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Traces of ancient range shifts in a mountain plant group (*Androsace halleri* complex, Primulaceae)

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Abstract

Phylogeographical studies frequently detect range shifts, both expansions (including long-distance dispersal) and contractions (including vicariance), in the studied taxa. These processes are usually inferred from the patterns and distribution of genetic variation, with the potential pitfall that different historical processes may result in similar genetic patterns. Using a combination of DNA sequence data from the plastid genome, AFLP fingerprinting, and rigorous phylogenetic and coalescence-based hypothesis testing, we show that *Androsace halleri* (currently distributed disjunctly in the northwestern Iberian Cordillera Cantábrica, the eastern Pyrenees, and the French Massif Central and Vosges), or its ancestor, was once more widely distributed in the Pyrenees. While there, it hybridized with *Androsace laggeri* and *Androsace pyrenaica*, both of which are currently allopatric with *A. halleri*. The common ancestor of *A. halleri* and the north Iberian local endemic *Androsace rioxana* probably existed in the north Iberian mountain ranges with subsequent range expansion (to the French mountain ranges of the Massif Central and the Vosges) and allopatric speciation (*A. rioxana*, *A. halleri* in the eastern Pyrenees, *A. halleri* elsewhere). We have thus been able to use the reticulate evolution in this species group to help elucidate its phylogeographical history, including evidence of range contraction.

Keywords: *Androsace*, chloroplast capture, hybridization, phylogeography, Pyrenees, range shift

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Introduction

Range shifts, both expansions, for instance via long-distance dispersal, and contractions, such as in the course of Pleistocene glaciations, can have profound consequences for lineage or species evolution, as they determine the possibility and extent of hybridization between different lineages, for example in suture zones (e.g. Green *et al*. 1996; Taberlet *et al*. 1998; Hewitt 2004). Range shifts are commonly inferred in phylogeographical studies, but since the paucity of the fossil record often prevents the direct inference of range shifts, these processes are usually deduced from patterns of genetic variation (e.g. Hellberg *et al*. 2001; Bartish *et al*. 2006; Zink *et al*. 2006).

Range expansions, either continuous or stepwise, can be statistically tested by nested clade analysis (NCA; Templeton 1998). It remains, however, controversial to what extent NCA is compromised by the interpretation of patterns produced by stochastic sorting of gene lineages as those produced by deterministic processes (see Knowles & Maddison 2002 vs. Templeton 2004). Other methods, such as mismatch distribution (Rogers & Harpending 1992) or neutrality tests (Fu 1997), can infer population growth, but their inferences are not always unambiguous because different processes can produce the same patterns and because population growth does not necessarily translate into actual range expansion.

Inferring population contraction is, in addition to the problems outlined for range expansions, hampered by the truism that extinct populations simply cannot be sampled, except in groups with well-preserved fossils, such as Pleistocene horses (Weinstock *et al*. 2005). Another exception can occur in groups that have undergone reticulate evolution (hybridization, introgression), where genetic markers of one population (or species) might be found in geographically distinct populations, suggesting gene transfer in a sympatric or parapatric phase in the history of the compared populations (Roelofs *et al*. 1997; Gielly *et al*. 2001; Álvarez & Wendel 2006). Although those patterns can arise from

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processes other than gene flow, especially incomplete lineage sorting (Maddison & Knowles 2006), the frequency of reticulate evolution and the scarcity of fossils in plants make this method a valuable alternative.

Range shifts of mountain plants typically lead to discontinuous (disjunct) distributions. A good model system for exploring the processes of range shifts and their significance for lineage evolution is *Androsace* sect. *Aretia* (Primulaceae). This group comprises *c.* 20 closely related, morphologically well-defined species with similar dispersal abilities and floral syndromes. It is distributed in the southern and central European mountain ranges with centres of species richness in the Alps and the Pyrenees, with many species possessing fragmented distribution areas both within and between mountain ranges.

A recent molecular phylogenetic study (Schneeweiss *et al*. 2004) has redefined both the circumscription of the whole group, and of several species groups. One such group, indicated by phylogenetic analyses using several plastid regions (C. J. Dixon, unpublished data) to be monophyletic comprises *Androsace laggeri* and *Androsace halleri* (corresponding to ssp. *laggeri* and ssp. *rosea* of a larger, polyphyletic *Androsace carnea* in Ferguson 1972) along with *Androsace rioxana* and *Androsace pyrenaica*. This close relationship is also reflected in the geography of the four species, all of which are centred around the mountains of northern Spain (Fig. 1). While *A. laggeri* and *A. pyrenaica* have compact distribution areas in the central Pyrenees, and *A. rioxana* is endemic to the northern Iberian Sierra de la Demanda, *A. halleri* in its current circumscription has a fragmented distribution range comprising four disjunct regions: Cordillera Cantábrica (northwestern Spain), eastern Pyrenees, Massif Central (central France) and Vosges (eastern France). Populations of *A. halleri* from the eastern Pyrenees differ morphologically from those elsewhere, and this has been suggested to result either from different habitat preferences (Kress 1997) or through introgression, probably from *A. laggeri* (Kress 1991). The latter hypothesis is supported by the fact that hybridization is quite common within the genus *Androsace*, wherever two or more species co-occur (Smith & Lowe 1997). It is, however, at odds with the current distribution of the taxa, since *A. halleri*, despite having the widest distribution area, does not co-occur with any of the other taxa. The highly fragmented distribution of *A. halleri* indicates that range shifts have played an important role in the evolution of this species, and therefore ancient para- or sympatric phases with other species providing the opportunities for gene flow might have existed.

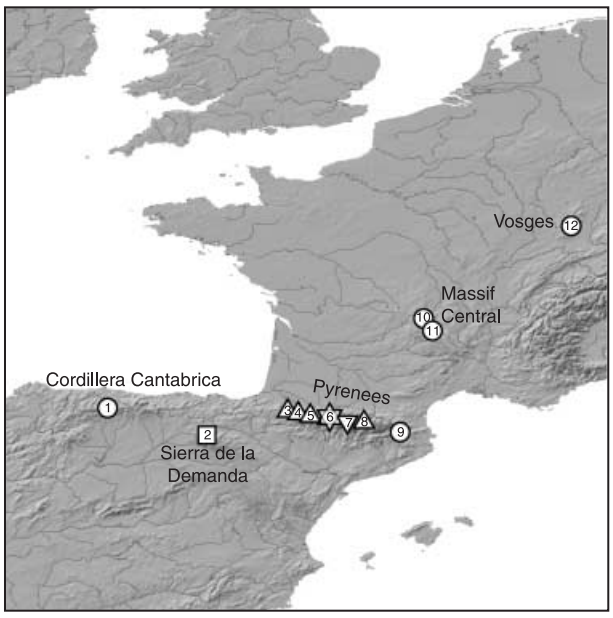

Fig. 1 Map showing the sampling locations of the four species in southwestern and central Europe. Further details are given in Table 1. Circles represent *Androsace halleri*, the square *Androsace rioxana*, triangles *Androsace laggeri* and inverted triangles *Androsace pyrenaica*. The six-pointed star represents both *A. laggeri* and *A. pyrenaica*.

In this study, we use chloroplast sequences, which are maternally inherited in the majority of angiosperms (Korpelainen 2004) including the Primulaceae (Harris & Ingram 1991), and amplified fragment length polymorphism (AFLP) markers, which are essentially nuclear and biparentally inherited (Bussell *et al*. 2005), to investigate range shifts and potential reticulate evolution involving *A. halleri*. Specifically, we address the following questions: what are the phylogeographical relationships of the four geographically distinct subgroups within *A. halleri* and which are the most likely processes responsible for the current highly fragmented distribution area of this species? Is there evidence for ancient hybridization of *A. halleri* with other species? If so, which species are involved and how does this affect the inference of a potential ancestral distribution area of *A. halleri*?

Materials and methods

Study group

Androsace halleri L. is a perennial caespitose herbaceous plant, typically found in the subalpine and lower alpine vegetation belts in juniper and *Vaccinium* communities on soils derived from silicaceous bedrock (Kress 1997). In the eastern Pyrenees, it is instead found in snowbed communities in the upper alpine or subnival vegetation belt (G. M. Schneeweiss and P. Schönswetter, personal observation). Across most of its range, its leaves are generally 10–25 mm long, with a ruffled upper surface, ending with a recurved hook and bearing branched hairs. Again, plants from the eastern Pyrenees differ from the others in having shorter and thinner leaves with no hooks (or only weakly developed

hooks) at their tips (Kress 1991, 1997). The flowers are always pink and arranged in a small umbel on the end of a leafless stalk. The population of *A. halleri* at the species' type locality in the Vosges mountains is critically endangered, having been reduced to a single location in the wild (Issler *et al*. 1965) and those plants that are currently propagated in botanical gardens.

Androsace laggeri A. Huet is endemic to the central Pyrenees, where it occurs in grassland communities in the subalpine and alpine vegetation belts over silicaceous bedrock. In its gross morphology, it closely resembles *A. halleri*, but differs in its shorter (5–15 mm long) and smoother, more upright leaves, which bear less complex hairs.

Androsace pyrenaica Lam. is also a central Pyrenean endemic species, with a distributional area overlapping with *A. laggeri*. It forms dense cushions on siliceous rocks and calciferous schists at up to 3000 m above sea level, often in rock fissures. Apart from the growth form, it differs from the other three species in having white flowers in single-flowered inflorescences and leaves up to 7 mm long. It is more widespread and numerous than has often been reported, with an estimated number of up to 10 000 individuals in the wild (García *et al*. 2002).

Androsace rioxana A. Segura is endemic to the Sierra de la Demanda mountains in La Rioja autonomous community (Spain) and, with only a few hundred individuals in two small populations (García-Baquero Moneo *et al*. 2001), is listed as Endangered in the Spanish Red Book (Medrano Moreno *et al*. 2004). It grows between 2000 and 2200 m above sea level in rocky grasslands on silicaceous soils that are dry in summer (Kress 1997). It resembles *A. halleri* morphologically, having scapes bearing umbels of pink flowers arising from a large basal leaf rosette and a similar type of branched hair on the vegetative parts (E. M. Sehr, E. M. Mayr, G. M. Schneeweiss, unpublished data). The leaves often bear small teeth towards the distal end and may be up to 4 mm wide. Although initially considered a close relative of *A. laggeri* and similar species (Montserrat in Segura Zubizarreta 1973), *A. rioxana* has been treated by some authors as a subspecies of *Androsace obtusifolia* (details in Kress 1997).

Plant material

Leaf material from 10 plants was collected at each of 12 sampling locations, representing the entire natural range of each of the four species (Table 1), and stored immediately in silica gel. The only exceptions were population 12 (Vosges), which comprises a single individual, population 10 (Massif Central) where only three individuals were found, each of which was sampled, and population 1 (Cordillera Cantábrica) where only five individuals were found, all these populations being from *A. halleri*. Voucher specimens (except for *A. rioxana*, which is already well documented in Spanish herbaria)

Table 1 Sampling locations, voucher information, numbers of individuals analysed, haplotypes and their GenBank Accession nos. All vouchers are deposited at University of Vienna and, unless otherwise noted, have been collected by G.M.S. and P.S.

	Location (voucher information)	Coordinates	*Androsace* Species	Individuals	Haplotype	GenBank Accession nos
	Šar, Macedonia (11080)	42°1′ N, 20°51′ E	*A. hedraeantha*	1	n. a.	EF189903, EF189904, EF189905
1	Pico Huevo, Cordillera Cantábrica (8899)	43°2′ N, 5°29′ W	*A. halleri*	5	C	EF152438, EF152451, EF152464
2	Pico San Lorenzo, La Rioja	42°14′ N, 2°57′ W	*A. rioxana*	10	E	EF152444, EF152457, EF152470
3	El Portalet, Pyrenees (8877)	42°48′ N, 0°26′ W	*A. laggeri*	10	H	EF152441, EF152454, EF152467
4	Picos del Infierno, Pyrenees (no voucher)	42°48′ N, 0°15′ W	*A. laggeri*	10	H	EF152442, EF152455, EF152468
5	Massif de Néouvielle, Pyrenees (8867)	42°46′ N, 0°07′ E	*A. laggeri*	10	D	EF152439, EF152452, EF152465
6	Sierra Marqués, Pyrenees (8865; 8864)	42°39′ N, 0°16′ E	*A. laggeri*	10	G	EF152440, EF152453, EF152466
			A. pyrenaica	10	F	EF152445, EF152458, EF152472
7	Macizo de Posets, Pyrenees (8839)	42°26′ N, 0°26′ E	*A. pyrenaica*	10	I	EF152446, EF152459, EF152473
8	Vall de Cardós, Pyrenees (8907)	42°42′ N, 1°18′ E	*A. laggeri*	10	H	EF152443, EF152456, EF152469
9	Puigmal d'Err, Pyrenees (8836)	42°22′ N, 2°08′ E	*A. halleri*	10	C	EF152437, EF152450, EF152463
10	Puy Mary, Massif Central (8816)	45°06′ N, 2°41′ E	*A. halleri*	3	B	EF152436, EF152449, EF152462
11	Mont Dore, Massif Central (8812)	45°31′ N, 2°50′ E	*A. halleri*	10	B	EF152435, EF152448, EF152461
12	Conservatoire Botanique de la Ville de Mulhouse *ex* Grand Ballon d'Alsace, Vosges	47°54′ N, 7°06′ E	*A. halleri*	1	A	EF152434, EF152447, EF152460

have been deposited in the herbarium of the University of Vienna (WU): see Table 1.

Molecular methods

Total genomic DNA was extracted from leaf material following the 2× cetyltrimethyl ammonium bromide (CTAB) method (Doyle & Doyle 1987) with minor modifications (Schönswetter *et al*. 2002). Three regions of the chloroplast genome were sequenced from four individuals per population (where available) using the following three primer pairs: ccmp3f (Weising & Gardner 1999) and trnRr (Dumolin-Lapegue *et al*. 1997); rpl20 and 5′-rps12 (both Hamilton 1999); trnS(UGA) and trnfM(CAU) (both Demesure *et al*. 1995). These regions are also being studied in other species of *Androsace* to enable interspecific comparison. Polymerase chain reaction (PCR) conditions for the first primer pair were 3 min at 94 °C followed by 40 cycles of 1 min at 94 °C, 30 s at 48 °C and 1 min at 72 °C, followed by 10 min at 72 °C. Reaction volumes were 25 μL, comprising 9 μL of Ready-Mix (Sigma-Aldrich), 1 μL of template DNA of unknown concentration and primers at a final concentration of 1 nM. For the other two regions, PCR conditions were 30 s at 96 °C followed by 35 cycles of 5 min at 94 °C, 45 s at 48 °C, and 10 min at 68 °C, finishing with 10 min at 68 °C. These reactions were carried out in 20-μL volumes, using Red Accu*Taq* Jump Start polymerase (Sigma-Aldrich), according to the manufacturer's instructions. These reactions each produced a single PCR product, as visualized on 0.5% agarose gels. The PCR products were cleaned with ExoSAP (USB Corporation) according to the manufacturer's instructions. All reactions were carried out on a GeneAmp 9700 thermocycler (Applied Biosystems). Cycle sequencing used BigDye Terminator chemistry (Applied Biosystems), according to the manufacturer's instructions, after which electrophoresis was carried out with an ABI 3100 capillary sequencer (Applied Biosystems). Sequences were edited with SEQMANTM II version 5.05 (DNAStar Inc.) and aligned by eye using BIOEDIT 7.0.4.1 (Hall 1999). Sequences have been submitted to GenBank (Table 1).

The AFLP procedure followed Gaudeul *et al*. (2000), but with PCR volumes halved. The following three selective primer combinations were chosen (fluorescent dye in brackets): (6-FAM)–*Eco*RI ACA and *Mse*I CAC; (NED)–*Eco*RI AAC and *Mse*I CTT; (VIC)–*Eco*RI AGG and *Mse*I CAA. For each individual, 1.2 μL 6-FAM–, 2 μL VIC–, and 3 μL NED–labelled selective PCR products were precipitated with 30 μL 96% ethanol and 1 μL sodium acetate (3N, pH 4.6), washed with 70% ethanol, dried, and re-suspended in 0.2 μL GeneScan ROX-500 (size standard; Applied Biosystems) and 9.8 μL formamide. Blind samples and replicates were routinely included to test for contamination and to assess reproducibility. Electrophoresis was carried out with an ABI 3100 capillary sequencer (Applied Biosystems); raw data were collected and aligned with the internal size standard using ABI PRISM GeneScan (Applied Biosystems). Since population (pop.) 5 (*A. laggeri*) and pop. 12 (*A. halleri*, Vosges) were erroneously omitted in this first analysis, a second set of AFLP data was generated following the protocol of Tremetsberger *et al*. (2003; see Supplementary material for further details).

The sample files were imported into GENOGRAPHER (version 1.6.0, Montana State University 1999; 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 to be irreproducible were excluded from the analyses.

Data analysis

Neighbour-joining trees were produced from the AFLP data in TREECON 1.3b (Van de Peer & De Wachter 1994) using Nei & Li (1979) distances.

As no intrapopulational sequence variation was encountered (see Results), phylogenetic analyses were conducted on a data set including one individual per population only, using *Androsace hedraeantha* as an outgroup (data matrix and phylogenetic trees available from TREEBASE under study no. S1676 and matrix accession no. M3034). Phylogenetic relationships were inferred using statistical parsimony as implemented in TCS 1.21 (Clement *et al*. 2000) to reconstruct a haplotype network, and maximum parsimony and maximum likelihood in PAUP* 4.0b10 (Swofford 2002) as well as Bayesian analysis as implemented in MRBAYES 3.1.2 (Ronquist & Huelsenbeck 2003) to reconstruct phylogenetic trees. For the network analysis, indels longer than 1 bp were re-coded as single base pairs and single-base-pair gaps were then treated as a fifth character state.

In the maximum-parsimony analysis, trees were sought employing a branch-and-bound strategy with furthest sequence addition. Characters were treated as unordered and of equal weight, and gaps were treated as missing data. Clade support was assessed by bootstrapping with 100 replicates, with the same search options. Maximum-likelihood trees were obtained using the Hasegawa–Kishino–Yano (HKY) substitution model (the best model as suggested by the Akaike information criterion as implemented in MRMODELTEST 2.2; Nylander 2004) with all parameters estimated in the course of the analysis through a heuristic search with 100 random addition sequence replicates, tree-bisection–reconnection branch swapping, MULTREES in effect, but the steepest descent option not in effect. Clade support was assessed via bootstrapping with 100 replicates with full optimization and using neighbour-joining trees as starting points.

In order to account for model uncertainty in the Bayesian analysis, we used an approach similar to that described

in Beier *et al*. (2004). Trees were sampled from each Markov chain Monte Carlo (MCMC) run in proportion to the Akaike weights of the models, the latter estimated as implemented in MRMODELTEST 2.2 (Nylander 2004), including models until the cumulative Akaike weight exceeded 0.95. The 95% credible set of models included the three main model types F81 (Felsenstein 1981), HKY (Hasegawa *et al*. 1985) and GTR (Tavaré 1986; Rodríguez *et al*. 1990), which are also available in MRBAYES and differ in the number of substitution types (1, 2 and 6, respectively). In order to reduce the number of models to be investigated, we included submodels of the three main model types. As the gamma shape parameter (Γ) and the proportion of invariable sites (I) are strongly correlated (Yang 1993; Sullivan & Swofford 2001), we subsumed models with a proportion of invariable sites (with or without an additional parameter for a gamma distribution) under models with a gamma distribution accounting for rate heterogeneity (Yang 1996), but increasing the number of discrete rate categories to 15 from the default value of 4. Submodels without rate heterogeneity were subsumed into the equivalent models with rate heterogeneity, because (potential) overparameterization appears to be less critical for tree topology estimation and clade support than underparameterization (Lemmon & Moriarty 2004; Nylander *et al*. 2004). The Bayesian analysis was eventually conducted on the models (with the submodels and their Akaike weights in parentheses) HKY+Γ (HKY: 0.2552, HKY+I: 0.0939, HKY+Γ: 0.0939, HKY+Γ+I: 0.0345), F81+Γ (F81: 0.1860, F81+I: 0.0684, F81+Γ: 0.0684, F81+Γ+I: 0.0252) and GTR+Γ (GTR: 0.0933, GTR+I: 0.0343, GTR+Γ: 0.0343, GTR+Γ+I: 0.0126), which contributed 47.75%, 34.80% and 17.45%, respectively, to the posterior set of trees. For the Metropolis-coupled Markov chain Monte Carlo (MC3) process, three runs with four chains each (one cold and three heated chains using the default incremental heating scheme) were run simultaneously for a number of generations proportional to the model's Akaike weight, with trees being sampled every 100th generation, using the default priors (flat Dirichlet priors for the substitution matrix and state frequencies; beta prior (1, 1) for the transition/transversion rate ratio; uniform prior (0.05, 50) for the shape parameter of the gamma distribution; all topologies equally probable; exponential prior (10) for the branch lengths). Convergence of independent runs was assessed (i) by comparing likelihood scores and means and variances across runs, and (ii) from the variance of split frequencies (mcmcdiagn = yes) calculated every 10 000th tree, discarding the first 20% (relburnin = yes burninfrac = 0.2). Convergence was considered to have been reached when the variance of split frequencies was < 0.01. The posterior probability (PP) of the phylogeny and its branches was determined from the combined set of trees, discarding the first 20% of trees of each run (i.e. the set of trees discarded for the convergence diagnostics when the runs were completed), each model contributing to the total of 50 000 trees in proportion to its Akaike weight (HKY+Γ: 23 875 trees, F81+Γ: 17 400 trees, GTR+Γ: 8725 trees).

Alternative phylogenetic hypotheses, specifically the monophyly of each of *A. pyrenaica* and *A. laggeri* (see Results for details), were tested in a phylogenetic framework using (i) Bayesian hypothesis testing (Huelsenbeck *et al*. 2002), and (ii) parametric bootstrapping [SOWH (Swofford–Olsen–Waddell–Hillis) test; Swofford *et al*. 1996]. Using the Bayesian test, alternative topologies with posterior probabilities of less than 0.05 (determined from the combined set of trees after the burn-in period) are considered significantly worse. The basic principle of the SOWH test is to compare the observed difference in likelihood scores between the null and the alternative hypothesis (that is, the tree constrained to the topology to be tested and the unconstrained maximum-likelihood tree, respectively) to a null distribution of differences in likelihood scores (Goldman *et al*. 2000). To this end, 500 data sets were simulated with SEQ-GEN 1.3.1 (Rambaut & Grassly 1997) on the null hypothesis topology (that is, the topology constraining both *A. pyrenaica* and *A. laggeri* to be monophyletic) using the maximum-likelihood parameters of the null hypothesis (using the HKY model with transition/transversion ratio of 1.00151 and base frequencies A = 0.332477 C = 0.166614 G = 0.172039 T = 0.32887), and each data set was analysed using full optimization maximum likelihood with PAUP* 4.0b10 (Swofford 2002) with and without the constraint enforced. The null hypothesis is rejected if the observed difference in likelihood scores is ≥ 95% of the (ranked) simulated differences in likelihood scores. As the SOWH test is very sensitive to model specifications with the potential pitfall that misspecified models will lead to high Type I error rates (Buckley 2002), it was repeated using a HKY+Γ model, as rate heterogeneity is thought to be one of the most important parameters to incorporate (Huelsenbeck *et al*. 1996; Buckley 2002). Data sets were simulated as described above, using the maximum-likelihood parameters from a HKY+Γ model (transition/transversion ratio of 1.00283, base frequencies A = 0.332461 C = 0.166636 G = 0.172055 T = 0.328848, shape parameter of the gamma distribution $\alpha = 0.012556$).

In order to take into account the stochastic population genetic processes that determine the shape of the genealogy of sampled gene sequences, we used the coalescence-based method of the Bayesian skyline plot as implemented in BEAST 1.3 (Drummond & Rambaut 2003). This method is an extension of the generalized skyline plot (Strimmer & Pybus 2001), which is itself a modification of the classic skyline plot (Pybus *et al*. 2000), taking phylogenetic uncertainty and the inherent stochastic error in the coalescence process into account (Drummond *et al*. 2005). An MCMC method is therefore employed, which co-estimates the ancestral genealogy, the parameters of the substitution process and the demographic parameters. We used the

same data set as for the phylogenetic analyses, but without the outgroup species *A. hedraeantha* (BEAST 1.3 employs a molecular clock and thus tree topologies are always rooted), using an HKY+Γ model with 10 discrete rate categories. The parameters of the MCMC run were successively tuned using the auto-optimization option, and the quality of the estimate of the posterior distribution of a given parameter was considered sufficient with effective sample sizes above 150 (assessed with TRACER 1.3, available from http://evolve.zoo.ox.ac.uk/). Final analyses were run for 3×10^7 generations, sampling every 1000th generation, and a burn-in of 3×10^6 generations (after a preburn-in of 3×10^5 generations). The analyses were repeated using different values for the number of grouped intervals (m = 5, 10) and different clock models (strict clock and relaxed clock with uncorrelated rates drawn from a log-normal distribution; Drummond *et al*. 2006), conducting two independent MCMC runs for each parameter combination. Alternative topologies with posterior probabilities less than 0.05 (determined from the combined set of 54 000 trees after the burn-in period) are rejected.

Results

AFLP

A total of 110 reproducible bands were produced for the accessions studied, of which 13 were monomorphic (i.e. found in all individuals) and four were found in only one individual each (see Supplementary material for information on the second AFLP data set). The error rate (Bonin *et al*. 2004) before the exclusion of unreliable characters was less than 5%. In the neighbour-joining tree (Fig. 2 and Supplementary material), three well-supported groups were discernible — *Androsace laggeri* (99% bootstrap support), *Androsace pyrenaica* (100%) and a third group comprising *Androsace halleri* and *Androsace rioxana* (99%). Within this latter group, three distinct lineages were observed, with the relationships between the three lineages being insufficiently supported. One lineage contained *A. rioxana* (100%), a second contained the Pyrenean population of *A. halleri* (100%), with all the remaining *A. halleri* accessions making up the third lineage (91%).

No fragments were found only in Pyrenean *A. halleri* and either *A. laggeri* or *A. pyrenaica*, while four fragments found in Pyrenean *A. halleri* were shared with *A. rioxana*, and three with the remaining *A. halleri*.

Plastid sequences

Five variable characters were found in the 988-bp long trnS–trnfM region, six in rpl20–5′-rps12 (687 bp) and six in ccmp3–trnR (492 bp), making a total of 17 characters, including indels, in 2167 bp (0.78% variability). Combining the three separate chloroplast sequences for each individual by simple concatenation, assuming the chloroplast genome to form a single linkage group, gave a total of nine haplotypes in the 48 individuals analysed, with no intrapopulational haplotype variation. Using the method described by Dixon (2006), which calculates a posterior probability distribution of the total number of haplotypes, including those which may not have been sampled, this gives a probability of 94% that all haplotypes have been sampled.

Statistical parsimony inferred three distinct lineages (Fig. 3): the '*laggeri* group' containing all accessions of *A. laggeri* with

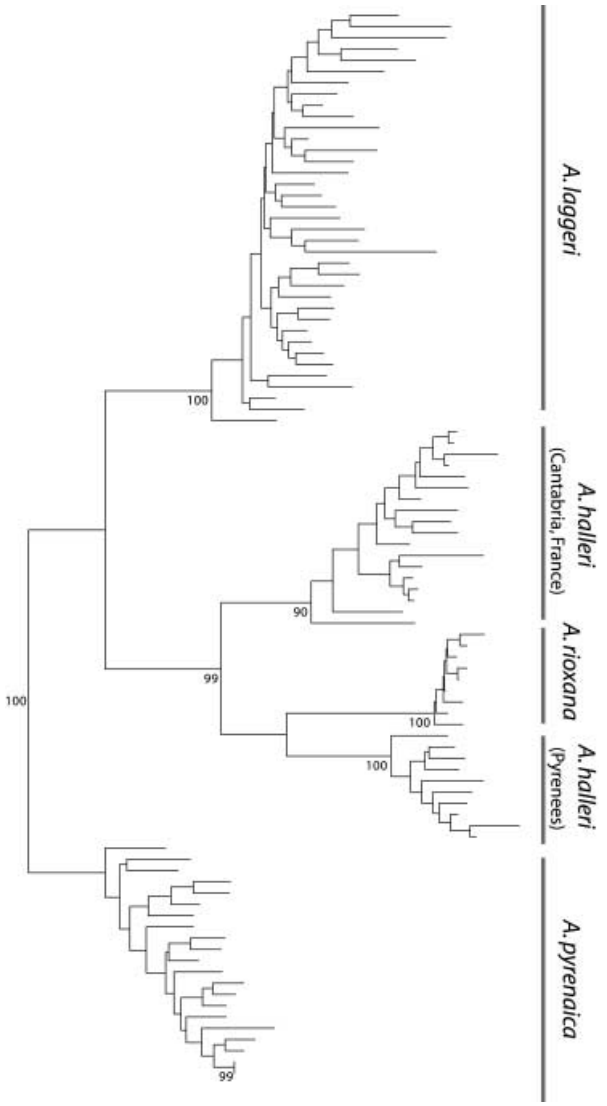

Fig. 2 Neighbour-joining tree based on a Nei–Li (1979) distance matrix derived from AFLP data. Values above branches are bootstrap percentages > 90%. The tree has been manually rooted at a point on the branch separating *Androsace pyrenaica* from the other taxa. Note that the branch leading from the internal node to '*halleri* 1, 9, 10, 11; *laggeri* 5' is of zero length.

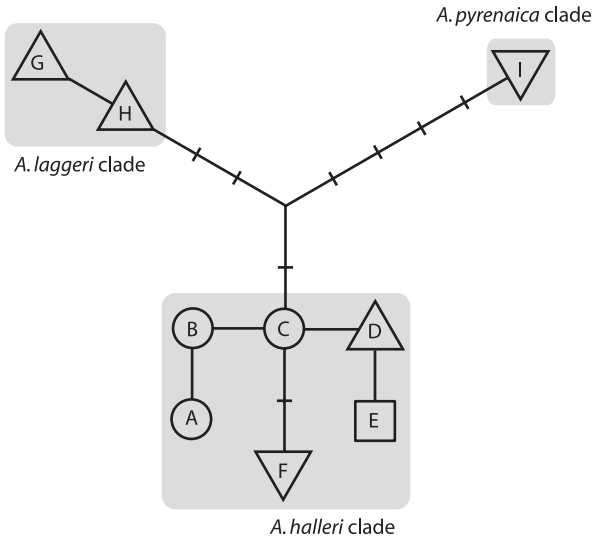

Fig. 3 Haplotype network for the combined chloroplast data. Haplotypes A, B and C occur in *Androsace halleri*, haplotypes G, H and D in *Androsace laggeri*, haplotypes I and F in *Androsace pyrenaica* and haplotype E in *Androsace rioxana*. Each branch and each additional bar represent one mutational change. The three main clades are shaded in grey.

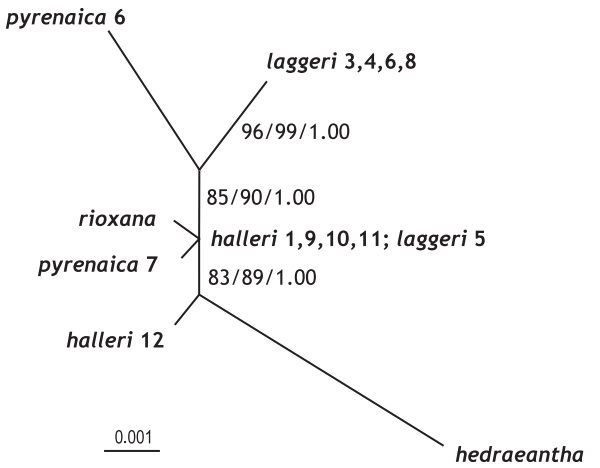

Fig. 4 Phylogenetic relationships of plastid haplotypes inferred from maximum likelihood. Numbers beside branches are maximum parsimony bootstrap/maximum-likelihood bootstrap/Bayesian posterior probabilities.

the exception of population 5 from the central Pyrenees; the '*pyrenaica* group' consisting of the eastern population of *A. pyrenaica* (pop. 7); and the '*halleri* group' comprising all populations of *A. halleri* (haplotypes A–C) and *A. rioxana* (haplotype E) plus one population each of *A. laggeri* (pop. 5: haplotype D) and *A. pyrenaica* (pop. 6: haplotype F). In order to be able to exclude the possibility of DNA contamination and/or mix-up, individuals of *A. laggeri* from population 5 and *A. pyrenaica* from population 6 were re-extracted from leaf material which was checked for the correct species determination, and re-sequenced. This confirmed the position of these two populations. Populations of *A. laggeri* united in the laggeri group possessed two haplotypes (haplotypes G and H) differing by one substitution only (a unique single base-pair insertion in *A. laggeri* from population 6), while successive mutations separated *A. halleri* from the Massif Central (haplotype B) and *A. halleri* from the Vosges mountains (haplotype A) from the Cantabrian and Pyrenean *A. halleri* (haplotype C). Using the closely related *Androsace hedraeantha* as an outgroup (Schneeweiss *et al*. 2004), the root connected along the branch leading to the *pyrenaica* group, suggesting *A. pyrenaica* to be the sister group to the remaining species (data not shown). The same three groups were found with the tree-building phylogenetic methods used. In contrast to the haplotype network, however, the root was placed at the branch separating *A. halleri* pop. 12 (Vosges) from all other populations of *A. halleri*, *A. laggeri*, *A. pyrenaica* and *A. rioxana* (Fig. 4), rendering the *halleri* clade paraphyletic. The *pyrenaica* and *laggeri* clades (maximum parsimony/maximum-likelihood bootstrap/Bayesian posterior probabilities 96/99/1.00) were inferred as sister taxa (85/90/1.00).

One haplotype each of *A. laggeri* (pop. 5) and *A. pyrenaica* (pop. 7) fell into the *halleri* group (Figs 3 and 4). The alternative hypothesis of monophyletic *A. laggeri* and *A. pyrenaica* (that is, that these accessions belong, respectively, to the *laggeri* and *pyrenaica* groups) is strongly rejected by the Bayesian test ($P < 0.001$) and by the SOWH test using the HKY model ($P = 0$), but not by the SOWH test using the HKY+Γ model ($P = 0.258$). The coalescence-based tests were conducted with models differing in the type of molecular clock (strict vs. relaxed clock) and number of grouped intervals (5 or 10) used. Models employing a strict molecular clock yielded better mean posterior log-likelihood scores than the equivalent models with a relaxed molecular clock (–2923.333 vs. –2953.281 and –2953.161 vs. –2978.12 for m = 10 and m = 5, respectively). The joint monophyly of *A. laggeri* and *A. pyrenaica* was rejected, which can be attributed nearly completely to the lack of monophyly for *A. laggeri*, while that of *A. pyrenaica* cannot be rejected (Table 2). As the Bayesian skyline plot indicated no detectable changes in effective population sizes, the analyses were repeated using a constant population-size model, but these gave essentially the same results as the Bayesian skyline plot analyses (data not shown).

Discussion

Phylogenetic relationships

In each of our analyses, the group of four species was split into three clear groups. In the AFLP analysis, these followed

Table 2 Probabilities of alternative hypotheses inferred using the Bayesian skyline plot with two different molecular clock models (strict and relaxed) and two different grouped intervals (m = 5 and m = 10) determined on unrooted and, in parentheses, rooted tree topologies (see text for details). Significant values (*$P < 0.05$; **$P < 0.01$) are indicated in bold

Molecular-clock type	Group size (m)	*Androsace laggeri* and *Androsace pyrenaica*	*A. laggeri*	*A. pyrenaica*
Strict	5	**0.0030** (0.0017**)**	**0.0030** (0.0019**)**	0.5578 (0.5146)
	10	**0.0043** (0.0030**)**	**0.0043** (0.0033**)**	0.6058 (0.5643)
Relaxed	5	**0.0341*** (0.0250*)**	**0.0341*** (0.0258*)**	0.9934 (0.9610)
	10	**0.0391*** (0.0311*)**	**0.0391*** (0.0316*)**	0.9960 (0.9738)

the species boundaries, with *Androsace laggeri* and *Androsace pyrenaica* being resolved as monophyletic, and *Androsace halleri* and *Androsace rioxana* being combined in the third group, which we call the *halleri* clade. This clade can in turn be divided into three distinct lineages, one containing *A. rioxana*, one containing *A. halleri* from the eastern Pyrenees, and one containing all the remaining *A. halleri* populations (Cordillera Cantábrica, Massif Central, Vosges). Analyses of the cpDNA sequence data essentially recovered the same groups but with some differences concerning the *halleri* clade. These are (i) the inclusion of one population each of *A. laggeri* and *A. pyrenaica*, and (ii) the lack of differentiation of *A. halleri* from the eastern Pyrenees from those elsewhere (see next section for a more detailed discussion).

The close relationship between *A. rioxana* and *A. halleri* is confirmed by both molecular data types. These two species share a similar growth form, identical hair types on the leaves, and pink flowers arranged in umbels on long stalks (Kress 1997; E. M. Sehr, E. M. Mayr, G. M. Schneeweiss, unpublished data), but *A. rioxana* differs from all other species considered here by its relatively broad leaves, which are often toothed. This latter feature probably caused some authors to consider a closer relationship to *Androsace obtusifolia* (details in Kress 1997), which is clearly erroneous (Schneeweiss *et al*. 2004; C. J. Dixon, P. Schönswetter, G. M. Schneeweiss, unpublished data).

The populations of *A. halleri* in the eastern Pyrenees have previously been recognized as different from *A. halleri* found elsewhere (Kress 1991, 1997). Since their ecology and morphology approach those of *A. laggeri*, it has been hypothesized that Pyrenean *A. halleri* has experienced introgression with *A. laggeri* (Kress 1991). We found no evidence to support this claim; indeed, Pyrenean *A. halleri* was found to share AFLP bands with other members of the *halleri* clade and not with *A. laggeri*, and to be just as distinct as *A. rioxana* from the remaining *A. halleri*. If its dissimilarity is not due to introgression, other processes such as genetic drift or different ecological profiles might be responsible for the differences. In any case, Pyrenean *A. halleri* should be recognized at the same taxonomic rank as *A. rioxana*, and will therefore be described as a new species in a forthcoming study.

Reticulate evolution and range shifts in Androsace halleri

In the plastid data set, one population each of *A. laggeri* (pop. 5: haplotype D) and *A. pyrenaica* (pop. 6: haplotype F) fall into the *halleri* clade with haplotypes clearly differing from those found in the remaining populations of these two species (Figs 3 and 4). As the AFLP profile unambiguously supports the morphology-based classification of these two populations, the aberrant chloroplast haplotypes require an explanation. The possibilities are incomplete lineage sorting of ancient polymorphisms, reticulate evolution or a combination of the two (Rieseberg & Soltis 1991; Wendel & Doyle 1998). Lineage sorting is increasingly being recognized as an important factor causing phylogenetic incongruence (Mason-Gamer *et al*. 1995; Bain & Jansen 2000; Comes & Abbott 2001; Linder & Rieseberg 2004; Jakob & Blattner 2006), and since both hybridization and lineage sorting are expected to be particularly active in young radiations, these processes might remain indiscernible. Although lineage sorting cannot be excluded with absolute certainty, the position of the two aberrant haplotypes of *A. laggeri* and *A. pyrenaica* at or close to the tips of the haplotype network instead of at a central position argue against this hypothesis (Schaal & Leverich 2001). Additionally, monophyly at least of the haplotypes of *A. laggeri* is clearly rejected, even if potential changes in effective population size are taken into account. With the exception of *A. halleri*, haplotype variation within species is very low and, if present, haplotypes are separated by single mutation steps only. If vertical transmission is assumed, this homogeneity is at odds with the presence of haplotypes differing from the others by several mutation steps (6 and 9 for *A. laggeri* and *A. pyrenaica*, respectively). The lack of geographical and ecological differentiation within *A. laggeri* and *A. pyrenaica* also makes selective forces unlikely as an explanation for the distinctness of these haplotypes.

The other possibility for explaining the placement of the two populations is chloroplast capture. Chloroplast capture is common in plants and has been suggested for many plant taxa (Soltis *et al*. 1991, 1996; Okuyama *et al*. 2005). In *Androsace*, hybridization is relatively common

where species co-occur (Smith & Lowe 1997) — the rarity of naturally occurring hybrids being caused more by the usually clear geographical and/or ecological separation of most species than by existing crossing barriers, as evinced by the successful glasshouse production of hybrids unknown in the wild — which potentially allows gene flow between species. Since the chloroplast genome is assumed to be maternally inherited in *Androsace*, as it is in other Primulaceae (Harris & Ingram 1991), gene flow via pollen can be excluded as explanation for the occurrence of *halleri*-type haplotypes in *A. laggeri* and *A. pyrenaica*. Recent hybridization with *A. halleri* in the central Pyrenees is unlikely, because (i) *Androsace halleri* is neither historically nor currently known from this area, and (ii) the *halleri*-type haplotypes in *A. laggeri* and *A. pyrenaica* differ from the single Iberian haplotype of *A. halleri* by one and two mutations, respectively, indicating more ancient hybridization. Although vertical transmission of plastid sequences cannot be rejected as explanation for the patterns observed in *A. pyrenaica*, the strong evidence for gene flow into *A. laggeri* makes this the more likely scenario for *A. pyrenaica* as well.

Although single long-distance dispersal events of seeds of *A. halleri* or its ancestor into the range of *A. laggeri* and *A. pyrenaica* with subsequent hybridization cannot be excluded, the most parsimonious explanation is that the *halleri* clade formerly inhabited a much wider area in the Pyrenees, where it was able to hybridize with both *A. laggeri* and *A. pyrenaica*, and has since become more restricted in its Pyrenean distribution. This tallies with the observation that habitats suitable for *A. halleri* are widespread in the Pyrenees, and can explain the curious distribution pattern of *A. halleri* to both the east and the west of the Pyrenees without invoking long-distance dispersal between the Cordillera Cantábrica and the eastern Pyrenees. The reasons for the range restriction, however, remain elusive and may include competitive replacement (e.g. Perry *et al*. 2001) by the ecologically similar *A. laggeri*, differential responses of *A. halleri* and *A. laggeri* to Pleistocene climatic fluctuations (Kadereit *et al*. 2004), or random sorting of these taxa into geographically distinct refugia during Pleistocene glaciations, which are well documented for the Alps (Schönswetter *et al*. 2005), and subsequent differential recolonization. After the disappearance of *A. halleri* from the central Pyrenees, repeated back-crossing of hybridogenic individuals with their paternal species would ultimately have resulted in a nearly pure but introgressant nuclear genome and morphology of the parental species (*A. laggeri* and *A. pyrenaica*), a mode suggested for the occurrence of a foreign plastid genome in some populations of the wild cotton species *Gossypium aridum* (Malvaceae) in Mexico (Álvarez & Wendel 2006). Inference of formerly wider distributions of species via their hybrid traces might also apply for other plant groups. For example, in Mediterranean species of *Cyclamen* (Myrsinaceae, formerly classified in Primulaceae), an ancient occurrence of *C. balearicum*, a species currently restricted to southern France and the Balearic islands, has been inferred by the presence of 'balearicum'-haplotypes in the closely related *C. repandum* (Gielly *et al*. 2001) on the island of Corsica.

The central position of the Iberian haplotype of *A. halleri* in the haplotype network suggests it to be ancestral (Castelloe & Templeton 1994). Occurrence of the same haplotype in the Cordillera Cantábrica and the eastern Pyrenees could be the result of a once less fragmented distribution (approaching a vicariance scenario), in agreement with the wider distribution of *A. halleri* (or its ancestor) inferred from the occurrence of chloroplast capture. Alternatively, it could be the result of long-distance dispersal between the Pyrenees and the Cordillera Cantábrica. The presence of a different species, *A. rioxana*, at a geographically intermediate position, tends to support the latter hypothesis. However, and again in agreement with a vicariance scenario, ecological divergence, both in *A. rioxana* towards drier alpine grasslands, and towards snowbed vegetation in the eastern Pyrenean *A. halleri* (ecological speciation), together with geographical isolation (allopatric speciation) might have jointly enhanced speciation (Schluter 2001). While the relatively large populations in the eastern Pyrenees retained the ancestral haplotype, molecular evolution in *A. rioxana* accelerated because of the small population sizes (Ohta 1992). Haplotypes of *A. halleri* from the Massif Central and the Vosges are separated from the Spanish ones by successive single mutations (Fig. 3). This is the expected pattern if the French mountain ranges were successively colonized from the ancestral area hypothesized to be in the northern Spanish mountains (Cann *et al*. 1987; Templeton 1998), and is supported by a statistically significantly negative value of Tajima's D (–1.6965, $P = 0.0067$). Overall, the current distribution area of *A. halleri* appears to have been shaped both by range expansion and range contraction.

Significance

Direct evidence for range shifts in alpine plants cannot usually be obtained, because of the lack of fossil evidence. Additionally, given the limited morphological differences between some of the taxa discussed here (*A. laggeri*, two species currently combined under *A. halleri*), unambiguous assignment of any macrofossils might be very difficult, leaving genetic methods the only alternative. Given sufficient sampling and genetic variation, several methods can be used to test for range expansions, but range contractions are much more difficult to detect. Here we show that traces of reticulate evolution such as chloroplast capture can be successfully used to infer the presence of taxa in areas where they no longer exist. It is anticipated that with an increasing number of studies which employ a sufficiently

dense sampling of both populations/individuals and genetic markers, more such cases will appear. As exemplified by *A. halleri*, range shifts in both directions — expansions and contractions — can significantly influence the formation of current distributions, suggesting that caution must be taken in drawing biogeographical conclusions from current distribution patterns alone.

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This work is part of a research project into phylogeographical patterns within *Androsace* sect. *Aretia*, which will form the basis of Christopher Dixon's Ph.D. thesis. Peter Schönswetter is interested in the evolution of the Alpine flora in a biogeographical context. Gerald M. Schneeweiss is interested in evolutionary aspects of holoparasitic and alpine plants.

Supplementary material

The following supplementary material is available for this article:

Figure S1. Neighbour-joining tree of the second AFLP data set.

This material is available as part of the online article from:
http://www.blackwell-synergy.com/doi/abs/10.1111/j.1365-294X.2007.03342.x
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