	0.390°W	42.675°N	WU 8874	4/5	XI (3)	EF512043, FJ606052–FJ606055; EF512071, FJ605923–FJ605926; EF512103, FJ692455–FJ692458; FJ654494
11	Port de Campbiell, Pyrenees	0.117°E	42.767°N	WU 8868	5/5	XII (3)	EF512044, FJ606048–FJ606051; EF512072, FJ605919–FJ605922; EF512104, FJ692451–FJ692454; FJ654493
12	La Maladeta, Pyrenees	0.689°E	42.585°N	WU 8851	4/6	IX, XI (3)	EF512045, FJ606043–FJ606047; EF512073, FJ605914–FJ605918; EF512105, FJ692446–FJ692450; FJ654492
13	Puerta de la Bonaigua, Pyrenees	1.117°E	42.633°N	369PV02	5/5	XI (3)	EF512046, FJ606064–FJ606067; EF512074, FJ605935–FJ605938; EF512106, FJ692467–FJ692470; EU109187
14	Puigmal d'Err, Pyrenees	2.148°E	42.365°N	WU 8826	4/5	XI (3)	EF512047, FJ606039–FJ606042; EF512075, FJ605910–FJ605913; EF512107, FJ692442–FJ692445; FJ654491
14a	Eastern Pyrenees			No voucher	–/–		–; –; –; EU109189
14b	Mt. Ventoux, south-western Alps			No voucher	–/–		–; –; –; EU109192
15	Courradour, south-western Alps	6.642°E	44.083°N	WU 8910	5/8	VI (3)	EF512048, FJ606008–FJ606014; EF512076, FJ605850–FJ605856; EF512108, FJ692382–FJ692388; EU109190
16	Cime des Trois Serrières, south-western Alps	6.846°E	44.342°N	WU 9207	4/6	VI (3)	EF512049, FJ606003–FJ606007; EF512077, FJ605845–FJ605849; EF512109, FJ692377–FJ692381; FJ654501
17	Cot de Serenne, south-western Alps	6.758°E	44.563°N	WU 9215	5/8	IV, V (2)	EF512050, FJ606032–FJ606038; EF512078, FJ605874–FJ605880; EF512110, FJ692406–FJ692412; EU109193
18	Col Agnel, south-western Alps	6.983°E	44.686°N	WU 9217	5/10	IV (2)	EF512051, FJ606023–FJ606031; EF512079, FJ605865–FJ605873; EF512111, FJ692397–FJ692405; FJ654503
19	Val Germanasca, south-western Alps	7.013°E	44.835°N	WU 9229	4/8	X (3)	n.a.; EF512080, FJ605903–FJ605909; EF512112, FJ692435–FJ692441; FJ654505
20	Val Germanasca, south-western Alps	7.088°E	44.860°N	WU 9228	5/8	X (3)	n.a.; EF512081, FJ605888–FJ605894; EF512113, FJ692420–FJ692426; FJ654500
21	Colle Lauson, south-western Alps	6.935°E	45.045°N	WU 9226	5/8	X (3)	n.a.; EF512082, FJ605881–FJ605887; EF512114, FJ692413–FJ692419; EU109194
22	Pic de l'Aiguille, south-western Alps	6.044°E	44.630°N	WU 9361	5/9	X (3)	n.a.; EF512083, FJ605895–FJ605902; EF512115, FJ692427–FJ692434; FJ654504
23	Col de Côte Plaine, south-western Alps	6.374°E	45.057°N	WU 9372	5/9	IV (2)	EF512052, FJ606015–FJ606022; EF512084, FJ605857–FJ605864; EF512116, FJ692389–FJ692396; FJ654502
24	Gornergrat, Pennine Alps	7.781°E	45.983°N	WU 9396	5/5	VI (3)	EF512053, FJ605990–FJ605993; EF512085, FJ605832–FJ605835; EF512117, FJ692364–FJ692367; EU109196
24a	Zermatt, Pennine Alps			No voucher	–/–		–; –; –; EU109191
25	Torrentalp, Pennine Alps	7.658°E	46.361°N	WU 9300	5/5	VI (3)	EF512054, FJ605999–FJ606002; EF512086, FJ605841–FJ605844; EF512118, FJ692373–FJ692376; EU109195
26	Mäderhorn, Pennine Alps	8.062°E	46.260°N	WU 9293	5/6	VI (3)	EF512055, FJ605994–FJ605998; EF512087, FJ605836–FJ605840; EF512119, FJ692368–FJ692372; FJ654499
27	Seiser Alm, south-eastern Alps	11.658°E	46.498°N	WU 9426	5/6	VII (3)	EF512056, FJ605976–FJ605980; EF512088, FJ605818–FJ605822; EF512120, FJ692350–FJ692354; FJ654498
28	Passo Pordoi, south-eastern Alps	11.823°E	46.476°N	WU 9418	5/5	VII (3)	EF512057, FJ605981–FJ605984; EF512089, FJ605823–FJ605826; EF512121, FJ692355–FJ692358; EU109198
29	Raudenspitze, south-eastern Alps	12.753°E	46.646°N	WU 8974	5/6	VI (3)	EF512058, FJ605985–FJ605989; EF512090, FJ605827–FJ605831; EF512122, FJ692359–FJ692363; EU109197

Table 1 (continued)

Number/ species	Location	Longitude	Latitude	Voucher specimen	Number of individuals ^a	Haplotype (haplotype lineage)	GenBank accession numbers ^b
30	Monte Amaro, central Apennines	14.059°E	42.100°N	WU 8937	4/9	VIII (3)	EF512059, FJ605968–FJ605975; EF512091, FJ605810–FJ605817; EF512123, FJ692342–FJ692349; EU109199
<i>A. wulfeniana</i>	Dreistecken, eastern Alps	14.397°E	47.457°N	WU 5213			EF512033; EF512061; EF512092; AY275048
<i>A. brevis</i>	Monte Legnone, Bergamo Alps	9.417°E	46.096°N	WU 4961			EF512032; EF512060; EF512093; AY275049

^a AFLP/cpDNA.

^b gene regions in the following order: trnG–trnR intergenic spacer; rpl20–rps12 intergenic spacer; trnS–ycf9–trnG–trnM intergenic spacers; ITS; regions not investigated in a given sample are indicated by a dash; in case of pops. 19–22, where the first region could not be amplified, this is indicated by n.a.

of Botany of the University of Vienna (WU) unless otherwise noted (Table 1). Total genomic DNA was extracted from similar amounts of leaf material according to the 2× cetyl trimethyl ammonium bromide (CTAB) method (Doyle and Doyle, 1987) with minor modifications (Schönswetter et al., 2002). Samples of *A. brevis* and *A. wulfeniana*, the putative sister-taxa to *A. vitaliana* (Schneeweiss et al., 2004), were included as outgroups.

AFLP fingerprints were produced for four to five individuals per population (Table 1), following the protocol of Tremetsberger et al. (2003), but with the restriction phase lasting three hours instead of two. Two pairs of selective primers were chosen (fluorescent dye in brackets): (VIC) – EcoRI AGG and MseI CA, and (6-FAM) – EcoRI ATC and MseI CT (Applied Biosystems, Foster City, USA). Five microliters of each selective PCR product were purified using Sephadex G-50 Superfine (GE Healthcare Bio-Sciences, Uppsala, Sweden) and 5 µl elution product was combined with 4.8 µl formamide and 0.2 µl GeneScan 500 ROX internal size standard (Applied Biosystems) and run on an ABI 3130 automated capillary sequencer. Blind samples and replicates (8% of individuals) were routinely included to test for contamination and to assess reproducibility. Fragment lengths were determined using ABI Prism GeneScan 3.7.1 (Applied Biosystems).

Supplementing the data set of Vargas (2003), ITS was amplified from, due to the limited variation (see Section 3.2), only one individual per population using 1.1× Reddy Mix PCR Master Mix (AB-gene, Epsom, UK) according to the manufacturer's instructions. PCR conditions were: 80 °C (5 min) followed by 36 cycles of 15 s at 98 °C, 30 s at 58 °C, 2 min at 72 °C and a final elongation of 72 °C for 8 min. The primers used for PCR were AB101 and AB102 (Schneeweiss et al., 2004), while for cycle sequencing either AB101 or ITS-3 (White et al., 1990; both forward) and ITS-6 (5'-ATG GTT CGC GGG ATT CTG CAA TTC ACA CC-3'; reverse) were used. Following PCR, single-stranded DNA was digested using ExoCIAP (MBI Fermentas, St. Leon-Rot, Germany), according to the manufacturer's instructions. All reactions were carried out on a GeneAmp 9700 thermocycler (Applied Biosystems). Cycle sequencing was performed using BigDye Terminator 3 (Applied Biosystems), according to the manufacturer's instructions. Electrophoresis was carried out on an ABI 3130 automated capillary sequencer (Applied Biosystems). Sequences were edited with SeqMan™ II v. 5.05 (DNA-Star, Madison, WI, USA) and aligned using BioEdit 7.0.4.1 (Hall, 1999). All sequences, including those used in the previous study of Vargas (2003) were deposited in GenBank (Table 1).

Three regions of the plastid genome were sequenced from 5 to 10 individuals per population (only three individuals for each of the three spatially close populations from the Sistema Central; Table 1). We chose to analyze a comparatively high number of individuals per population in order to check for the presence of potentially rare interior haplotypes (see Section 3.3). The following three primer pairs were used: ccmp3f (Weising and Gardner, 1999) and trnR (Dumolin-Lapegue et al., 1997), rpl20 and 5'-rps12 (both Hamilton, 1999), and trnS(UGA) and trnM(CAU) (both Demesure

et al., 1995). These regions are also being studied in other species of *Androsace* thus enabling interspecific comparisons. For four populations in the south-western Alps (populations 19–22), the first region failed to amplify either with the primers ccmp3f and trnR or with a pair of externally located primers (NTCP8-f, Bryan et al., 1999; atpA-P3MD, Heinze, 2007). Since it is likely that this failure to amplify is caused by, e.g., an inversion over primer binding regions, no further efforts were made to amplify this region for those populations. They were treated as missing data. PCR was carried out in 25 µl volumes, containing 1 µl DNA of unknown concentration, 1.5 U EcoTaq (Ecogen, Madrid, Spain), 2.8 mM MgCl₂, 0.64 µM dNTPs, 0.2 µM each primer, and 1× polymerase buffer as supplied with the enzyme. Reactions were carried out in a GeneAmp 9700 thermocycler (Applied Biosystems), with the following cycling conditions: 5 min at 94 °C followed by 30 cycles of 1 min at 94 °C, 30 s at 60 °C, 1 min at 72 °C and a final elongation phase of 5 min at 72 °C. Amplified products were cleaned using the PCR Clean-up kit (MoBio Laboratories, Carlsbad, CA, USA) following the manufacturer's protocol. Sequencing was performed by the Unidad de Genómica (University of Madrid, Spain).

2.3. Data analyses

AFLP sample files were imported into Genographer 1.6.0 (<http://hordeum.msu.montana.edu/genographer/>) for scoring, and the results were exported as a presence/absence matrix. Only bands which could be scored unambiguously were included, and those found by comparing replicate runs not to be reproducible were excluded from the analyses. Analysis of molecular variance (AMOVA) was carried out using Arlequin 3.11 (Excoffier et al., 2005). We used Structure 2.2, which implements a Bayesian clustering approach for dominant markers (Pritchard et al., 2000; Falush et al., 2007), with an admixture model with uncorrelated allele frequencies and recessive alleles. Ten replicate runs for each *K* (number of groups) ranging from 1 to 9 were carried out at the Bioportal of the University of Oslo (<http://www.bioportal.uio.no/>), using a burn-in of 10⁵ generations and sampling from the following 10⁶ generations. Similarity among results of different runs for the same *K* was calculated according to Nordborg et al. (2005) using AFLPsum (Ehrlich, 2006). We identified the optimal number of groups as the value of *K* where the increase in likelihood started to flatten, the results of replicate runs were identical, and no empty groups were encountered. The replicate runs of the best *K* were merged with Clumpp 1.1.1 (Jakobsson and Rosenberg, 2007) using the greedy algorithm with 1000 random input orders. The relative 'cluster membership coefficients' of all individuals were then averaged for each population. To aid visualization of AFLP data, we additionally produced a neighbor-net diagram based on Nei-Li distances using the program SplitsTree 4 (Huson and Bryant, 2006). This is a valuable method when reticulate evolution may have played a role in the evolution of the study group, likely to be the case in intraspecific studies (Posada and Crandall, 2001).

Sequences from the three plastid regions were concatenated based on the assumption that the plastid genome forms a single linkage group. In both nuclear (including sequences from five populations investigated by Vargas, 2003; Fig. 1) and cpDNA sequence data sets insertions/deletions longer than 1 bp were re-coded as single base pair indels, and variation in the length of mononucleotide repeats were excluded, since these are prone to homoplasy at larger geographic scales (Ingvarsson et al., 2003). Haplotype networks with and without outgroups were constructed using TCS 1.21 (Clement et al., 2000) treating sequence gaps as a fifth character state. The haplotype network construction with TCS can be misled by missing data (Joly et al., 2007), therefore, for the cpDNA sequence data set, we employed the following *ad hoc* procedure: As the position of the four populations (pops. 19–22) where one DNA region was missing could be unambiguously determined from the characters in the other two regions, and the variable positions in the third region only affected other parts of the network, for the final analysis the variable positions in the third region were re-coded to agree with the haplotypes found in the very clade populations 19–22 belong to. Due to the unknown nature of the mutation causing the amplification failure, this mutation was not coded separately.

Phylogenetic analyses of the nuclear and cpDNA sequence data (including *A. wulfeniana* and *A. brevis*) were carried out employing maximum parsimony with PAUP* 4.0b10 (Swofford, 2002) and Bayesian inference using MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003). For the maximum parsimony analyses, heuristic searches were conducted employing 1000 random sequence addition replicates and TBR branch swapping with maximally 500 trees saved per replicate. Branch support was assessed via non-parametric bootstrapping with 500 pseudo-replicates, each employing 10 random sequence addition replicates and TBR branch swapping with 100 or fewer trees saved per replicate. As the best-fit substitution model had low Akaike weight (<0.22) in ModelTest 3.6 (Posada and Crandall, 1998) and the set of models with a cumulative Akaike weight >0.95 also included more complex models, we used a GTR+ Γ model (nst = 6 rates = gamma), subsuming a proportion of invariable sites in the gamma distribution, which models rate heterogeneity across sites. For the Bayesian inference, four runs of four chains each were run in MrBayes 3.1.2, with incremental heating (temperature 0.1), for 5×10^6 generations, sampling every 1000 generations, using the default priors and estimating model parameter values during the runs. The first 10% of trees were discarded as burn-in, being well after tree likelihoods and model parameters had reached stationarity. Clade posterior probabilities were calculated from the combined set of 18,000 trees.

Alternative topologies concerning the relationships of Pyrenean and Apennine populations to populations from the Alps (see Section 3.3 for details) were tested in a parsimony framework using the Kishino-Hasegawa-test as implemented in PAUP* 4.0b10 and in a Bayesian framework using Bayes factors calculated from the posterior likelihood obtained with MrBayes 3.1.2. Bayes factors (BF) measure the change in support for one model versus another given the data (Suchard et al., 2001). Marginal likelihoods (incl. their Monte Carlo error; Suchard et al., 2003; Redelings and Suchard, 2005) and BFs were calculated with Tracer 1.4 (<http://tree.bio.ed.ac.uk/software/tracer/>). As a test statistics we used $2 \times \ln BF_{12}$, considering $2 \times \ln BF_{12} > 10$ as strong support for and $2 \times \ln BF_{12} < -10$ as strong evidence against hypothesis 1 (Kass and Raftery, 1995).

Bayesian hypothesis testing was performed on the more variable cpDNA data using the approach implemented in the program BEAST 1.4.8 (Drummond et al., 2006; Drummond and Rambaut, 2007). The prior distribution of the mutation rate was given as a normal distribution with a mean of 2.2×10^{-3} per site per million years (as suggested for the *matK* gene in perennial herbs; Yamane

et al., 2003; Paun et al., 2005) and a wide standard deviation of half the mean. Although *matK* evolves rapidly for a coding region (Johnson and Soltis, 1995), we still expect it to evolve more slowly than non-coding regions such as the ones that we have used, biasing our estimates towards older ages. Additionally, substitution rates derived from phylogenetic studies with usually deeper time coverage (older than 1–2 million years) will often be gross underestimates of more recent substitution rates (Ho et al., 2005, 2007), introducing an additional bias towards older ages for recent (Pleistocene) diversifications. In order to keep the number of parameters to be estimated to a minimum, we used a strict clock rather than a relaxed molecular clock. Following age estimates for *Eu-Aretia*, the clade to which *A. vitaliana* belongs (Schneeweiss et al., 2004), the root node was constrained to a maximum age of 5 million years. Stationarity of the Markov chain, which was run for 3×10^7 generations, was determined using Tracer 1.4. The first 10% of sampled generations was discarded as burn-in, and stationarity was reached when all effective sample size values were greater than 100. A second run was conducted to confirm convergence of the Markov chain on the stationary distribution. All parameter estimates were based on these two runs combined (54,000 sampling points). An initial analysis using a Bayesian skyline plot (Drummond et al., 2005), the most general demographic model, with 10 to 30 population size classes detected no signal of population size changes (data not shown). Additionally, models allowing exponential population growth for either all populations or only the Alpine ones were not significantly better than those with constant population sizes, and the 95% highest posterior density intervals (HPDs) of the growth parameter included zero (data not shown). Hence, for the final analyses we assumed constant population sizes through time.

Several *a priori* hypotheses were tested against an unconstrained analysis (necessary genealogical constraints are shown in Fig. 2; BEAST input files are available as Supplementary material). Based on preliminary results (data not shown), we used a simplified coding of mountain ranges, specifically, the spatially complex Iberian mountain ranges were treated as single region and the Apennines were always considered jointly with the Alps. All hypotheses which involve colonization steps assumed that after long-distance dispersal the founding populations were sufficiently small to prohibit the retention of polymorphism and/or that colonizers were genetically homogeneous, in agreement with haplotype patterns of present day populations (see Section 3.3). Additionally, we assume that *A. vitaliana* has not been extirpated in any other major mountain range so that we can use the most recent common ancestor of extant populations as surrogate for the origin of the whole species. The first hypothesis is one of geographical separation of genetic lineages among the Iberian mountain

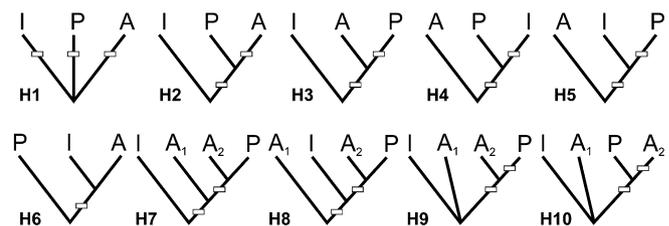


Fig. 2. Genealogical constraints of tested *a priori* (H1–H6) and *a posteriori* (H7–H10) phylogeographic hypotheses (see text for details): H1 vicariance, H2 stepwise eastward migration, H3 reverted eastward migration, H4 stepwise westward migration, H5 reverted westward migration, H6 Pyrenean origin, H7 Iberian origin, H8 Alpine origin, H9 Alpine colonization, H10 Pyrenean colonization. White bars indicate monophyly constraints, which in H2–H10 are connected to colonization events of the respective region(s). A, Alps; A₁, A₂, Alpine haplotypes of haplotype groups 2 and 3, respectively (see Fig. 5); I, Iberian mountain ranges; P, Pyrenees.

ranges, Pyrenees, and Alps plus Apennines (vicariance hypothesis). Schwarz (1963) suggested that *A. vitaliana* originated on the Iberian Peninsula. From there, the Alps might have been colonized via the Pyrenees, simply following the linear spatial arrangement of the major mountain ranges (stepwise eastward migration), or the Pyrenees might have been colonized from the Alps (reverted eastward migration), a pattern known from other high mountain species (Schönswetter et al., 2002). Alternatively, *A. vitaliana* could have originated in the Alps, where its closest relatives occur (*A. wulfeniana* and *A. brevis*: Schneeweiss et al., 2004). Analogous to the previous scenarios, *A. vitaliana* might have colonized the Iberian mountain ranges via the Pyrenees (stepwise westward migration) or the Pyrenees via the Iberian mountain ranges (reverted westward migration); relationships between the Alps and the Iberian mountain ranges sparing the Pyrenees are known from other *Androsace* taxa (Dixon et al., 2008). Finally, although it has not previously been suggested, we also tested an origin of *A. vitaliana* in the Pyrenees (Pyrenean origin). Based on the inferred haplotype structure, we also formulated and tested several *a posteriori* hypotheses (Fig. 2), which take the deep phylogeographical split within the Alps into account (see Section 3.3). One concerns the position of the root either between the Iberian mountain ranges and the other regions (Iberian origin) or within the Alps (Alpine origin). The second deals with the colonization direction between the Alps and the Pyrenees, which, as already described before, might have been either from the Alps to the Pyrenees (Alpine colonization) or vice versa (Pyrenean colonization). To quantify the support for each of these hypotheses we used Bayes factors as described above.

3. Results

3.1. AFLP

A total of 175 AFLP bands were scored from 143 individuals, 40 of which were monomorphic. The error rate was 4.8% before the exclusion of irreproducible bands, using the method of Bonin et al. (2004). Structure analysis gave $K=4$ as the best number of groups. Higher values of K had higher likelihoods, but the clustering results differed strongly among runs and were consequently not considered (Fig. S1 available as Supplementary material). These

groups corresponded to the four geographical areas: (1) Iberian mountain ranges, (2) Pyrenees, (3) south-western Alps and (4) Pennine to south-eastern Alps plus Apennines. Most individuals showed unambiguous cluster membership, the most prominent exception being the Apennine population, which showed cluster membership coefficients between 0.2 and 0.3 with the south-western Alpine group (Table S1 available as Supplementary material). Results from the neighbor-net analysis (Fig. 3) were congruent with those from Structure, but showed stronger differentiation within the Iberian mountain ranges and the eastern Alpine plus Apennine group. Specifically, the Iberian mountain ranges were separated into the four constituent mountain ranges Sierra Nevada, Sierra de Javalambre, Sistema Central and Cordillera Cantábrica, while the Apennines and Pennine Alps were well separated from the south-eastern Alps, which themselves were divided into their constituent populations. Except for the separation of the Iberian mountain ranges and the Pyrenees from the remaining groups, the relationships among those lineages were poorly resolved and weakly supported (Fig. 3). In an AMOVA with the four groups derived from Structure analysis, 41% of the variance was accounted for by variation among main geographical areas, 23% between populations within the regions, and 36% within populations ($P < 0.001$ in all cases). An AMOVA with six groups, as suggested by the neighbor-net (Fig. 3), gave similar results with 46% of the variance accounting for among group variation, 16% for variation among populations within a group, and 38% for variation within populations (again $P < 0.001$ in all cases).

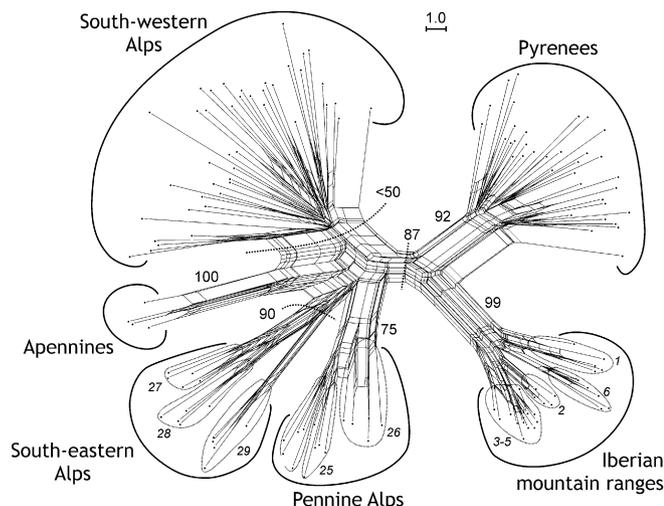


Fig. 3. Neighbor-net of AFLP data of *Androsace vitaliana*. Numbers (normal font) are bootstrap values from a neighbor-joining analysis using Nei-Li distances over 1000 bootstrap pseudo-replicates. Circled areas represent cohesive populations and are marked with population numbers (in italics). Scale bar is percent distance.

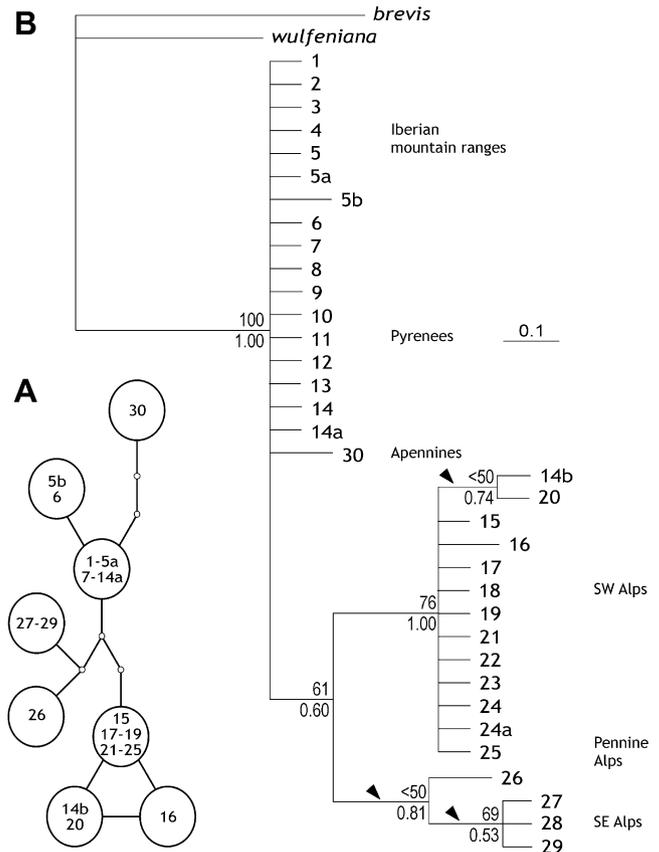


Fig. 4. Relationships of ITS ribotypes of *Androsace vitaliana* (indicated by their population numbers given following Table 1) shown as (A) haplotype network and (B) majority rule consensus tree of the Bayesian analysis. In (B), branches collapsing in the maximum parsimony strict consensus tree are indicated by arrowheads; numbers at branches are maximum parsimony bootstrap and posterior probabilities; scale bar is substitutions per site.

3.2. ITS

The aligned sequences from the 37 analyzed individuals (35 from *A. vitaliana*) were 618 bp long including 33 variable and 18 parsimony-informative characters (10 variable and 6 parsimony-informative characters when outgroups are excluded). ITS ribotypes from *A. vitaliana* and its sister-taxa *A. brevis* and *A. wulfeniana* grouped in three unconnected networks according to species delimitation (data not shown), and only the *A. vitaliana* network is shown (Fig. 4A). The Pyrenees and the central and southern Iberian mountain ranges share the same ITS ribotype, from which those from the Cordillera Cantábrica and the Apennines are separated by one and three steps, respectively. The main Alpine ITS ribotype and its two derivatives are found in the south-western and parts of the Pennine Alps and are separated by at least four mutational steps from those found in the south-eastern Alps and Pennine Alps. Maximum parsimony analyses resulted in 1874 equally most parsimonious trees (length = 34, consistency index [C.I.] excluding uninformative characters = 0.9474, rescaled C.I. = 0.9533). The strict consensus tree from the maximum parsimony analysis was topologically nearly identical to the majority rule consensus tree obtained from the Bayesian analysis (harmonic mean of log-likelihoods = -1225.09; Fig. 4B). Populations from the western Alps (Pennine and south-western Alps; bootstrap [BS]/posterior probability [PP] 76/1.00) were separated from a clade (BS/PP <50/0.81) or grade of the clade of south-eastern Alpine populations (BS/PP 69/0.53) plus one population from the Pennine

Alps. All Alpine populations (BS/PP 61/0.60) were sister to a basal grade including the remaining populations (Iberian mountain ranges, Pyrenees, Apennines). Alternative hypotheses of Pyrenean or Apennine populations grouping with populations from the Alps, as suggested by cpDNA data (see below), are at least marginally significantly rejected (Kishino–Hasegawa test $P = 0.0455$ and $P = 0.0833$, respectively; $2 \times \ln BF = -20.45$ and -13.21 , respectively, the Monte Carlo error not exceeding 0.16).

3.3. cpDNA

The aligned sequences from the 191 individuals (189 individuals of *A. vitaliana*, 3–10 per population, mean 6.3) were 2256 base pairs long including 27 variable and 23 parsimony-informative characters (23 variable and 23 parsimony-informative characters with outgroups excluded). Within *A. vitaliana*, 12 haplotypes were found. Using Dixon's (2006) method, this situation had a probability of completeness of 1.0, suggesting that all haplotypes present in this species were sampled. Most populations contained only one haplotype, while three populations harbored two haplotypes separated by a single mutational step (Table 1, Fig. 5). The 12 haplotypes fell into three distinct groups, each separated from the others by at least eleven mutational steps (Fig. 5A). Haplotype group 1 included three haplotypes from the Iberian mountain ranges, the central one (I) in the Sierra Nevada and the Sierra de Javalambre and two, each derived by a single mutational step, in the Sierra de Javalambre and Sistema Central (II) and the Cordillera

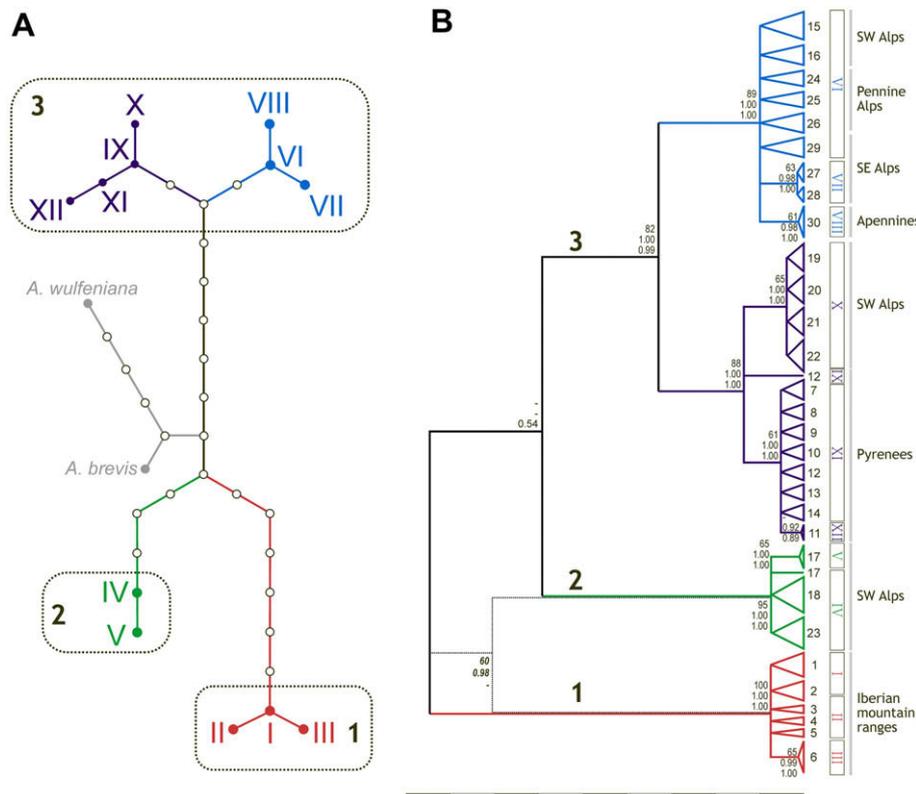


Fig. 5. Relationships of plastid DNA haplotypes of *Androsace vitaliana* (indicated by roman numerals) shown as (A) haplotype network and (B) majority rule consensus tree from strict clock Bayesian analysis (with BEAST; the alternative topology from the maximum parsimony strict consensus tree and the outgroup Bayesian analysis with MrBayes is shown by dotted lines). Node heights correspond to median ages (see text for details). Different lineages are marked in different colors, which match those used in Fig. 1 (outgroup haplotypes in (A) are indicated in gray), and the circumscription of haplotype groups 1–3 is indicated. In (B), numbers along branches are maximum parsimony bootstrap values, Bayesian posterior probabilities with outgroups (MrBayes) and Bayesian posterior probabilities without outgroups (BEAST); identical individuals of the same population (population numbers as in Table 1) are collapsed as triangles, their vertical extension being proportional to the number of individuals; scale bar 0.9 my with increments of 0.1 my.

Cantábrica (III), respectively (Fig. 1). Haplotype group 2 comprised the two haplotypes IV and V separated by a single mutational step and was restricted to the south-western Alps (populations 17, 18, 23; Fig. 1). Haplotype group 3 was composed of seven haplotypes from the Pyrenees, the Alps and the Apennines (VI–XII), which themselves were divided into two groups separated by four mutational steps. The first group included in an interior position a haplotype (VI) found in the Pennine Alps (pops. 24–26), two populations from the south-western Alps (pops. 15 and 16) and one population from the south-eastern Alps (pop. 29) and, derived from it by a single mutational step each, the haplotype from the remaining south-eastern Alpine populations (pops. 27–28: VII) and the Apennines (VIII; Fig. 1). The second group comprised a haplotype (IX) in an interior position found in a single individual from the Pyrenees (pop. 12) and, each derived by a single mutational step, the main Pyrenean haplotype (XI) and its derivative (XII; pops. 7–14) and the haplotype found in four populations from the south-western Alps (pops. 19–22: X; Fig. 1). The outgroups join the network on the edge connecting haplotype groups 1 and 2 with haplotype group 3, *A. brevis* being closer to any of the haplotype groups than these are among each other (Fig. 5A).

Phylogenetic analysis of the plastid sequence data both with outgroup taxa (maximum parsimony: length = 27, both C.I. excluding uninformative characters and rescaled C.I. = 1.000; Bayesian inference: harmonic mean of log-likelihood = -3692.07) and without them (Bayesian method implemented in BEAST: 95% highest posterior density interval/mean of the model posterior and the tree likelihood, respectively: -3409.531 to -3008.159/-3195.763; -3226.707 to -3209.917/-3217.925) congruently revealed three well-supported clades (BS/PP 82–100/1.00), corresponding to haplotype groups 1–3 (Fig. 5B). In agreement with the haplotype network (Fig. 5A), tree-based methods using an outgroup (maximum parsimony, Bayesian inference in MrBayes) placed the root between the clade of haplotype group 3 and a clade including haplotype groups 1 and 2 (BS/PP 60/0.98), while the method implemented in BEAST, which does not require an outgroup, placed the root between a clade of haplotype group 1 and a clade comprising haplotype groups 2 and 3, but with negligible support (PP 0.54). The clade of haplotype group 3 consisted of two well-supported subclades (BS/PP 88–89/1.00), which corresponded to the ones described for the haplotype network. The date estimates for all nodes were strongly right-skewed, the earliest diversification within the species having a mean/median of 1.05/0.85 million years before present (95% highest posterior density interval: 0.25–2.48 million years; age estimates for the other nodes summarized in Table 2).

The estimation of Bayes factors was not affected by the uncertainties connected to the calculation of the marginal likelihoods, as the Monte Carlo errors were always around 0.1, which would

translate into a decrease of the test statistic $2 \times \ln BF$ of c. 0.4 units only. Of the six a priori phylogeographical hypotheses tested (vicariance, Pyrenean origin, stepwise or reverted eastward or westward migration), all except for the reverted eastward migration hypothesis were strongly rejected as indicated by $2 \times \ln BF < -25$ (Table 3). When taking the deep phylogeographical structure within the Alpine populations into account (Fig. 5), evidence for an Iberian origin compared to an origin in the Alps (hypotheses Iberian origin and Alpine origin, respectively) was negligible ($2 \times \ln BF$ 1.47, Table 3). Additionally, a colonization of the Pyrenees from the Alps (Alpine colonization) is strongly favored over one of a colonization of the Alps from the Pyrenees (Pyrenean colonization; $2 \times \ln BF$ 31.19, Table 3).

4. Discussion

4.1. The origin of *A. vitaliana*

An origin of *A. vitaliana* in the Pyrenees, although compatible with the ITS sequence data (Fig. 4; see Section 4.3 for further discussion), could be rejected ($2 \times \ln BF$ -25.86, Table 3) as is expected from the terminal position of the clade including the Pyrenean haplotypes (Fig. 5A) and the resulting young ages (Fig. 5B, Table 2). The ambiguity concerning the position of the root of the intraspecific genealogy, which was also reflected by indecisively small Bayes factors when comparing hypotheses of explicit root positions (Iberian vs. Alpine origin, Table 3), rendered it, however, difficult to determine whether *A. vitaliana* originated in the Iberian mountain ranges or in the Alps. This uncertainty not only stemmed from discrepancies between different markers (ITS vs. cpDNA data: Fig. 4 vs. Fig. 5), but also from differences between outgroup rooted and clock-rooted phylogenies (Fig. 5). Clock-based rooting has the advantage of not relying on a potentially too distant or even misleading outgroup (Huelsenbeck et al., 2002). This may, however, come at the expense of having too little signal for reliably placing the root of the ingroup, as appears to be the case for *A. vitaliana*. A similar pattern had been observed by Renner et al. (2008), who used the relaxed clock-approach implemented in BEAST for rooting purposes in maples and relatives.

An origin in the Iberian mountain ranges was in agreement with the nuclear ITS data (Fig. 4) and the clock-rooted plastid data (Fig. 5B) and, because it requires one dispersal event less between the Alps and the Iberian mountain ranges, it is also more parsimonious than an origin in the Alps. An Iberian origin with subsequent eastward migration has been inferred for *Anthyllis montana* (Fabaceae; Kropf et al., 2002), *Pritzelago* (Brassicaceae; Kropf et al., 2003), and for rock lizards of the genus *Iberolacerta* (Crochet et al., 2004). While the lizards' diversification has been dated to the Miocene (5.8–12.6 Mya), that of *Anthyllis* and *Pritzelago* has been suggested to be maximally 0.7 and 0.9 Mya, respectively (Kropf et al., 2002, 2003). The Pleistocene diversification ages found for *Anthyllis* and *Pritzelago* are in line with that of *A. vitaliana* (Vargas, 2003; this study), thus rendering the hypothesis of a Tertiary diversification within this species unlikely (Lüdi, 1927; Chiarugi, 1930; Schwarz, 1963).

4.2. Vicariant haplotype differentiation and subsequent long-distance dispersal

Irrespective of whether *A. vitaliana* originated in the Iberian mountain ranges or in the Alps, the simple scenarios of stepwise colonizations, regardless of whether these involved long-distance dispersal or successive isolation by extirpation in the lowlands, i.e., successive vicariance (Kropf et al., 2006), were clearly rejected by cpDNA data and at least one colonization in the opposite

Table 2
Divergence dates of cpDNA haplotype clades (as in Fig. 5; haplotype numbers following Table 1) in million years ago given as mean/median (95% highest posterior density interval).

Clade comprising haplotype(s)	Divergence date
I + II + III	0.020/0.013 (0.001–0.592)
IV + V	0.104/0.074 (0.007–0.291)
VI – XII	0.417/0.329 (0.077–1.010)
VI – VIII	0.130/0.099 (0.015–0.332)
VII	0.022/0.015 (0.001–0.064)
VIII	0.019/0.013 (0.001–0.057)
IX – XII	0.174/0.136 (0.023–0.173)
X	0.056/0.042 (0.007–0.147)
XI + XII	0.068/0.052 (0.009–0.173)
XII	0.009/0.006 (0.000–0.028)

Table 3
Marginal likelihoods and their Monte Carlo error as well as the test statistic $2 \times \ln \text{BF}$ for the tested *a priori* (H1–H6) and *a posteriori* hypotheses (H7–H10; see Section 2 and Fig. 2 for details) in *Androsace vitaliana*. The compared hypotheses (H and H') are arranged in rows and columns, respectively. $2 \times \ln \text{BF}_{H \text{ vs. } H'} > 10$ is considered strong support for H, while $2 \times \ln \text{BF}_{H \text{ vs. } H'} < -10$ is considered strong support for H'.

Hypothesis	Marginal likelihood \pm Monte Carlo error										
	H0	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10
H0: unconstrained		58.274	57.040	0.874	64.476	64.850	25.860	0.378	1.848	1.020	32.210
H1: vicariance	-58.274		-1.234	-57.400	6.202	6.576	-32.414	-57.896	-56.426	-57.254	-26.064
H2: stepwise eastward migration	-57.040	1.234		-56.166	7.436	7.810	-31.180	-56.662	-55.192	-56.020	-24.830
H3: reverted eastward migration	-0.874	57.400	56.166		63.602	63.976	24.986	-0.496	0.974	0.146	31.336
H4: stepwise westward migration	-64.476	-6.202	-7.436	-63.602		0.374	-38.616	-64.098	-62.628	-63.456	-32.266
H5: reverted westward migration	-64.850	-6.576	-7.810	-63.976	-0.374		-38.990	-64.472	-63.002	-63.830	-32.640
H6: Pyrenean origin	-25.860	32.414	31.180	-24.986	38.616	38.990		-25.482	-24.012	-24.840	6.350
H7: Iberian origin	-0.378	57.896	56.662	0.496	64.098	64.472	25.482		1.470	0.642	31.832
H8: Alpine origin	-1.848	56.426	55.192	-0.974	62.628	63.002	24.012	-1.470		-0.828	30.362
H9: Alpine colonization	-1.020	57.254	56.020	-0.146	63.456	63.830	24.840	-0.642	0.828		31.190
H10: Pyrenean colonization	-32.210	26.064	24.830	-31.336	32.266	32.640	-6.350	-31.832	-30.362	-31.190	

direction was required (Table 3). This can be attributed to the position of the Pyrenean haplotypes nested within haplotypes from the Alps and the Apennines (haplotype group 3), while the above colonization scenarios would predict the Pyrenean haplotypes to be either sister to or paraphyletic to all Alpine or all Iberian haplotypes (Fig. 2). Replacement of a resident Pyrenean haplotype by invading ones is unlikely, because organelle genomes are expected to readily introgress into invading lineages even under low levels of interbreeding, especially when the invading population is far away from its source populations (Currat et al., 2008). Therefore, it is possible that the Pyrenees were circumvented, a pattern known from the disjunction between the Cordillera Cantábrica and the western Alps seen in *A. adfinis* s. l. and *A. cantabrica* (Schneeweiss et al., 2004; Dixon et al., 2008) and in *Primula pedemontana* (Zhang et al., 2004). The latter example is important, because it indicates that heterostyly does not prevent successful establishment after long-distance dispersal. The degree of self-compatibility in *A. vitaliana* is unknown, since corresponding experiments failed (Schaeppi, 1935).

The strong distinctness of the three main plastid lineages (haplotype groups 1–3; Fig. 5) and the lack of any observed intermediate haplotypes suggest massive extinction of haplotypes in phases of small population sizes and geographical separation. The causes of this geographical separation remain unclear, but might be connected to range contractions resulting from Pleistocene climatic fluctuations (Table 2), thus following a vicariance scenario. Except for the location of the refugium of haplotype group 2 in the south-western Alps, we cannot be certain where these isolated regions may have been. For the Iberian peninsula (haplotype group 1), the interior position of haplotype 1 points to a location in one or more of the southern and central Iberian mountain ranges (Sierra Nevada, Sierra de Javalambre). The precise location of the third refugium (haplotype group 3) is unclear, although, based on the stronger evidence for the hypothesis of colonization of the Pyrenees from the Alps ($2 \times \ln \text{BF}$ 31.19 in Alpine colonization vs. Pyrenean colonization, Table 3), it is likely that this was also situated in the Alps. While haplotype group 2 has remained exclusively in the south-western Alps, haplotype group 1 more recently (Table 2) expanded its range northwards to the Cordillera Cantábrica. Despite the geographical proximity, there is no evidence for a colonization of the Pyrenees from any of the Iberian mountain ranges. A similar connection between the Sistema Ibérica and Sistema Central and the Cordillera Cantábrica, but not the Pyrenees, is also found in the alpine butterfly *Erebia triaria* (Vila et al., 2005). Range shifts have been particularly prominent in haplotype group 3 (Fig. 5). These include connections between the south-western Alps and the Pyrenees, as previously found in other alpine plants, such as *Phyteuma globulariifolium* (Schönswetter et al., 2002), *Hypericum nummularium* (Gaudeul, 2006) and *Rhododendron ferrugineum* (Wolf et al., 2004), or between the Alps and the Apennines, a less widely reported pattern (but see, for example, Randi et al., 2003), as well as within the Alps, as suggested by the presence of haplotype VI in the southern south-western Alps, while otherwise distributed exclusively in the Pennine and the south-eastern Alps (Fig. 1).

The presence of very distinct haplotype lineages and the resulting high haplotype diversity within the south-western Alps (Figs. 1 and 5) may be the result of retaining ancestral polymorphism or of gene flow into that region. Despite recent methodological and statistical progress (Noor and Feder, 2006; Slotte et al., 2008), these processes remain difficult to distinguish especially in relatively recently diverged groups (Jakob and Blattner, 2006). The weaker glaciation of the south-western Alps (Ehlers and Gibbard, 2004) left many potential refugial areas (Schönswetter et al., 2005), which would facilitate survival of different lineages. However, with the exception of *Eritrichium nanum* (Stehlik et al., 2002), no other

widespread Alpine taxon has been shown to have survived over the long term in separate refugia at such a local scale (Schönswetter et al., 2005). Furthermore, ancestral polymorphisms are expected to occur predominantly at interior nodes in a haplotype network (Schaal and Leverich, 2001), and not at the tips, as is the case with our data. Therefore, we consider the high haplotype diversity within the south-western Alps to be the result of gene flow from other, more easterly regions of the Alps into that region rather than of retained ancestral polymorphism.

4.3. Tracing range dynamics at different time levels

There is clear evidence from the plastid data for long-distance dispersal events, even if these were rare. The seeds lack obvious morphological adaptations for long-distance dispersal, but such dispersals may be mediated by non-standard vectors, i.e., dispersal vectors, which are different from those that can be inferred from the phenotype of the diaspores, and/or chance events (Nathan et al., 2008). In contrast, there is no signature of long-distance dispersal between major mountain ranges in the nuclear data, which instead show good congruence with geographical proximity (Figs. 3 and 4) in agreement with a vicariance pattern (strongly rejected by the cpDNA data: Table 3) as found in Iberian mountain plants (Kropf et al., 2006) or the montane caddis fly *Drusus discolor* (Pauls et al., 2006). This is particularly pronounced in the pattern inferred from AFLP data, where Iberian mountain ranges and the Pyrenees are well separated from the Alps and the Apennines (Fig. 3). AFLPs are, however, susceptible to genetic homogenization, and are, therefore, expected to reflect more recent events than plastid sequence data (Bussell et al., 2005; Meudt and Clarke, 2007). Consequently, AFLPs likely reflect isolation in different high mountain ranges caused by post-glacial (Quaternary) climate changes (Kropf et al., 2008, 2009), whereas plastid data indicate an older (Pleistocene) pattern of ties between major mountain ranges via long-distance dispersal.

The rather local pattern of gene flow via pollen as expected from insect foraging ranges (up to 10 km for hymenoptera: Goulson and Stout, 2001; Pasquet et al., 2008; long-distance pollen transfer capabilities of lepidoptera are controversial: Jennersten, 1984) will promote regional genetic differentiation, especially in geographically isolated and small populations, as is the case for the Iberian mountain ranges and probably also the south-eastern Alps (Figs. 3 and 4). Additionally, remigration from different refugia, as is probably the case for the differentiation between the Pennine Alps and the south-western Alps (Fig. 3) along the “Aosta line”, one of the most prominent biogeographical divisions within the Alps both at the inter- and intraspecific level, is expected to cause phylogeographical breaks (Merxmüller, 1954; Schönswetter et al., 2005).

Genetic homogenization between invading and resident genotypes via hybridization (Bänfer et al., 2006; Currat et al., 2008) is also well known for nuclear ribosomal DNA (Álvarez and Wendel, 2003). This would explain the striking and statistically significant incongruence between the plastid and the nuclear sequence data on the Iberian Peninsula, where populations from the Iberian mountain ranges and the Pyrenees have essentially identical ITS sequences (Fig. 4), but diametrically different plastid haplotypes (Fig. 5A). According to this hypothesis, Pyrenean *A. vitaliana* had been introgressed from populations in the Iberian mountain ranges with subsequent convergence of the resident ITS towards the invading type. Similar processes might be responsible for the incongruent position of the Apennine population. This would imply gene flow between the southern European mountain ranges sparing the Alps as known from other plants (Csergő et al., 2009), but this finds no support in the AFLP data (Fig. 3). Evidently, further data are necessary to test these hypotheses.

4.4. Taxonomy

With the exception of the south-eastern Alpine subsp. *sesleri* and the Apennine subsp. *praetutiana*, current infraspecific taxonomy only poorly reflects distinct genetic lineages. This is true for subsp. *cinerea* and subsp. *vitaliana*, which both are reported from the Pyrenees as well as the south-western Alps (Kress, 1967, 1997a,b). In contrast, genetic data (Figs. 3 and 4) suggest a simple bipartition following the geographic distinctness between these two mountain ranges. Elsewhere in the Alps, diversity has been underestimated, because populations from the Pennine Alps, currently subsumed under subsp. *vitaliana*, are well separated from those in the south-western Alps (Fig. 3). In contrast, the variability within the Iberian mountain ranges has been overestimated taxonomically, because all four currently recognized subspecies collectively form a single entity of comparable genetic distinctness with the other subspecies (Figs. 3–5). A forthcoming paper will address a variety of taxonomic and nomenclatural issues arising from this work, but which are beyond the scope of this publication.

5. Conclusions

The phylogeographical history of *A. vitaliana* is more complicated than could be inferred from current distribution. Instead of a simple and ancient vicariance pattern with diversification dating back to the Tertiary, the history of *A. vitaliana* is significantly shaped by a combination of vicariance and long-distance dispersal events between and within major mountain ranges during the Quaternary. Despite the desirable statistical rigor of Bayesian hypothesis testing, its reliance on *a priori* hypotheses may render its application to complex phylogeographical histories such as the one of *A. vitaliana* difficult. Methods that statistically estimate phylogeographical history without relying on *a priori* hypotheses, as described by Lemmon and Lemmon (2008), will, once extended to non-continuous distribution areas, become a promising alternative.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jympev.2009.07.016.

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