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Phylogeography of *Primula allionii* (Primulaceae), a narrow endemic of the Maritime Alps

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Primula allionii is endemic to a tiny area of the Maritime Alps and has one of the narrowest distribution ranges in this hotspot of biodiversity. Phylogeographical patterns in *P. allionii* were studied using plastid DNA markers and dominantly inherited markers (AFLP and ISSR) to verify any admixture between *P. allionii* and the sympatric *P. marginata* and to detect the phylogeographical history of the species. Morphometric measurements of flowers and admixture analysis support the hypothesis that hybridization occurs in nature. Species distribution models using two climate models (CCSM and MIROC) suggested a reduction in habitat suitability during cold periods. Phylogeographical analysis suggested an old allopatric divergence during the mid-Pleistocene transition (about 0.8 Mya) without recolonization/contraction cycles. The Alps watershed does not act as a strong barrier between the two main areas of the distribution range, and moderate gene flow by pollen seems to create the admixture recorded among the stands. According to our results, the persistence of *P. allionii* throughout the Ice Age appears to be linked to the capacity of the Maritime Alps to provide a wide diversity of microhabitats consistent with the recent biogeographical pattern proposed for the Mediterranean Basin. *Botanical Journal of the Linnean Society*, 2013, **173**, 637–653.

ADDITIONAL KEYWORDS: biogeography – disjunct distribution – endemicity – natural hybridization.

The Maritime and Ligurian Alps are one of the most important biodiversity hotspots in the Mediterranean Basin (e.g. Médail & Quézel, 1997; Mucciarelli & Fay, 2013) and one of the most important centres for endemism in the whole Alps (Pawlowski, 1970; Tribsch, 2004; Schönswetter *et al*., 2005). Situated at the crossroads of the Mediterranean Basin and the Alps, this region is a hotspot for plant biodiversity (Médail & Verlaque, 1997; Casazza, Barberis & Minuto, 2005) given its possible role as an ancestral area for some plant species (Merxmüller, 1965; Pawlowski, 1970) and populations (Garnier *et al*., 2004). This particular biodiversity richness is probably due to two main factors: (1) landscape and ecological heterogeneity; and (2) vicariance events due to climatic oscillations. The landscape and ecological heterogeneity allowed the long-term persistence and divergence of species, when survival was made possible by locally high diversity in ecological niches. It is well known that the varied geological and topographical conditions favoured the isolation of populations, therefore making them, in general, highly divergent even over short geographical distances (Hewitt, 2001, 2011; Parisod & Besnard, 2007). Recent molecular investigations of the endemic plants of this region have shown that vicariance events are probably the most important factor explaining the distribution of some endemic plants in the area (Conti *et al*., 1999; Diadema *et al*., 2005; Minuto, Grassi & Casazza, 2006; Grassi *et al*., 2009; Kadereit *et al*., 2011). In particular, these dispersal/vicariance processes can be seen in the fragmented distribution area of some endemic species, such as *Phyteuma cordatum* Balb. and *Primula allionii* Loisel. (Martini, 1992, 1994; Casazza *et al*., 2008).

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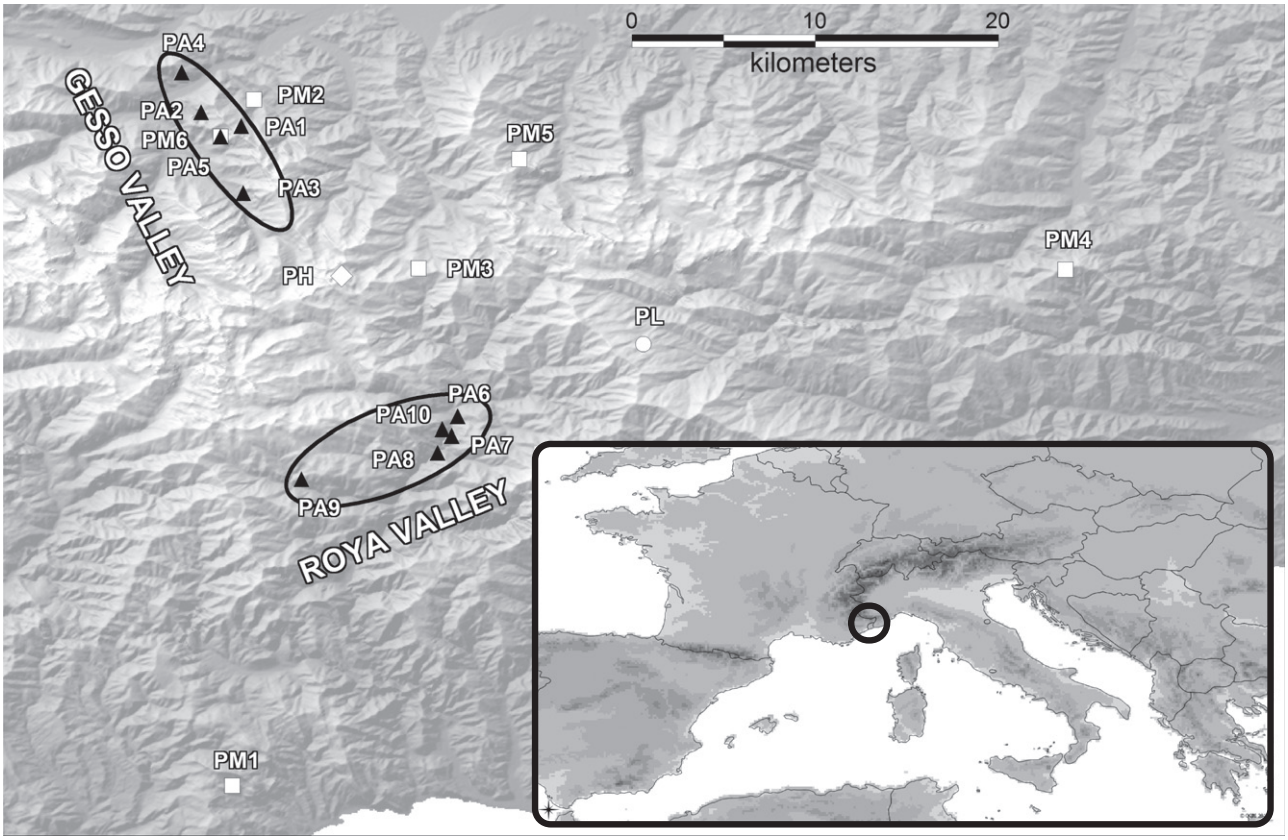

Figure 1. Geographical locations of the *Primula allionii* (black triangle), *P. marginata* (white squares), *P. latifolia* (white circle) and *P. hirsuta* (diamond) stands sampled. The borders of the two *P. allionii* distribution areas (Gesso and Roya Valleys) are indicated with a continuous black line. The stands are coded according to Table 1.

Primula allionii belongs to *Primula* L. section *Auricula* Duby, endemic to the high mountains of Europe and showing highest species diversity in the Alps (Zhang & Kadereit, 2004). The range size of these species differs enormously; whereas some species are widespread, others are limited to narrow areas. Section *Auricula* originated from an Asian ancestor at the end of the Late Tertiary (about 3.6 Mya) and diversified into two lineages ('eastern' and 'western') at the Plio-/Pleistocene boundary (about 2.4 Mya), probably as a result of an early glaciation in the Alps, forcing the ancestor into western and eastern refugia (Zhang, Comes & Kadereit, 2004). The western clade shows evidence that the species originated in isolated glacial refugia and re-colonized areas during interglacial periods (Kadereit, Griebeler & Comes, 2004).

During post-glacial expansion, these species came into contact in areas of sympatry where interspecific hybridization probably occurred (Casazza *et al*., 2012; Kadereit *et al*., 2011). A study of some partly sympatric species of this section [*P. marginata* Curt./*P. latifolia* Lapeyr. and *P. daonensis* (Leyb.) Leyb./*P. hirsuta* All.] suggested that at present the species are well isolated and there is no evidence for any genetic assimilation of rare by widespread species (Kadereit *et al*., 2011). Nevertheless, interspecific hybridization with *P. allionii* seems to have played a role in the evolution of *P. marginata* polyploids (Casazza *et al*., 2012) and hybrids between *P. allionii* and *P. marginata* have been suggested in nature (Richards, 2003). *Primula allionii* is endemic to a tiny area of the Maritime Alps and is a species with one of the narrowest distribution ranges in this hotspot of biodiversity. In the past, the species suffered from excessive collecting (Burnat, 1897) and is currently listed in the Italian and French Red Books (Conti, Manzi & Pedrotti, 1992, 1997; Olivier, Galland & Maurin, 1995). It occurs in two distinct areas on both sides of the French and Italian border (Fig. 1): one area is located in France, south of Col di Tenda, in the Roya Valley; the other is in Italy, north of Col di Tenda, around the Gesso Valley (Minuto *et al*., 2012). On the basis of the distributional data, some decades ago Martini (1982) suggested that the species originated in the southern population and later colonized the northern area during its post-glacial range expansion.

In the present study, we investigated the level and geographical structuring of plastid DNA and nuclear variation in *P. allionii* over its range, based on sequencing of two non-coding plastid DNA regions, AFLP and inter-simple sequence repeat (ISSR). We combined species distribution models (SDMs) with phylogeographical methods to infer glacial refugia and to reconstruct the phylogeographical pattern of this species. Additionally, because hybridization is an important issue in phylogeographical studies as it may compromise the interpretation of species histories, we included the sympatric species *P. marginata* in our analyses. More specifically, the present study aimed to: (1) infer the relationship between *P. allionii* and *P. marginata*; (2) test Martini's hypothesis of a southern origin of *P. allionii*; and (3) infer the phylogeography of the species.

MATERIALS AND METHODS

Morphometric measurements

As considerable reductions in sexual organ reciprocity between species may lower interspecific pollen flow, with potential effects for reproductive isolation (Keller, de Vos & Conti, 2012), the degree of reciprocity between sexual organs was analysed. To describe the extent and specificity of variation in the reciprocal herkogamy expression, stigma height and anther height were recorded according to Fey (1929) and Kálmán *et al*. (2007). Intra- and interspecific sexual organ reciprocity was calculated using the single-value reciprocity index (SI) of Sanchez, Ferrero & Navarro (2008). This index compares stigma–stamen height gaps for all potential crosses (i.e. stamen of each morph versus stigma of each complementary morph), also considering the dispersion of data, and is not skewed by the more frequent sex organ (stamens). The index can be interpreted as a measure of the average deviation from perfect reciprocity (SI = 0), and distyly (SI < 0.05). One hundred flowers per species (Table 1, $N = 50$ per sexual morph) were sampled at anthesis, stored at 4 °C and handled in the laboratory within 24 h. The flowers were longitudinally dissected and then photographed with a Leica M205 C stereomicroscope (Leica Microsystems). Measurements were taken from the digital photographs with the image analyser software Leica LAS EZ (version 1.6.0).

Sampling strategy and DNA extraction

Primula allionii is locally abundant and, in each of the two main distribution areas (the Roya and Gesso

Table 1. Details of the stands of *Primula allionii* and *P. marginata* sampled for the analyses

Stand name	Code	N_{IA}	N_{cp}	N_{SI}	Lat	Long	Elev
P. allionii							
Val Roaschia (I)	PA1	4	1		44°15′10″	7°26′32″	1557
Comba Infernetto (I)	PA2	4	3		44°15′44″	7°24′53″	1041
Caire Porcera (I)	PA3	3	1		44°12′24″	7°26′37″	1255
Vallone Saben (I)	PA4	5	2		44°17′22″	7°24′05″	1300
Gorge della Reina (I)	PA5	8	2		44°14′44″	7°25′41″	1191
St Dalmas de Tende (F)	PA6	5	2		44°03′13″	7°35′26″	723
Terris (F)	PA7	5	1	50	44°02′25″	7°35′11″	850
Pointe de Traya (F)	PA8	5	1	50	44°01′44″	7°34′ 37″	807
Vallon de Merim (F)	PA9	5	3		44°00′39″	7°29′01″	485
Mont Bonsapée (F)	PA10	5	2		44°02′42″	7°34′48″	850
P. marginata							
Mont Baudion (F)	PM1	4	2	50	43°48′02″	7°26′10″	1141
Val Roaschia (I)	PM2	6	2		44°16′15″	7°27′04″	858
Rocca dell'Abisso (I)	PM3	5	2		44°09′19″	7°33′50″	2085
M. Galero (I)	PM4	5	2		44°09′15″	8°00′25″	1501
Val Cravina (I)	PM5	4	2		44°13′48″	7°37′58″	1410
Gorge della Reina (I)	PM6	2	2	50	44°14′44″	7°25′41″	1191
P. hirsuta							
Rocca dell'Abisso (I)	PH		1		44°08′24″	7°31′37″	2085
P. latifolia							
Riserva delle Navette (I)	PL		1		44°06′11″	07°43′04″	1874

Population code (Code), number of samples used in ISSR and AFLP analyses (N_{IA}), number of samples used in plastid DNA analysis (N_{cp}), number of flowers sampled for Sanchez et al. (2008) reciprocity index calculation (N_{SI}), latitude north (Lat), longitude east (Long) and elevation as m a.s.l. (Elev) are reported. I, Italy; F, France.

Valleys), the plants are so frequent on the cliffs that it is impossible to detect distinct populations. However, based on the literature, herbaria and from the field data, 70 GPS points were globally recorded. Forty-nine specimens were sampled for molecular analyses from ten stands randomly selected at a reciprocal distance of at least 500 m (Fig. 1; Table 1): five stands in Gesso Valley (24 specimens) and five in Roya Valley (25 specimens). In addition, 26 specimens of the co-occurring species *P. marginata* were sampled from six stands surrounding the *P. allionii* distribution range. For plastid DNA analysis, a subset of 18 specimens of *P. allionii* (nine in Gesso Valley and nine in Roya Valley) and 12 specimens of *P. marginata* was selected (Table 1; Fig. 1). A specimen of each from *P. latifolia* and *P. hirsuta* were used as an outgroup. One to two leaves per specimen were dried *in situ* in silica gel; total genomic DNA was extracted using a DNeasy Plant Mini Kit (Qiagen), following the manufacturer's instructions.

Molecular analyses

Molecular analyses were performed as follows:

1. For plastid DNA analysis, the *trnL* intron was amplified with primers c and d (Taberlet *et al*., 1991); the *psbA-trnH* intergenic spacer was amplified with primers *psbA*F and *trnH*R (Sang, Crawford & Stuessy, 1997). A PCR was performed in a 30-μL reaction volume containing 10 ng plant DNA, the PureTaq Ready-To-Go PCR Beads Kit (Healthcare) and 10 pmol of the specified primers. PCR amplification was carried out in a Mastercycler Gradient thermal cycler (Eppendorf) using the following profile: 3 min at 94 °C; 35 cycles of denaturation (30 s at 94 °C), annealing (35 s at 51.5 °C) and extension (75 s at 72 °C); then a final step for 7 min at 72 °C. To prepare for DNA sequencing, PCR products were purified using the QIAquick PCR Purification Kit (Qiagen Inc.). Sequencing was performed in an automated DNA Sequencer by Macrogen Inc. Sequences were aligned with the Staden Package (http://staden.sourceforge.net/).
2. The ISSR-PCR mixture (12 μL) contained 25 ng total plant DNA, GoTaq Master Mix (Promega) and 10 pmol primer. Amplification was carried out in a Mastercycler Gradient thermal cycler (Eppendorf). The following thermal profile was used: initial denaturation at 94 °C for 3 min, then 35 cycles of denaturation at 94 °C for 45 s, primer annealing for 50 s [$(CT)_8G$, $(GACA)_4$, $(CA)_8YG$, $(TC)_8C$, $(CT)_8RG$, $(GA)_8C$, $(ATG)_6$, 47.5 °C; $(CA)_8T$ and $(AC)_8G$ 48 °C; (AC)YC 51 °C] and elongation at 72 °C for 130 s. Amplification products were resolved electrophoretically on 2.0% agarose gels run at 100 V in TAE buffer, visualized by staining with ethidium bromide and photographed under ultraviolet light. Of the various molecular markers, ISSR has some advantages: (i) primers anneal directly to simple sequence repeats and thus, unlike SSR markers, no prior knowledge of target sequences is required for ISSR; and (ii) ISSR markers, which have longer primers than RAPD (random amplified polymorphic DNA) markers, allow more stringent annealing temperatures and reveal more polymorphic fragments, can be highly variable within a species and have the advantage over RAPD markers (Esselman *et al*., 1999).
3. AFLP reactions were conducted following the protocol of Vos *et al*. (1995) with some modifications. Two AFLP primer combinations were used: *MseI*-CAG/*EcoRI*-ACA and *MseI*-CAT/*EcoRI*-ACC. Genomic DNA (200 ng) was digested (3 h) with *EcoRI* (0.5 U) and *MseI* (0.5 U). DNA fragments were ligated (with T4 DNA-ligase) to *EcoRI* (5 pmol) and *MseI* (50 pmol) adapters in a final volume of 50 μL. The ligation reaction was performed at 37 °C for 6 h. Amplification was carried out for 36 cycles under the following conditions: denaturation for 30 s at 94 °C; annealing for 30 s at 65 °C for the first cycle, followed by lowering of the temperature (0.7 °C) in the next 12 cycles, then 56 °C for the remaining 23 cycles; and extension for 60 s at 72 °C. Selective fluorescence amplification was conducted using custom forward primers, 5′-labelled with 6-FAM (Applied Biosystems). Selective amplification products were diluted tenfold and 2 μL of each dilution was mixed with 10 μL deionized formamide and 0.15 μL GeneScan 1200-LIZ internal size standard (Applied Biosystems) and separated by capillary electrophoresis on an ABI 3730xl DNA analyzer (Applied Biosystems). Raw data were analysed using GeneMapper 4.0 software (Applied Biosystems) and then exported in Microsoft Excel to conduct phylogenetic and statistical analyses.

Combined AFLP and ISSR datasets have proved to be robust in the resolution of relationships both within and among species (Xu & Sun, 2001; Mitka *et al*., 2007; Toklu *et al*., 2009). For this reason, we combined the results of the two DNA fingerprinting methods (125 markers). To verify any correlation between the two matrices, a Mantel test was performed between ISSR and AFLP distance matrices based on a Jaccard similarity index using PASSaGE software (Rosenberg & Anderson, 2011) and 1000 permutations. The methodological error rate was assessed by comparing original AFLP and ISSR profiles with replicates for the eight (about 10% of the

total sample size) individuals replicated. The genotyping error rate was calculated, according to the method described by Pompanon *et al*. (2005), as the ratio of the total number of mismatched genotypes (band presence vs. band absence) to the number of replicated genotypes. The genotyping error rate in this study was 3.12% for AFLP and 2.16% for ISSR.

Admixture analysis

To infer the membership probabilities of each individual for the two species we used discriminate analysis of principal components (DAPC), a multivariate method free of assumptions about Hardy–Weinberg equilibrium or linkage disequilibrium (Jombart, Devillard & Balloux, 2010). DAPC analysis was performed in the R package Adegenet (Jombart, 2008) to identify and describe clusters from the same dataset. To assess how admixed the clusters are, DAPC drives group membership probabilities on the basis of retained discriminant functions, which are linear combinations of variables that optimize the separation of individuals into pre-defined groups (Jombart, 2012). We used the a-score to measure the trade-off between finding a space with a good power of discrimination using DAPC and retaining too many dimensions.

The a-score is computed as (Pt – Pr), where Pt is the reassignment probability using the true cluster and Pr is the reassignment probability for randomly permuted clusters. The a-score measures the proportion of successful reassignments of the DAPC analysis compared with k-means clustering (observed discrimination) and random clustering (random discrimination) and can be seen as the proportion of successful reassignments corrected for the number of retained principal components (Jombart, 2012).

We also conducted a non-metric multidimensional scaling (NMDS) analysis of the Jaccard distance matrix. We first calculated the pairwise distances in PAUP 4.0b10 (Swofford, 2002); we then calculated the axes values using the software SYN-TAX 2000 (Podani, 2001). We drew the three-dimensional graphic using Statistica 8.0 (StatSoft Inc., 2007). This method positions accessions in a reduced ordination space that best reflects their original distances and is especially useful for exploring non-hierarchical relationships associated with hybridization and introgression (Edwards & Sharitz, 2000).

Species distribution models

In *Primula*, SDMs have proven to be useful in supporting or modifying hypotheses derived from phylogeography, even in narrow endemic species (Schorr *et al*., 2012). At the local scale, models provide excellent spatial predictions by also using non-climatic predictors (Lomba *et al*., 2010). For this reason, we added the specific substrate (Triassic and Jurassic limestones) where the species grows as a further predictor variable. A layer reporting the presence/absence of suitable substrate was elaborated, starting from the geological data available on the BRGM (http://www.brgm.fr/numerique.jsp) and ISPRA (sgi.isprambiente.it/geoportal) websites. The bioclimatic and altitudinal layers for the present conditions (resolution 30 arcsec) and Last Glacial Maximum (LGM) data (resolution 2.5 arcmin) were downloaded from the WorldClim database website (http://www.worldclim.org). We used data from both the Community Climate System Model (CCSM) and the Model for Interdisciplinary Research on Climate (MIROC) to predict the LGM. To reduce the multicollinearity between predictors in order to minimize model overfitting, we performed a pairwise Pearson correlation between 19 bioclimatic predictors and one topographic predictor (altitude) using ENMTools 1.3. We used the predictors that are considered physiologically important for plants (Körner, 1999) and that were not strongly correlated with each other (Pearson correlation coefficient, $r^2 < |0.80|$). In modelling species distributions, we used eight variables: BIO2, mean diurnal range; BIO3, isothermality (monthly/annual temperature range); BIO7, annual range in temperature; BIO9, mean temperature of driest quarter; BIO15, precipitation seasonality; BIO18, precipitation of warmest quarter; BIO19, precipitation of coldest quarter; and the presence/absence of suitable substrate. We removed exactly matching occurrence records using ENMTools (Warren, Glor & Turelli, 2010; see also Warren & Seifert, 2011). Finally, 54 presence records were applied in SDM analysis.

To account for model-based uncertainties in the modelling process, we applied an ensemble forecasting approach based on ten statistical algorithms: generalized linear models (GLM); generalized boosting models (GBM); generalized additive models (GAM); classification tree analysis (CTA); artifical neural networks (ANN); surface range envelop (SRE); flexible discriminant analysis (FDA); multiple additive regression spline (MARS); random forest (RF); and maximum entropy (MAXENT). Consensus methods, especially those based on the average function, significantly improve the accuracy of species distribution predictions (Marmion *et al*., 2009). We therefore implemented ensemble forecasts using the average consensus of the model predictions.

Analyses were implemented with the BIOMOD2 v. 1.3.5 package (Thuiller *et al*., 2009) for R (R Development Core Team, 2005). Because most modelling techniques implemented in BIOMOD2 require that species distribution data are presence and

absence, we generated three different sets of 500 pseudo-absences randomly chosen. Even if it has been demonstrated that the number of replicate sets of pseudo-absences may influence the predictive accuracy of the models, the better strategy in the pseudo-absence choice changes according to the modelling techniques (Barbet-Massin *et al*., 2012) and currently no guidelines exist for ensemble forecasting frameworks. The predictive performance of the models was evaluated for each pseudo-absence run by repeating a split-sample cross-validation 20 times, using a random subset (80%) of the initial dataset each time to calibrate the models while the remaining 20% were used to evaluate the models. Three different evaluation measures were calculated: the area under the curve (AUC) of an ROC plot; the true skill statistic (TSS); and the Kappa statistic. The models outlined prediction suitability values between 0 and 1 at each site. To transform continuous probability values from model to binary presence–absence form we used AUC. This threshold corresponds to the point on the ROC curve (sensitivity against 1 – specificity) which has the shortest distance to the top-left corner (0,1) in an ROC plot (Liu *et al*., 2005).

Phylogeographical analysis

Sequences of the two plastid DNA regions were concatenated to form a single matrix. We analysed phylogenetic relationships based on the plastid DNA sequences with Bayesian analyses using MRBAYES v.3.1.2 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003) at the freely available Bioportal server (http://www.bioportal.uio.no). Sequences were aligned with MAFFT v. 6.903 (Katoh & Toh, 2008); the resulting alignments were adjusted manually. Indels were coded as binary characters using simple indel coding (Simmons & Ochoterena, 2000) as implemented in SEQSTATE 1.4.1 (Müller, 2005). Best-fit models of nucleotide substitution were determined to be an F81 model for the *trnL* intron and an F81 + I model for the *psbA-trnH* spacer by the Akaike information criterion using the program MRMODELTEST 2.3 (Nylander, 2004). Bayesian analysis was performed over 10×10^6 generations with one cold and three incrementally heated Monte Carlo Markov chains (MCMCs).

To detect genealogical relationships among sequences with shallow genetic divergences, we also constructed plastid DNA haplotype networks using a statistical parsimony algorithm described by Templeton, Crandall & Sing (1992), as implemented in TCS v.1.21 (Clement, Posada & Crandall, 2000). We ran the software with default settings and gaps treated as missing data. According to Olsson *et al*. (2009), as TCS cannot run on a mixed matrix containing DNA and binary data, the two binary coded indels were manually exchanged to A (1) and T (0), respectively.

A Bayesian skyride plot (Drummond *et al*., 2005) of changes in effective population size (N_e) through time based on plastid DNA concatenated sequences data was estimated using BEAST 1.7.4 (https://www.bioportal.uio.no/appinfo/show.php?app=BEAST). Best-fit models of nucleotide substitution were determined to be a GTR + G + I model for the *trnL* intron and the *psbA-trnH* spacer concatenated, by the Akaike information criterion using MRMODELTEST 2.3 (Nylander, 2004). We chose the GTR + G + I substitution model with empirical base frequencies, a strict molecular clock and a coalescent Gaussian Markov random field (GMRF) Bayesian skyride tree prior. Four runs of 1×10^9 were compared to ensure convergence. Outputs were visualized in TRACER 1.5 (http://tree.bio.ed.ac.uk/software/tracer/) and combined in LOGCOMBINER 1.7.4 [effective sample size (ESS) > 200]. In the study, an evolutionary rate for the chloroplast of 2×10^{-9} substitutions per site per year (prior: Normal; Stdev 0.001) was used. These values approximate to the evolutionary rates of introns and non-coding spacers of organelle DNA (Wolfe, Sharp & Li, 1987; Graur & Li, 2000; Wang *et al*., 2009).

To infer the biogeographical history of *P. allionii* we employed a recently developed Bayesian approach (Lemey *et al*., 2009) as implemented in BEAST 2.0 applied to a comprehensive data set of plastid sequences. Under this model, rates of diffusion between a priori defined discrete locations are estimated using a continuous-time Markov chain model (Lemey *et al*., 2009). Starting from the unobserved location at the root of the tree derived from a uniform distribution over all sampled locations, dispersal proceeds along each branch according to this model and gives rise to the observed locations at the tips. We defined two distribution regions for *P. allionii* stands in this analysis: Gesso Valley and Roya Valley (Fig. 1). We used the default setting according to the tutorial (Bouckaert, 2012) and the GTR + G + I nucleotide substitution model.

Combined AFLP and ISSR datasets were analysed using BAPS 5.2 (Corander *et al*., 2008) to detect clusters of genetically similar individuals. The method determines clusters of population samples, minimizing Hardy–Weinberg and linkage disequilibria within the clusters and treating the number of clusters as an unknown parameter. We adopted the spatial option in this study, which uses population locations when estimating the number of clusters (K) and considers that genetically distinct clusters of individuals are likely to be spatially separated. We ran ten replicates for values of $K_{max} = 10$ (K_{max} = maximum number of clusters).

In addition, a DAPC (Jombart *et al*., 2010) was performed to infer clusters of genetically similar individuals. DAPC uses sequential K-means and model selection to infer genetic clusters: the Bayesian information criterion (BIC) allowed us to determine the optimal number of clusters. To describe the identified clusters, DAPC relies on data transformation using principal component analysis (PCA) as a prior step to DA (discriminant analysis), which maximizes the separation between groups. We tested values of K = 1–20, with ten runs at each value of K. Once the best number of genetic clusters was selected, the DAPC function was executed using this grouping, retaining the axes of the PCA that were sufficient to explain ≥ 90% of the total variance of the data.

The hierarchical distribution of genetic variation was described using molecular analysis of variance (AMOVA). Components of variance partitioned among the Gesso and Roya Valley groups, among stands within groups and within stands were estimated using ARLEQUIN 3.11 (Excoffier, Laval & Schneider, 2005) with 1000 permutations. All data sets has been uploaded to the Dryad Digital Repository (http://dx.doi.org/10.5061/dryad.mm7kk).

Table 2. Morphometric characteristics (mm, mean ± SD) of *P. allionii* and *P. marginata* flowers

Floral characteristic	Morph	*P. allionii*	*P. marginata*
Stamen height	L	2.79 ± 0.59	3.36 ± 0.69
	S	9.11 ± 1.41	8.80 ± 1.57
Stigma height	L	8.25 ± 1.16	9.03 ± 1.50
	S	3.17 ± 0.56	3.43 ± 0.62

L, long styled morph; S, short styled morph.

RESULTS

Natural hybridization

Average values of the measurements of the floral traits for *P. allionii* and *P. marginata* are reported in Table 2. The sexual organs were arranged in the same position in the corresponding morphs of both species, as shown in Figure 2. In particular, anthers and stigma of long-styled flowers were positioned *c*. 9 mm from the bottom of the corolla tube, whereas the same sexual organs of short-styled flowers were 3 mm from the bottom of the corolla tube. The indices of reciprocity calculated according to Sanchez *et al*. (2008) were < 0.05 both within and between species

Table 3. Results of intra- and interspecific sexual organ reciprocity based on Sanchez *et al*. (2008) reciprocity index in *P. allionii* (PA) and *P. marginata* (PM)

	PM (S)	PA (S)
PM (L)	0.046	0.041
PA (L)	0.048	0.044

L, long styled morph; S, short styled morph.

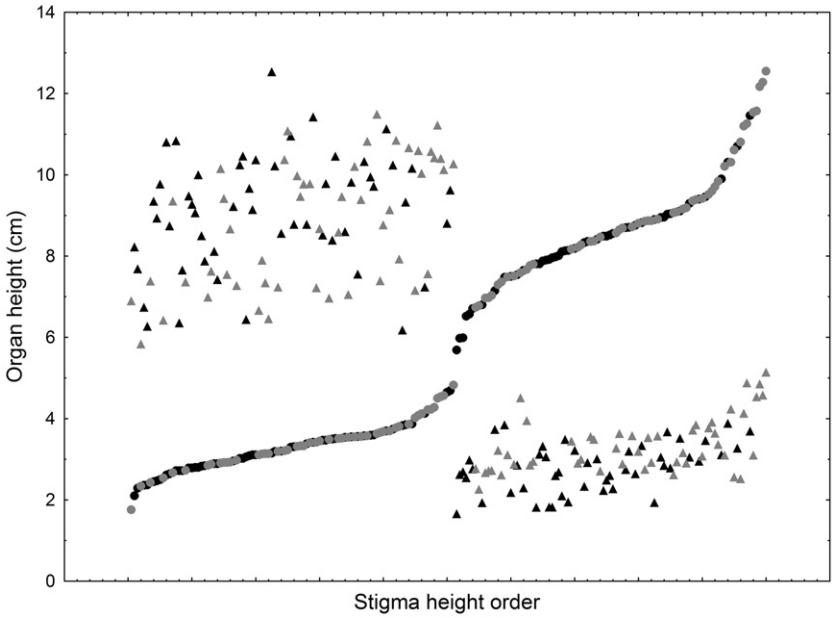

Figure 2. Variation in stigma (circles) and anther (triangles) position in *P. allionii* (black) and *P. marginata* (grey) flowers. Values are ordered by increasing stigma height.

(Table 3), indicating that sexual organs always fit spatially in all reciprocal morph combinations.

A Mantel test showed a statistically significant correlation ($r = 0.57$; $P = 0.001$) between the genetic distance matrices based on ISSR and AFLP analyses. In total, 121 polymorphic fragments (76 in ISSR and 45 in AFLP) were detected: 113 in *P. allionii* (70 in ISSR and 43 in AFLP) and 106 in *P. marginata* (68 in ISSR and 38 in AFLP). Of all the fragments, 23 were private (15 in ISSR and eight in AFLP): 15 (12.40%) in *P. allionii* [eight (10.53%) in ISSR; seven (15.56%) in AFLP]; eight (6.61%) in *P. marginata* [six (7.89%) in ISSR; two (4.44%) in AFLP]. In *P. allionii* one private AFLP fragment was found in the Gesso Valley group. DAPC analysis identified eight individuals of *P. allionii* and two individuals of *P. marginata* as admixed (Fig. 3A) with a probability of being assigned to the other species > 0.2. The results of DAPC analysis were also supported by the NMDS results, showing admixture between the two species (Fig. 3B).

Centre of origin and diversification of the species

Under current climate conditions, according to Thuiller, Lafourcade & Araujo (2008), AUC, TSS and Kappa (Supporting Information, Table S1) indicated an excellent model performance (AUC > 0.9; Kappa/TSS > 0.8) for all modelling techniques with the exception of CTA, which had a lower value for the Kappa statistic only (Kappa = 0.65). The predicted current potential distribution of *P. allionii* was wider than its known distribution range (Fig. 4). Suitable habitat occurred mainly in the Roya and Gesso Valleys (where the species occurs) and eastward along the ridge of the Ligurian Alps (Fig. 4). The predicted potential niche during the LGM was strongly reduced, even if different in the two climates applied (MIROC and CCSM). According to the MIROC climate, the predicted suitable areas were smaller than currently and completely nested within them. A narrow area of overlap between the known and LGM-predicted distributions was found in both the Roya and the Gesso Valleys (Fig. 4). In contrast, no suitable areas were predicted using the CCSM climate.

According to the Bayesian phylogenetic tree (Supporting Information, Fig. S1), *P. allionii* accessions formed a strongly supported clade [posterior probability (PP) = 1.00]. All stands from the Roya Valley and one stand from the Gesso Valley (PA3.1), that nearest to the Roya Valley, formed a well-supported clade (PP = 1.00). All remnant stands of the Gesso Valley fell into an unresolved polytomy.

Inspection of the final BEAST outputs with Tracer version 1.5 revealed a sufficient level of mixing and convergence for a stationary distribution and a good ESS for all estimated parameters, with ESS largely > 200 (ESS posterior 408 and ESS prior 397). The estimated median [95% highest posterior density (HPD)] time to the most recent common ancestor (tMRCA) for the main split within the *P. allioni* clade was 0.781 (0.089–3.99) Myr (Supporting Information, Table S2). The GMRF Bayesian skyride plot for the plastid DNA sequences identified a past population decline throughout the Pleistocene. Moreover, we identified a weak expansion signal starting between the conclusion of the Pleistocene and the beginning of the Holocene (Fig. 5).

The discrete phylogeography analysis postulated that the ancestors of *P. allionii* originated in the Roya Valley with a probability of 49.85% (Fig. 6A); the probability that the *P. allionii* ancestral range was in the Gesso Valley was 50.15%.

The haplotype network (Fig. 6B, C) revealed four haplotypes exclusive to the Roya Valley (RV1–4) and three to the Gesso Valley (GV1–3). GV1 was separated by a lower number of steps from the PM1–PL haplotype than from other *P. allionii* haplotypes (RV1–4 and GV3). The frequent GV2 was directly derived from GV1, whereas the GV3 was derived directly from the southern RV3. RV2 and RV1 from GV1 and RV3 from GV2 were separated by five steps. All the RV haplotypes were directly connected to another RV haplotype.

Among the polymorphic fragments detected in *P. allionii*, 101 (63 in ISSR and 38 in AFLP) were polymorphic in the Gesso Valley stands and 108 (67 in ISSR and 41 in AFLP) were polymorphic in the Roya Valley stands. BAPS assigned individuals to four genetic clusters. Two genetic clusters of individuals (grey and dark grey in Fig. 7A) occurred both in the Gesso and in the Roya Valleys. The other two genetic clusters occurred only in the south (light grey and black in Fig. 7A). In the DAPC analysis, using model selection based on the BIC values, the optimal number of clusters was three but clustering solutions for K = 2 and 4 had a nearly equivalent BIC (Supporting Information, Fig. S2). The three genetic clusters occurred in both the Gesso and the Roya Valleys (Fig. 7B). AMOVA (Table 4) showed the lower variation component 'between groups' (Gesso and Roya Valleys), progressively increasing for 'among the stands within groups' and 'within stands'.

DISCUSSION

Natural hybridization

Natural hybridization events in the genus *Primula* are assumed to be possible in many regions (Richards, 2003) but, because *Primula* species are morphologically distinguishable, it is possible to suppose that

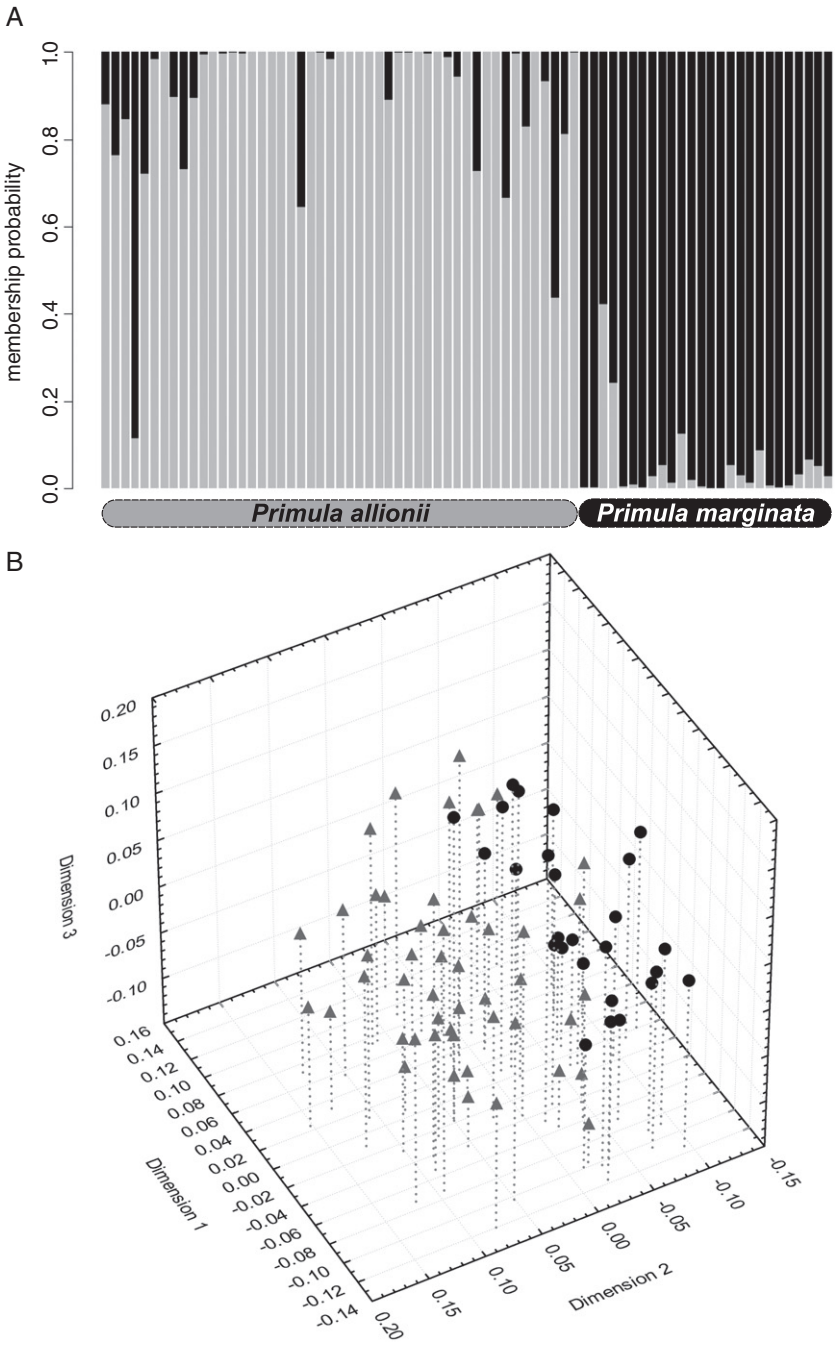

Figure 3. A, admixture coefficients for *P. allionii* and *P. marginata* based on ISSR and AFLP data estimated using DAPC. Bar plots show membership of individual genotypes to K clusters (each cluster is represented by a different colour and each vertical bar represents an individual). B, NMDS scatterplot of *P. allionii* and *P. margianta* individuals based on Jaccard index: *P. allionii*, grey triangles; *P. marginata*, black circles.

hybridization among individuals is quite rare, as observed in different plants and animals (Mallet, 2005). Our admixture analysis revealed the existence of some individuals with evidence of admixture (Fig. 3A, B), differing from recent observations on some other species of *Primula* section *Auricula* which indicated that hybrids may be rare in nature (Kadereit *et al*., 2011). In this study, DAPC and NMDS analyses highlighted the existence of hybrid individuals in *P. allionii* and in *P. marginata*, suggesting a bi-directionality of hybridization, as observed in other *Primula* species (Zhu *et al*., 2009; Kadereit

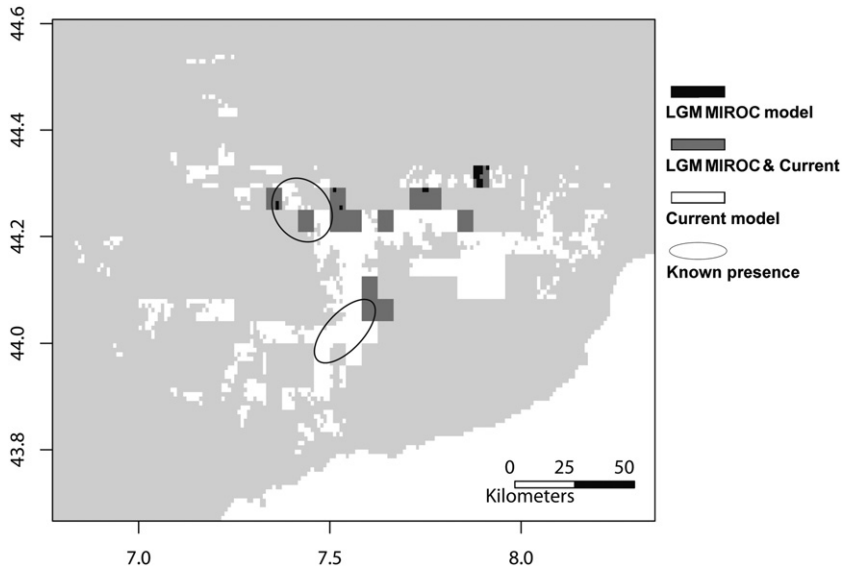

Figure 4. Species distribution models depicting potential distribution for *P. allionii* during the present time and Last Glacial Maximum (LGM, ~21 kya). White areas indicate suitable areas at present; dark grey areas indicate areas suitable during both the present and LGM according to MIROC climate; black areas indicate areas suitable only during the LGM according to MIROC climate. Black lines indicate the current known distribution. CCSM climate does not show any suitable areas during the LGM.

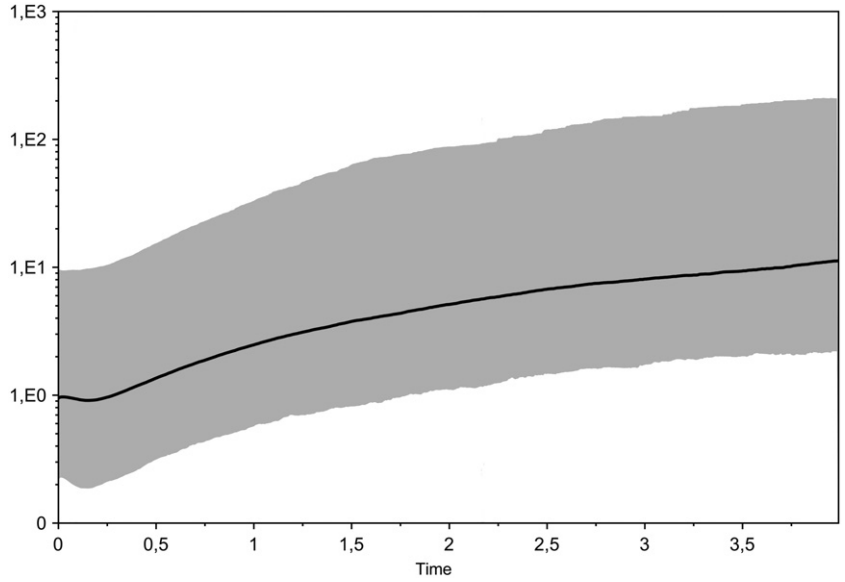

Figure 5. Bayesian skyride plot of *P. allionii* using plastid DNA sequences. The *x* axis is time before present (Mya), and the *y* axis is the expressed population size estimated in units of *Ne m* (*Ne*, effective population size; *m*, mutation rate per haplotype per generation). The dark line represents median inferred *Ne*·*m*, and grey bands mark the 95% highest probability density (HPD) intervals.

et al., 2011). The DAPC and NMDS results are congruent with a previous study involving the two species in which dodecaploids of *P. marginata* were assumed to be hybrids between *P. marginata* and *P. allionii* (Casazza *et al*., 2012).

In *Primula* section *Auricula*, geographical isolation and edaphic differentiation among species are recognized as important reproductive barriers, but floral phenology is not likely to contribute to reproductive isolation among species (Kadereit *et al*., 2011). In

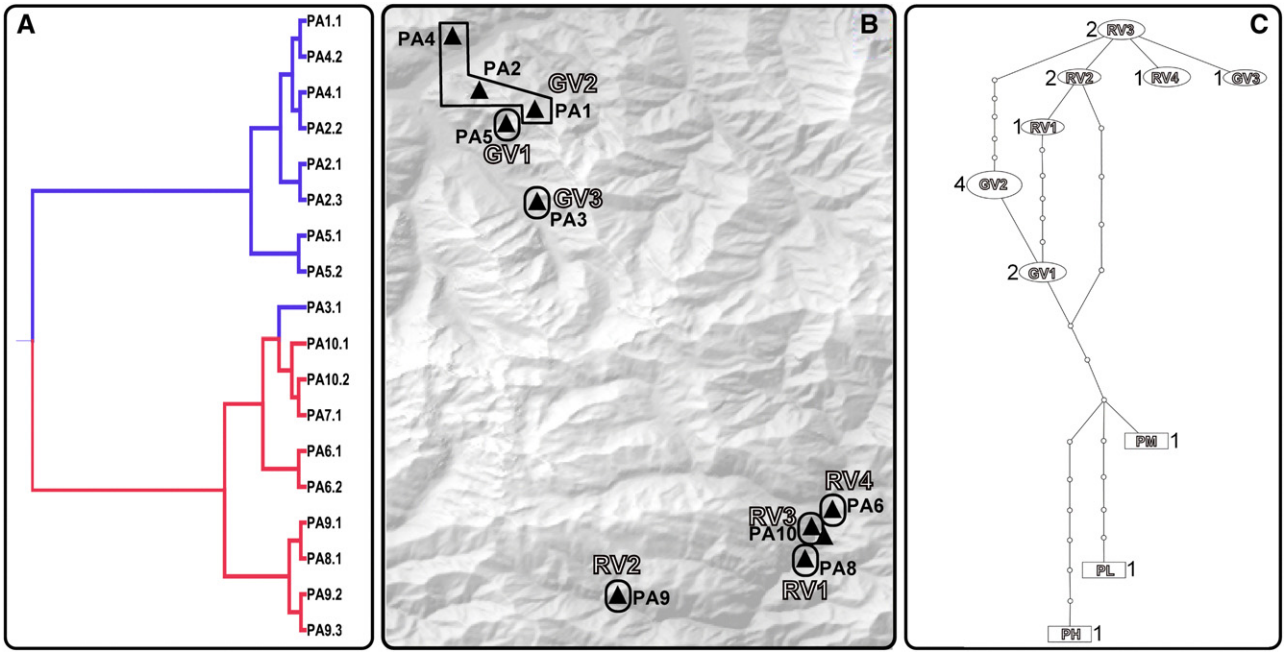

Figure 6. A, Bayesian reconstruction of ancestral distribution areas. Branches are coloured according to location: blue (dark grey in print version), Gesso Valley; red (pale grey in print version), Roya Valley. Branch widths represent posterior support for the branch. Stand codes are as reported in Table 1. B, geographical representation of stands used in plastid DNA analysis and haplotypes inferred by TCS analysis. Codes as in Table 1 and Figure 5B. C, haplotype network inferred by TCS analysis from *trnL* intron and *psbA-trnH* intergenic spacer data for *P. allionii* plus outgroup taxa (PM, *P. marginata*; PL, *P. latifolia*; PH, *P. hirsuta*). Mutational steps separating the haplotypes are indicated by open circles. GV1–3, haplotypes located in Gesso Valley; RV1–4, haplotypes located in Roya Valley. The number of individuals exhibiting the different haplotypes is also given.

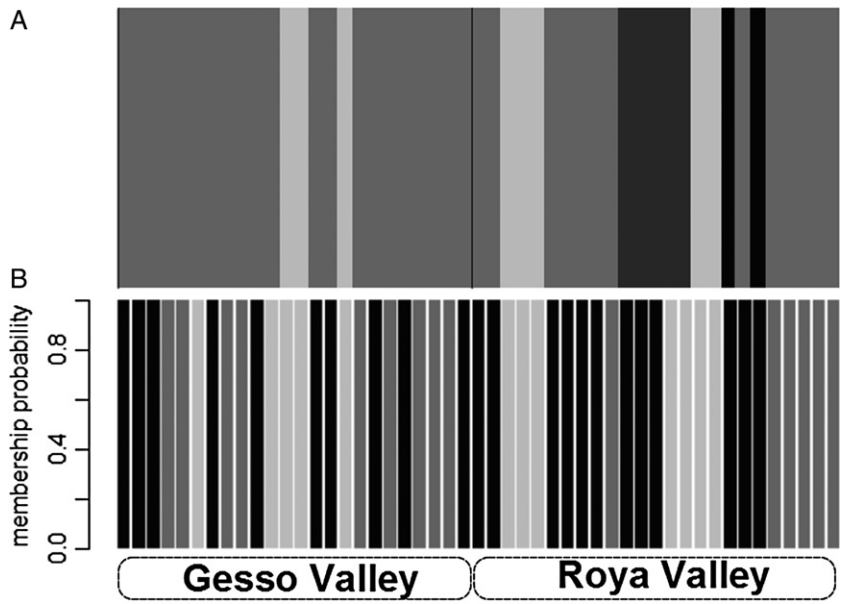

Figure 7. Inferred clusters of individual genotypes based on ISSR and AFLP data for *P. allionii* stands using (A) BAPS and (B) DAPC. Each colour represents the most likely ancestry of the cluster from which the genotype or partial genotype was derived. The geographical division of stands is reported at the foot of the figure.

Table 4. AMOVA among Gesso and Roya Valley groups, among stands within groups and within stands of *Primula allionii* based on ISSR and AFLP data

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Between groups	1	1.806	0.01413^{NS}	1.45
Among stands within groups	8	11.307	0.11928**	12.28
Within stands	39	32.683	0.83803**	86.27
Total	48	45.796	0.97145	100

^{NS}, not significant, $*P \leq 0.1$, $**P \leq 0.05$.

agreement with these observations, the flowering periods of *P. allionii* and *P. marginata* overlap (December–April and March–June, respectively; Richards, 2003; our personal observations). Nevertheless, in Val Roaschia (PA1/PM2 in Table 1) and Gorge della Reina (PA5/PM6 in Table 1) stands, where *P. allionii* and *P. marginata* grow on the same cliff, we found few hybrid individuals, suggesting that co-occurrence does not increase hybridization. In addition, the reciprocal arrangement of the stigmas and anthers between mutually compatible morphs of *P. allionii* and *P. marginata* seemed not to be a limitation to pollen transfer between species (Table 2; Fig. 2). However, as already observed for *Narcissus* L. (Marques *et al*., 2007), no single isolation mechanism can be fully effective in preventing hybridization and the relative importance of pre- and post-zygotic barriers differs among species (Widmer, Lexer & Cozzolino, 2009). In *P. allionii*, pollination barriers seem to contribute weakly to reproductive isolation from *P. marginata*, but post-pollination and pre-zygotic barriers cannot be excluded. Further studies on the mechanism of reproductive isolation in *Primula* section *Auricula* will be necessary to understand the reasons for hybrid formation.

Centre of origin and diversification of the species

Palaeodistributional models indicate a strong reduction of habitat suitability for *P. allionii* during the LGM. This decrease is more marked in the CCSM than in the MIROC climate (Fig. 4), probably because the LGM climate simulated by the former in the Alps is colder and drier than that produced by the latter (Schorr *et al*., 2012). For the LGM, the CCSM model does not predict any potential refugial areas. By contrast, the MIROC model predicts potential refugial areas nested within the current predicted range and they are mainly located in the north-eastern part of it where the species currently does not occur. The reduction of predicted habitat suitability is congruent with palaeoecological evidence for the study area. Vegetation changes reconstructed from pollen data show the presence of steppe that is dominated by *Artemisia* L. during the LGM, whereas forest development was observed to start from 11 000 years BP during the early Preboreal (Renault-Miskovsky, 1986; Ortu, David & Caramiello, 2003; Renault-Miskovsky & Lebreton, 2006; Ortu *et al*., 2008). The increase in lower elevation of taxa such as *Artemisia* suggests extremely dry and cold climate conditions, and it justifies the reduction of habitat suitability detected by BIOMOD models. This reduction is in accordance with the GMRF Bayesian skyride plot, which identifies a population decline throughout the Pleistocene and a weak expansion starting from the beginning of the Holocene. These results indicate that *P. allionii* did not shift its distributional range during the glacial period and it probably survived in microniches (i.e. on sunny cliffs) located both on the northern and on the southern sides.

The reason why some species have a highly restricted geographical range has long fascinated ecologists and evolutionary biologists. Many historical and ecological factors and their interaction are invoked to explain rarity and endemism (Kruckeberg & Rabinowitz, 1985), but reproductive traits (e.g. a lower investment in pollen and seed production) are also involved in determining dispersal capability (Lavergne *et al*., 2004). The predicted current potential niche of *P. allionii*, definitely wider than its known distribution (Fig. 4), suggests that its distribution is not mainly determined by habitat suitability.

Disjunctions are well documented in the Alps (Briquet, 1906; Pawlowski, 1970; Schönswetter *et al*., 2003) and *Primula* section *Auricula* provides an excellent example of this phenomenon (Zhang *et al*., 2004). Notably, vicariance caused by ecological (e.g. substrate specificity) and historical (e.g. Pleistocene glaciation) factors is the main explanation for the disjunction in mountain plant species (Vargas, 2003; Tribsch, 2004; Chen *et al*., 2008). In *P. allionii*, the separation into two groups probably dates back to the beginning of the Early/Mid Pleistocene border (about 0.780 Mya), during the mid-Pleistocene transition; the absence of any ancient demographic increase detected by BEAST analysis (Fig. 5) suggests the

absence of any subsequent re-colonization events. During the mid-Pleistocene transition (*c*. 1.2–0.5 Mya) the global climate changed; low-amplitude 41-kyr obliquity-dominated cycles gave way progressively to the high-amplitude, quasi-periodic (about 0.1 Mya) fluctuations that characterize the later Pleistocene and Holocene (Head, Pillans & Farquhar, 2008). Beginning *c*. 0.9 Mya, successive major glaciations profoundly affected the biota and physical environment. During approximately the middle of the mid-Pleistocene transition (*c*. 0.8 Mya), a major glacial event occurred. The extreme glacial conditions in this period appear to have played a major role in the extirpation of a large number of Tertiary relicts, causing a shift in the vegetational composition (Tzedakis, Hooghiemstra & Pälike, 2006). The same climate conditions probably caused the separation of *P. allionii* into two groups.

In the plastid phylogenetic tree (Supporting Information, Fig. S1), *P. allionii* forms a strongly supported clade, but this monophyly does not correspond to the haplotype lineages recognized by the haplotype network. More specifically, in the TCS network (Fig. 5C), the Val Gesso haplotypes GV1 and GV2 were nearer to the *P. marginata*/*P. latifolia* haplotype than to the remainder of *P. allionii*. This result suggests a first ancient separation of *P. marginata*-like and *P. allionii*-like ancestors, followed by the vicariance event causing a range disjunction in *P. allionii* and finally an early secondary contact between *P. allionii* and *P. marginata* in the Gesso Valley. Nevertheless, incomplete lineage sorting and transpecific polymorphism (Templeton, 2001) cannot be discarded as an explanation of the network structure. In general, plastid DNA results are consistent with the interpretation of two glacial refugia located in the Gesso and Roya Valleys. The Roya Valley has already been recognized as one of the main glacial refugia (Diadema *et al*., 2005; Minuto *et al*., 2006; Grassi *et al*., 2009, Zecca *et al*., 2011) and as an area of endemism in the Maritime Alps (Casazza *et al*., 2008; Casazza & Minuto, 2009). Our study also suggested the Gesso Valley as a potential refugium where secondary contact between species may have taken place.

Discrete phylogeography analysis (Fig. 5A) was unable to definitely indicate any centre of origin for the *P. allionii* lineage, and suggested that one dispersal event occurred from the Roya Valley to the Gesso Valley (PA3.1). The wallcreeper (*Tichodroma muraria*) might be the putative vector for seed dispersion, as has already been suggested by Martini (1982). This bird preferentially inhabits limestone rock faces in the high mountain ranges of Europe and Asia, with a short-distance seasonal migration like some other Alpine birds (Berthold, 2001).

Bayesian clustering and DAPC analysis (Fig. 6A, B) resolved the ten stands into four and three differentiated genetic clusters, respectively, which did not correspond geographically to the Gesso and Roya Valley. BAPS results supported the hypothesis of a greater diversification in the Roya Valley, detecting two private genetic clusters in the south. The lack of any separation between the two parts of the distribution range was also confirmed by AMOVA based on dominantly inherited markers (AFLP and ISSR), detecting the lower value between geographical areas (Table 4). The level of separation among populations was directly correlated with the strength of the barriers, and the differentiation among populations increased when the proportion of migrant genes that cross the barrier became small (Wilkinson-Herbots & Ettridge, 2004). In the case of *P. allionii*, the Alpine watershed does not seem to be a strong barrier for gene flow by pollen. The movements of genes is influenced by species-specific life-history strategies, such as the breeding system and spatial structure of the population, and by a large number of external factors, including numerous stochastic ones which can interact with each other (Washitani *et al*., 2005). Unfortunately, at present there are no complete data on the reproductive system of the species, although the partial data might be useful in interpreting any historical events (i.e. genetic drift, bottleneck, founder effects, gene flow, etc.; Hamrick & Godt, 1996; Sosa *et al*., 2010) acting on the present genetic structure. The strength of a phylogeographical break that is caused by a barrier to gene flow tends to increase with time after the barrier disappears. A recent past expansion, which could have facilitated gene flow by pollen dispersal, may explain this lack of genetic separation. However, the hypothesis of a past range expansion was not supported by ecological niche modelling and demographic reconstruction postulating a reduction in habitat suitability and a population decline throughout the Pleistocene.

In conclusion, neither the Gesso Valley nor the Roya Valley can be rejected as a centre of origin of *P. allionii* and, for this reason, the hypothesis (Martini, 1982) of a southern origin of the species is not supported or refuted by our results. The phylogeographical history of *P. allionii* is consistent with an early allopatric divergence in two refugia (the Gesso and Roya valleys) during the mid-Pleistocene transition, followed by introgression with *P. marginata* and recent demographic expansions. The Alpine watershed has low strength as a barrier between the two geographical parts of the distribution range, and a moderate level of gene flow by pollen seems to have created the admixture recorded among the stands.

The former paradigm of three main glacial refugia in southern European peninsulas (Taberlet *et al*.,

1998) constitutes an oversimplified biogeographical pattern (Gomez & Lunt, 2007; Médail & Diadema, 2009), probably due to the intensity and accumulation of a number of processes in a patchy landscape across a varied topography (Nieto Feliner, 2011). Our results agree with this more complex point of view of the biogeographical pattern in the Mediterranean Basin. As Médail & Diadema (2009) proposed, the persistence of *P. allionii* throughout the last ice age seems to be linked to the capacity of the mountain range to provide a wide diversity of microhabitats (e.g. sheltered and relatively humid gullies versus exposed and relatively dry ridges; south- versus north-facing slopes). According to this point of view, the gorges of the Roya and Gesso valleys seem to have ensured the survival of some endemic species [e.g. *Acanthoprasium frutescens* (L.) Spenn], whereas the surrounding cliffs have allowed the persistence of other species, including *P. allionii*.

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SUPPORTING INFORMATION

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

Figure S1. cpDNA bayesian 50% majority-rule consensus tree of *Primula allionii*, *P. marginata*, *P. latifolia* and *P. hirsuta* accessions; posterior probability values are indicated. Stands are coded according to Table 1.
Figure S2. Goodness of fit (Bayesian Information Criterion, BIC) computed for each k.
Table S1. Evaluation of individual modelling techniques for *P. allionii*. Statistics given are the mean values for area under the curve (AUC), the true skill statistic (TSS) and Kappa statistic (KAPPA). Values given in parentheses are the associated standard deviations. Accuracy classification for AUC: 1 > excellent >0.9 > good > 0.8 > fair > 0.7 > poor > 0.6 > fail; accuracy classification for TSS/KAPPA: 1 > excellent > 0.8 > good > 0.6 > fair> 0.4 > poor > 0.2 > fail (see BIOMOD Manual; Thuiller *et al*., 2008).
Table S2. Statistical results for each single run and for all runs (comb).