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ORIGINAL ARTICLE

Differential effects of historical migration, glaciations and human impact on the genetic structure and diversity of the mountain pasture weed *Veratrum album* L.

U. A. Treier* and H. Müller-Schärer

Department of Biology, Ecology & Evolution, University of Fribourg, Chemin du Musée 10, CH–1700 Fribourg, Switzerland

ABSTRACT

Aim Today's genetic population structure and diversity of species can be understood as the result of range expansion from the area of origin, past climatic oscillations and contemporary processes. We examined the relative importance of these factors in *Veratrum album* L., a toxic weed of mountain grasslands.

Location Continental Europe.

Methods Forty populations from the Asian border (Urals and Caucasus) to Portugal were studied using amplified fragment length polymorphisms (AFLPs) combined with selected plant and population measures. The data were analysed with phylogenetic, population genetic and regression methods inferring both genetic structure and diversity from geographic and ecological factors.

Results Fragment frequency clines together with genetic distance clustering and principal coordinates analysis indicated an east–west direction in the genetic structure of *V. album*, suggesting ancient migration into Europe from a proposed Asian origin. However, the strong geographic pattern in the genetic structure, pronounced isolation by distance ($R^2 = 0.74$) and moderate overall population differentiation ($F_{ST} = 0.13$) suggests high historical gene flow, possibly during glacials, and vicariance into mountainous regions during interglacials. Occurrence of *V. album* during the last glaciation in several areas along the periphery of the Alps and recolonization of this mountain range from both eastern and central–western areas was indicated. Genetic diversity was highest in central Europe, a pattern that did not agree with the expectations from east–west migration into Europe. Furthermore, managed habitats showed higher levels of genetic diversity compared to unmanaged habitats. Stepwise linear regression determined shoot density and soil phosphorus as the main predictors of within-population genetic diversity ($R^2 = 0.40$).

Main conclusions Our results showed that *V. album* retained genetic imprints of historical range expansion into Europe, although this was alleviated by the influence of climatic oscillations and contemporary processes. For example, genetic population structure was strongly affected by post-glacial vicariance while patterns of genetic diversity seemed mainly to be influenced by human land use. Our findings highlight the importance of applying a synthetic approach, testing the influence of both historical and contemporary processes on genetic structure and diversity in order to understand complex phylogeographic patterns. This may especially apply to widespread species, such as weeds. Implications of our findings for biological control are briefly discussed.

*Correspondence: Urs Treier, Department of Biological Sciences, Ecoinformatics and Biodiversity, Aarhus University, Ny Munkegade 114, Building 1540, DK–8000 Aarhus C, Denmark.
E-mail: urs.treier@gmail.com

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INTRODUCTION

Historical processes leave footprints in the genetic structure and diversity of species. Such processes include migration from the area of origin or effects of past climatic changes, for example Quaternary glacial–interglacial oscillations. Recolonization patterns after the retreat of the last ice age's glaciers have been the focus of many molecular studies (e.g. reviewed in Brochmann *et al.*, 2003; Stehlik, 2003; Hewitt, 2004; Schönswetter *et al.*, 2005), and glacial isolation and interglacial range expansion are regarded as important drivers of present genetic population structure and diversity (Hewitt, 1999, 2000). Legacies of earlier events may, however, have persisted in the species' genetic pattern (Neigel & Avise, 1993; Koch *et al.*, 2006; Magri *et al.*, 2007), although so far they have received less attention (Hampe & Petit, 2007). Such ancient dynamics may include species' migration from their region of origin. Generally, clines in the genetic structure or diversity along species' migration routes are expected. A successive pattern of differentiation in the genetic structure may indicate the area of origin or refugia (Comes & Kadereit, 2003; Schönswetter *et al.*, 2006). Additionally, newly founded populations generally harbour only a subset of their source gene pools, leading to higher genetic diversity in source compared to colonized areas (Barrett & Husband, 1990; Hewitt, 1996; but see Comps *et al.*, 2001; Widmer & Lexer, 2001). Likewise, higher levels of genetic diversity in refugia relative to their likely descendant populations are expected (Hewitt, 1996; Comes & Kadereit, 1998). However, convergence of colonization routes and subsequent admixture in so-called hybrid zones (Hewitt, 1996), can lead to genetic diversity similar to or higher than in refugia (Petit *et al.*, 2003; Walter & Epperson, 2005). Furthermore, some putative southern refugial populations may rather be genetically depauperate relicts without having served as sources for northern expansion (Petit *et al.*, 2005). Even though some general patterns exist, refugia and migration routes have been shown to be species-specific (Comes & Kadereit, 1998; Taberlet *et al.*, 1998; Brochmann *et al.*, 2003; Stehlik, 2003; Schönswetter *et al.*, 2005).

Genetic patterns are further expected to be shaped by recent processes that influence the balance between gene flow and genetic drift. Firstly, smaller size and isolation of populations at the edge of species' ranges favour genetic drift over gene flow. This may cause the often observed decline in within-population genetic diversity and increase of among-population differentiation from the centre to the periphery of geographic ranges (Lesica & Allendorf, 1995; Eckert *et al.*, 2008). Secondly, land use can have important genetic consequences. Many species suffer from habitat fragmentation and destruction, causing population decline and isolation, again decreasing genetic diversity and increasing interpopulation genetic distances (Young *et al.*, 1996; Aguilar *et al.*, 2008). Others, however, may have increased their range and population sizes due to an increase in suitable habitats caused by changes in land use. As a consequence, a shift in favour of gene flow over drift would decrease among-population differentiation and increase within-population genetic diversity. Such species are often characterized as good colonizers and successful founders of populations in previously unoccupied regions or habitats and many are recognized as weeds (Brown & Marshall, 1981).

Exploring the genetic structure of weed populations and identifying their geographic origin may be essential for successful weed control (Barrett, 1992). Biological control programmes, which are often the only environmental and economical option for large-scale weed control in semi-natural and extensively managed habitats, depend on such knowledge. Populations that exhibit high genetic diversity may limit the epidemic spread of highly specific biological control agents due to limited numbers of suitable hosts and reduce long-term control success because of their increased potential to evolve resistance to pests or pathogens (Hufbauer & Roderick, 2004; Sterling *et al.*, 2004; Müller-Schärer & Schaffner, 2008; Le Roux & Wieczorek, 2009). Finally, effective and highly specific biological control agents are most probably found in the species' area of origin (Goolsby *et al.*, 2006).

Veratrum album L. (white false hellebore) is a toxic weed in Europe's extensively managed and generally species-rich mountain grasslands, especially in pastures of the Alps and the Massif Central (Schaffner *et al.*, 2001). The genus *Veratrum* (Liliales: Melanthiaceae) comprises 17–45 species depending on the taxonomic treatment (Mathew, 1989; Zomlefer *et al.*, 2003; Liao *et al.*, 2007). Notably, the *V. album* complex circumscribes several closely related taxa, which are probably not distinct at the species level. The distribution pattern as well as taxonomic and phylogenetic evidence strongly indicates an East Asian origin of *Veratrum* (Liao *et al.*, 2007). While most *Veratrum* species are limited to Asia, *V. album* and the taxonomically distinct *V. nigrum* L. are the only species in the genus to occur in Europe and *V. album* occurs in North America as well, implying that they originated in Asia, with subsequent range expansion (Mathew, 1989). In Europe, *V. album* probably occurred well before the Last Glacial Maximum (21,000 years ago), indicated by a pollen record from Switzerland dated to the second early Würmian interstadial (*c.* 100,000 years ago; Burga & Perret, 1998). Interestingly, however, the herbivore community of *V. album* in central Europe comprises only a low number of specialists and therefore resembles those of introduced plant species

(Schaffner *et al.*, 2001). Thus, biological control may offer a way to manage this weed, especially because congeners are generally lacking in the species' weedy range (Pemberton, 2000).

While many phylogeographic studies traditionally have focused on effects of Quaternary range dynamics, the main objective of the present study was to examine the genetic population structure and diversity of *V. album* using a synthetic approach designed to disentangle three main processes: migration from the area of origin, range dynamics due to Quaternary temperature fluctuations, and contemporary influences on the species' range and abundance. In general, vicariance and recolonization caused by climatic oscillations and contemporary processes are expected to have obscured genetic patterns generated by migration from Asia. In this study we therefore ask the following question: (1) Is the hypothesized westward migration from the proposed area of origin in Asia evident in the observed genetic pattern, i.e. do we find east–west clines in the genetic structure and diversity? We further ask: (2) to what degree have climatic oscillations shaped the observed genetic pattern of *V. album* in Europe, and (3) are contemporary processes, e.g. human land use, evident?

MATERIALS AND METHODS

Study species

Veratrum album is a tall herbaceous, unpalatable and toxic weed of mountain grassland. Its distribution is limited to the Northern Hemisphere, ranging from coastal Alaska through Japan, China, Siberia and the Caucasus, to its western border in Europe (see Appendix S1 in Supporting Information; Mathew, 1989). In Europe it occurs in most mountainous areas, generally from 800 to 2500 m a.s.l., while in north-eastern Fennoscandia it also grows at lower elevations along the coast (Fig. 1; Alm, 2002). Currently, *V. album* is typically found in man-made grasslands below the tree line, but its natural habitats may have been open forests, the dwarf shrub zone above the timberline, and fens. Probably due to strong preference for moist conditions, it mainly occurs on north-facing slopes. In the drier areas of the Iberian Peninsula it is closely linked to streams, lakes or fens. *Veratrum album* is very long-lived, predominantly cross-pollinating, most likely by flies, and clonal with a hemicryptophytic growth form (Kleijn & Steinger, 2002). Each year during snow-melt new shoots are formed, and in late summer above-ground plant parts die and rot quickly. Only the wooden stems of flowering shoots persist during winter, retaining fruits and seeds. The number of seeds per plant is highly variable (700 on average), with the seeds showing adaptations for wind dispersal (flattened, winged, ellipsoid in shape of *c.* 9.9 × 3.7 mm in size and *c.* 3.2 mg in weight; Hesse *et al.*, 2007). Although exact data are lacking, it probably takes decades until a plant initiates flowering, which occurs only sporadically every 4–8 years (cf. Hesse *et al.*, 2008).

Study populations and sampling

Forty populations were selected within 11 predefined sampling regions located from the westernmost distribution limits of *V. album* in Portugal, to the Urals and Caucasus on the western border of Asia, including major mountainous areas of Europe (Table 1, Fig. 1). In each region at least three populations were sampled, except on the Iberian Peninsula (region 1 and 2) where *V. album* is rare (Table 1). Sampling sites within the same region were located at least 10 km apart.

Generally only large populations were sampled (> 500 *V. album* shoots, for exceptions see below). In each population ten plants were sampled along a 50 × 2 m sampling transect by selecting the plant closest to predefined sampling points spaced at 5-m intervals along the transect (starting at 2.5 m). This avoided multiple sampling of the same clone as the maximum clone extension has been shown to be only 75 cm (Kleijn & Steinger, 2002). Pieces of an uninfected leaf of the selected plant were cut and immediately dried on silica gel (Chase & Hills, 1991). The very rare populations on the Iberian Peninsula did not allow the same sampling scheme: in region 1 leaf pieces from all 31 shoots of a small population were sampled and in region 2 leaf pieces from two plants in each of two populations were sampled. Thus, the total sample size included 405 samples (37 populations with 10 samples, two populations with 2 samples, and one population with 31 samples).

To examine effects of contemporary processes on genetic diversity, managed sites (pastures/hayfields) and sites with no obvious management were distinguished. Additionally, soil cores (2.5 cm diameter, 10 cm deep) were taken at each sampling point, pooled and immediately air dried. Soil samples were analysed for total nitrogen (soil-N), total phosphorus (soil-P), and pH-H_2O (soil-pH) as described in Kleijn & Müller-Schärer (2006). To investigate a possible influence of population size on genetic diversity, total area covered by *V. album* shoots and population density was estimated by counting all *V. album* shoots within the 100-m^2 sampling transect. For each population, elevation and geographic coordinates were recorded (Table 1 & Appendix S2).

DNA extraction and amplified fragment length polymorphism fingerprinting

DNA was extracted using a modified cetyl trimethyl ammonium bromide (CTAB) procedure (Steinger *et al.*, 1996) but with an additional polyethylene glycol (PEG) purification step (Nicoletti & Condorelli, 1993). DNA samples were diluted to concentrations of 45 ng μL^{-1} prior to AFLP analysis (Vos *et al.*, 1995), which was carried out as described by Handley *et al.* (2008). However, selective multiplex polymerase chain reaction (PCR) amplifications combined fluorescence-labelled 5-FAM–*Eco*RI+AGG, JOE–*Eco*RI+ACC and TAMRA–*Eco*RI+AGC primers with the *Mse*I+CAC primer in a first reaction, and with the *Mse*I+CAG primer in a second reaction. All primers were synthesized by Microsynth (Microsynth AG,

Balgach, Switzerland). Preliminary tests showed that the primers chosen produce highly reproducible, clear, and good quality amplified fragment length polymorphism (AFLP) banding patterns. Fragments were separated on an ABI PRISM 310 Genetic Analyzer equipped with GeneScan v. 3.1 software using GeneScan-500 ROX size standard (Applied Biosystems, Foster City, CA, USA). Electropherograms were scored for presence (1) or absence (0) of fragments in the range 100–500 base pairs using GelCompar II v. 3.50 (Applied Maths, 2003). A detailed protocol for fragment

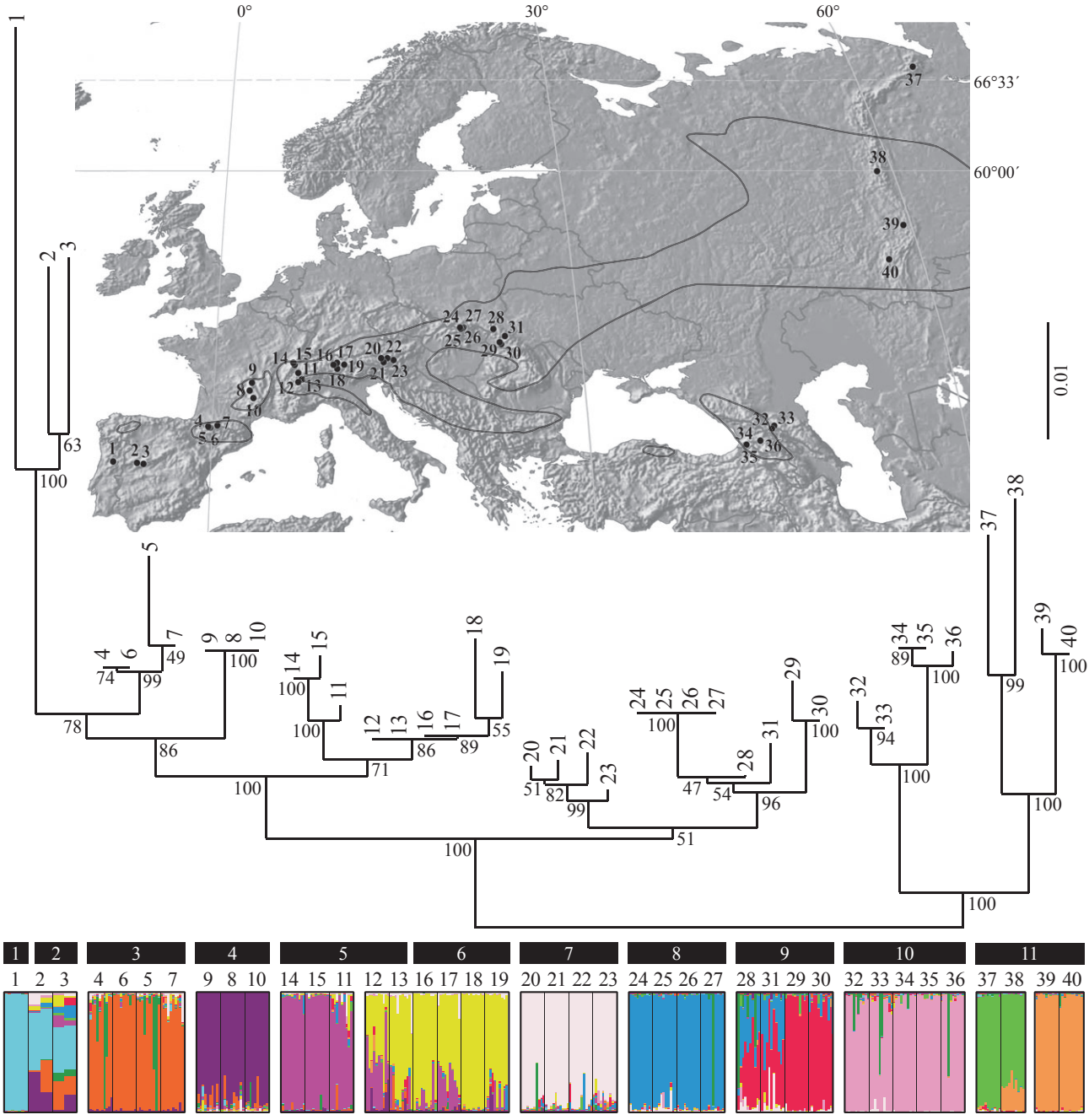

Figure 1 The geographic location and genetic structure of the 40 investigated *Veratrum album* populations. The European distribution of *V. album* according to Hultén & Fries (1986) is outlined (see Appendix S1 for the global distribution). The Fitch tree was based on Nei's genetic distances among populations and at each node the percentage support from 1000 bootstraps is given. The structure plot shows the membership probability of each individual for 12 clusters (population 1 with seven individuals; populations 2 and 3 with two individuals and all other populations with ten individuals, clusters in different colours; see also Appendix S3). Spacing in the structure plot indicates 11 genetically maximally differentiated and geographically homogeneous groups as revealed by SAMOVA (see also Appendix S3). Black bars with white numbers delimitate *a priori* regions. See Table 1 for further details on populations and regions.

Table 1 Geographic description of the 40 investigated *Veratrum album* populations.

Region	Population	Coordinates °E/°N	Elevation m a.s.l.
1 Serra da Estrela	1 Lagoa Comprida (PT)	−7.636/40.364	1647
2 Sistema Central	2 Laguna del Daque (ES)	−5.750/40.333	2000
	3 Hoyos del Espino (ES)	−5.250/40.283	1900
3 Pyrenees	4 Cabane d'Arnousse (FR)	−0.532/42.828	1457
	5 Astün (ES)	−0.495/42.807	1872
	6 Ei Batallero (ES)	−0.404/42.783	1657
	7 Toue de la Pègue (FR)	0.120/42.881	1912
4 Massif Central	8 Col du Pas de Peyrol (FR)	2.674/45.114	1537
	9 Le Barbier (FR)	2.820/45.583	1204
	10 Prairie d'Eglise (FR)	2.991/44.626	1309
5 SW Alps	11 La Chapelle d'Abondance (FR)	6.776/46.287	1260
	12 Bourg-St. Maurice (FR)	6.813/45.686	2074
	13 Prax de Fareox (IT)	7.144/45.857	2140
Jura	14 La Petite-Ronde (CH)	6.462/46.937	1178
	15 Les Prax (CH)	6.478/46.787	1293
6 Central Alps	16 Ochsenalp (CH)	9.648/46.807	1929
	17 Wätterweid (CH)	9.857/46.909	1703
	18 Alp Proliebas (CH)	9.900/46.584	2052
	19 Alpe Egg (IT)	10.462/46.790	2057
7 Eastern Alps	20 Obertauern (AT)	13.546/47.251	1673
	21 Karneralm (AT)	13.788/47.009	1935
	22 Sölkpass (AT)	14.084/47.240	1240
	23 Winterleitenhütte (AT)	14.572/47.095	1783
8 High Tatra	24 Temnosmrečinská dolina (SK)	20.018/49.195	1511
	25 Pliesko pod Skokom (SK)	20.048/49.151	1685
	26 Sliezsky dom (SK)	20.159/49.159	1677
	27 Skalnaté pleso (SK)	20.230/49.188	1761
9 Carpathians	28 Uzhansky Pass (UA)	22.887/49.004	863
	29 Dolina Narzisy (UA)	23.362/48.185	186
	30 Bushtyno (UA)	23.462/48.072	200
	31 Synevyr National Park (UA)	23.765/48.592	1154
10 Caucasus	32 Djvari Pass (GE)	44.461/42.490	2277
	33 Krestovyi Pass (GE)	44.614/42.668	2105
	34 Shuakhevi (GE)	42.453/41.485	1933
	35 Chirukhi (GE)	42.485/41.444	2364
	36 Tskhratskharo Pass (GE)	43.520/41.685	2457
11 Urals	37 The Sob River (RU)	65.740/66.906	139
	38 Konzhakovsky Kamen Mountain (RU)	59.285/59.616	889
	39 Mramorskiy Village (RU)	60.436/56.534	205
	40 Nurgoosh Ridge/Mountain (RU)	59.141/54.825	1162

Region: 11 predefined mountainous areas, the south-western Alps and the Jura mountains are combined into region 5; Population: numbering, site name, and country (PT, Portugal; ES, Spain; FR, France; Italy; CH, Switzerland; AT, Austria; SK, Slovakia; UA, Ukraine; GE, Georgia; RU, Russia). See Appendix S2 for ecological and genetic population parameters.

scoring with GelCompar II is available upon request. DNA extractions, AFLP reactions, and fragment scoring were carried out using randomized and anonymized samples assuring randomly distributed scoring errors across individuals, populations and regions.

Data analysis

Statistical analyses of AFLP patterns were based on the following assumptions (Despres *et al.*, 2002): (1) AFLP markers behave as diploid, dominant markers with alleles either present (amplified) or absent (non-amplified), (2) co-migrating fragments represent homologous loci, and (3) populations are at the Hardy–Weinberg equilibrium. These assumptions appear justified because sampling of small populations was generally avoided, *V. album* is predominantly cross-pollinating (Kleijn & Steinger, 2002) and diploid ($2n = 32$; Zomlefer *et al.*, 2003), and departures from Hardy–Weinberg equilibrium may generally be modest for dominant markers in outcrossing species (Krauss, 2000).

The number of 'rare' fragments per individual in each region/population ($R_{reg/pop}$) was estimated according to Winkler *et al.* (2010) using the AFLPdat rarity R-function (Ehrich, 2006; http://www2.uit.no/ikbViewer/page/ansatte/organisasjon/ansatte/person?p_document_id=41186&p_dimension_id=88165&p_lang=2; version used: 20 June 2010). The proportion of polymorphic loci for each population (P_{pl}) and the number of 'private' fragments (confined to one region/population, N_{pf}) were additionally extracted from the binary data matrix. Population genetic parameters (H_t, total gene diversity; H_w, mean gene diversity within populations; H_j, Nei's gene diversity in the jth population; H_b, average gene diversity among populations; F_{ST}, population differentiation; f_{Fpop}, frequency of each AFLP fragment for each population and f_{Ftot}, for the total sample) were computed with AFLP-SURV v. 1.0 (Vekemans, 2002), using a Bayesian method with non-uniform prior distribution of allele frequencies, following Zhivotovsky (1999), to estimate allele frequencies at AFLP loci. Further computations and denotation of parameters followed Lynch & Milligan (1994). In addition to the matrix of genetic distances (Nei's D) among populations, another 1000 distance matrices were computed by bootstrapping over AFLP loci. The matrices were used as input for the procedures FITCH and CONSENSE from the PHYLIP software package v. 3.66 to construct a population tree (Felsenstein, 2004). For the FITCH procedure, global rearrangement was enabled and the tree building process was repeated 100 times with randomized input of populations (Jumble option) to search for the best tree. For bootstrapped matrices only 10 such repetitions were made to reduce computation time. The tree was built both unrooted and rooted with *V. nigrum*. The inferred relationship among populations in the two trees was identical and only the former is shown. The genetic distance matrix was also subjected to a principal coordinates analysis (PCoA) using CANOCO v. 4.53 (PRCOORD program implemented; ter Braak & Šmilauer, 2004).

Rare fragments may get lost during colonization while common fragments may get fixed. These expectations were tested by investigating clines in fragment frequencies (f_{Fpop}) along the suggested colonization path out of Asia for each locus with Spearman's rank correlations between f_{Fpop} at each locus and distance to 'Asia'. The latter was expressed as the longitudinal distance of each population to the western border of the Asian distribution range of *V. album* (80° longitude, see Appendix S1). Mann–Whitney U-tests were used to assess if fragments with decreasing (negative Spearman's rho) or increasing (positive rho) frequencies towards western Europe were significantly less or more common in the total sample (f_{Ftot}), respectively. Tests were done on loci with significant ($P < 0.05$) or marginally significant ($P < 0.10$) clines. Computations were performed in the R environment (R Development Core Team, 2009).

Genetic differentiation was assessed in different ways. Firstly, F_{ST}-values were computed and tested against a distribution of 10,000 F_{ST}-values obtained through random permutation of individuals among populations or regions using AFLP-SURV. Secondly, a hierarchical analysis of molecular variance (AMOVA) was run based on squared Euclidean distances among populations using ARLEQUIN v. 3.11 (Excoffier *et al.*, 2005). The total molecular variance was partitioned into three levels: among regions, among populations within regions, and within populations. Variance components were tested with 100,000 permutations. Thirdly, spatial analysis of molecular variance (SAMOVA) (Dupanloup *et al.*, 2002) was applied to characterize geographic patterns of genetic divergence. SAMOVA combines genetic and geographic information to define maximally differentiated but geographical homogeneous groups. For various group numbers 100 simulated annealing steps were run. Finally, individual-based STRUCTURE v. 2.3.3 analysis (Pritchard *et al.*, 2000; Falush *et al.*, 2007) was used to assess patterns of admixture in pre-defined mountainous regions. Various group numbers were tested by applying the program's standard settings (admixture model with recessive alleles and correlated allele frequencies), using various iteration schemes (e.g. 10^5 burn-in followed by 10^6 sampling) with at least five replicated runs each (see Appendix S3 for details).

The Mantel test (Mantel, 1967) implemented in ARLEQUIN was used to test for a correlation between genetic (F_{ST}-values computed with AFLP-SURV) and geographic distances, by running 100,000 permutations. Furthermore, within-region genetic distances between pairs of individuals ('$1 - r$'-values computed with AFLP-SURV following Lynch & Milligan, 1994) were correlated with their respective geographic distances. Because distances among study populations within regions differed, correlation coefficients (r_i) were standardized across the European core regions (Pyrenees to Carpathians), among which distances were comparable. Firstly, mean among individual geographic distances of each region (d_i) were divided by the overall mean ($\bar{d}$), and subsequently r-values were divided by the standardized regional sampling extent ($r_i/(d_i/\bar{d})$).

Relationships among genetic (P_{pl}, H_j, R_{pop}) and ecological parameters (*V. album* shoot density, soil-N, soil-P, soil-pH, longitude, latitude, and elevation) were tested with Spearman's rank correlations. Stepwise multiple linear regression determined those parameters that best explained genetic diversity. Parameters were transformed to improve normality when needed. Differences in parameters between habitat types were assessed with t-tests. Analyses were performed using SPSS v. 16.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

AFLP phenotyping

On all 405 *V. album* samples the six primer combinations generated 877 scoreable AFLP fragments of which 17 (1.9%) were monomorphic. Scoring reproducibility was 92.4%, revealed by 29 'blindly' included replicates. Our protocol assured randomly distributed scoring errors making it unlikely that the highly supported phylogeographic pattern represents systematic biases arising from sample processing or scoring.

Contrariwise, random noise would rather obscure underlying biological patterns making tests conservative (Herrera & Bazaga, 2008). Only the 860 polymorphic fragments were used for analyses. In the very small and therefore completely sampled population 1 (Serra da Estrela, Portugal) AFLP patterns revealed seven individuals (genets) among 31 sampled shoots reducing the total sample size to 381 sampled individuals (seven in population 1, two each in populations 2 and 3, and 10 each in the remaining 37 populations). On average 43% of the fragments were polymorphic at the population level (Appendix S2), only six fragments were private, and none of them were fixed (i.e. appearing in all individuals of a population).

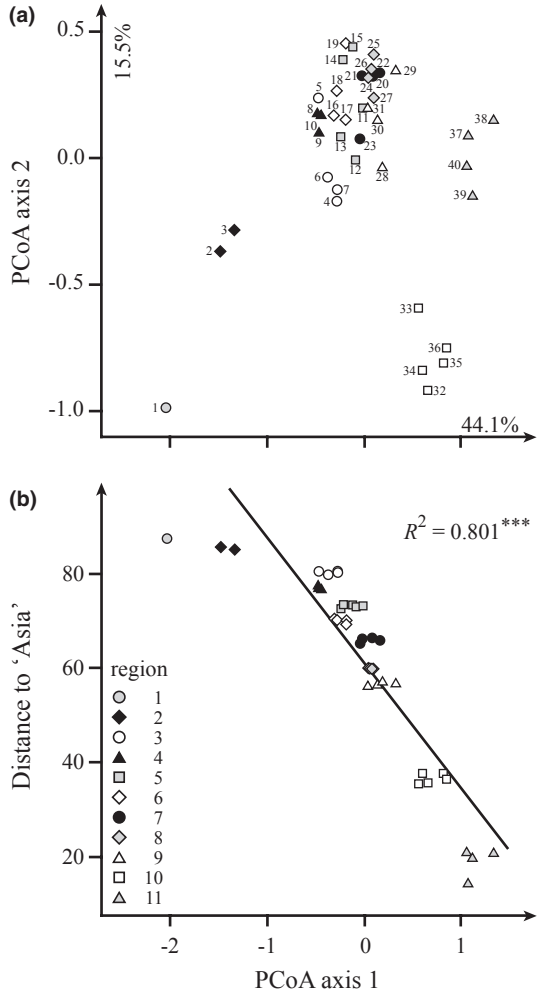

Figure 2 Scatter plots of the 40 investigated *Veratrum album* populations, principal coordinates analyses (PCoA) based on Nei's genetic distances among populations: (a) first and second axis with the percentage variation explained by each of the coordinates; (b) first axis and distances of each population to 'Asia' measured in longitudes (degrees) to 80° E; regression line, $F_{1,38} = 153.3$, $^{***}P < 0.001$; $R^2 = 0.943^{***}$ with region 1 and 2 excluded. For population numbers and geographic regions (different symbols) see key and Table 1.

Phylogeographic structure

The FITCH tree, STRUCTURE, SAMOVA and PCoA reflected the geographic pattern of the studied populations well (Figs 1 & 2a). In the PCoA the westernmost (Iberian Peninsula) and easternmost (Caucasus and Ural) populations clustered into regions while the remaining European core populations clustered together (Fig. 2a). The first two axes in the PCoA explained about 60% of the variation. Further, there was a strong linear relationship between the first axis' coordinates and the distance of each population to 'Asia' ($R^2 = 0.80$, Fig. 2b). Multiplying the explained variation of this correlation (80%) with the variation of the first PCoA axis (44%) indicated that about 35% of the total variation could be attributed to an east–west orientation in the genetic structure.

On a large scale the FITCH tree extracted three main clusters consisting of populations from: (1) the Caucasus and Urals, (2) eastern Europe, and (3) central–western Europe (Fig. 1). In contrast to the PCoA, the FITCH tree furthermore grouped most of the populations into predefined regions with generally high bootstrap support (Fig. 1). Only populations from the south-western Alps and Jura (region 5) and Carpathians (region 9) did not cluster into their region. The eastern alpine populations (region 7) clustered, although with weak bootstrap support, together with the eastern European populations. A neighbour joining analysis revealed the same overall pattern (data not shown), but placed the eastern alpine populations in a separate cluster, in close proximity of the eastern European populations. STRUCTURE analyses confirmed these sequential patterns of genetic clusters (Appendix S3). Only the Sistema Central (region 2), the southern-central Alps (regions 5 and 6) and the Carpathians (regions 8 and 9) showed important levels of admixture (Fig. 1).

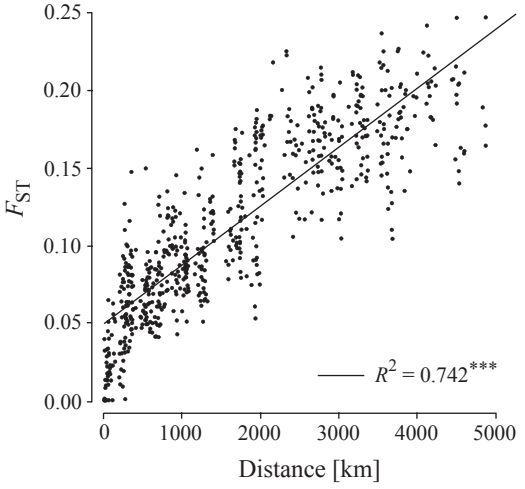

Figure 3 Relationship between genetic and geographic distances of all pairwise combinations of 37 *Veratrum album* populations covering an area from the Pyrenees to the Urals and Caucasus, excluding the three very small westernmost Iberian populations (for details see text). The significant positive relationship shows isolation by distance. $^{***}P < 0.001$, Mantel test.

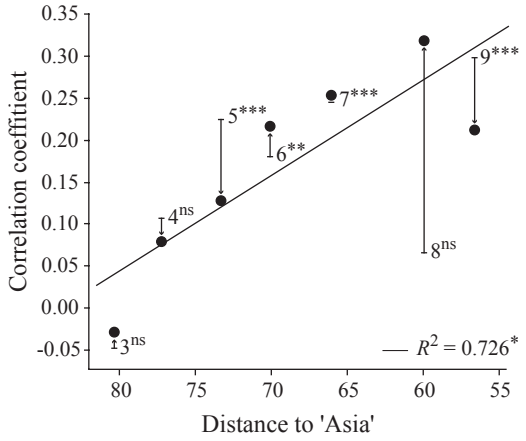

Figure 4 Intra-regional correlations between genetic and geographic distances among individuals of European *Veratrum album* core populations (regions 3–9) in relation to the distance of each region to 'Asia' (***$P < 0.001$, **$P < 0.01$, ns, not significant, Mantel tests, *P*-values Bonferroni corrected). Original correlation coefficients (bars) were standardized for differences in extents of the regional sampling area (indicated by arrows to the standardized values, dots; see methods for standardization). Isolation by distance increased in importance towards the east ($F_{1,5} = 13.3$, *$P = 0.015$). Distance to 'Asia' measured in longitudes (degrees) to 80° E.

Analyses of fragment frequency clines showed that loci with significantly decreasing fragment frequencies along the suggested colonization path from Asia to western Europe (L_{rare}: 105 loci, 12%) had a significantly lower overall fragment frequency (median $f_{Ftot} = 0.19$) than the loci that showed increasing frequencies (L_{common}: 137 loci, 16%, $f_{Ftot} = 0.35$; Wilcoxon rank sum test, $p = 0.006$ Bonferroni corrected). This result was robust when also marginally significant clines were included (L_{rare}: 141 loci, 16%, $f_{Ftot} = 0.16$; L_{common}: 172 loci, 21%, $f_{Ftot} = 0.29$; $P = 0.003$).

Isolation by distance

Genetic and geographic distances among populations showed a significant positive relationship (Fig. 3), and interpopulation geographic distances explained more than half of the variation in genetic distance ($R^2 = 0.55$). The three westernmost populations (Iberian Peninsula) showed extraordinarily large genetic distances relative to other populations (Fig. 1). Excluding these three populations, the explained variation increased strongly ($R^2 = 0.74$, Fig. 3). However, using regions instead of populations, the explained variation was as high ($R^2 = 0.76$). Within Europe, correlations were still significant but explained less variation (eastern Europe: $R^2 = 0.49$, populations 20–31; western Europe: $R^2 = 0.40$, populations 4–19; all European core populations: $R^2 = 0.43$; using regions: $R^2 = 0.48$). Within regions none of the population-based correlations were significant. However, correlating inter-individual genetic distances with the respective geographic distances, some regions showed isolation by distance and this association decreased from east to west within the European core area (Fig. 4).

Table 2 Analysis of genetic diversity and genetic differentiation of the 40 investigated *Veratrum album* populations.

Comparison	*n*	H_t	$H_w \pm$ SE	$H_b \pm$ SE	F_{ST}	*P*-value	R_{reg}	N_{pf}	*KM*
Among regions	11	0.2036	0.1733 ± 0.0047	0.0303 ± 0.0072	0.1496	<0.0001	NA	NA	1922.4
Among all populations	40	0.2111	0.1830 ± 0.0026	0.0282 ± 0.0027	0.1335	<0.0001	NA	NA	1769.0
Among European core population (4–31)	28	0.2048	0.1897 ± 0.0022	0.0152 ± 0.0009	0.0741	<0.0001	NA	NA	774.1
Among populations within regions									
1 Serra da Estrela†	1	0.1342	0.1342 ± 0.0060	NA	NA	NA	2.43	0	NA
2 Sistema Central‡	2	0.2013	0.1775 ± 0.0100	0.0238 ± 0.0000	0.1179	0.3292	2.91	1	42.7
3 Pyrenees	4	0.2013	0.1974 ± 0.0112	0.0040 ± 0.0016	0.0195	<0.0001	2.48	1	28.6
4 Massif Central	3	0.1881	0.1873 ± 0.0027	0.0008 ± 0.0000	0.0044	0.0006	2.28	0	73.5
5 South–western Alps & Jura Mt.	5	0.1969	0.1911 ± 0.0020	0.0058 ± 0.0013	0.0297	<0.0001	2.11	1	82.0
6 Central Alps	4	0.1957	0.1910 ± 0.0024	0.0047 ± 0.0022	0.0242	<0.0001	2.18	3	40.9
7 Eastern Alps	4	0.1856	0.1820 ± 0.0035	0.0037 ± 0.0001	0.0198	<0.0001	2.11	0	47.9
8 High Tatra	4	0.1841	0.1846 ± 0.0048	−0.0005 ± 0.0003	−0.0030	0.0055	2.21	0	10.0
9 Carpathians	4	0.2001	0.1935 ± 0.0081	0.0066 ± 0.0000	0.0328	<0.0001	2.34	1	69.8
10 Caucasus	5	0.1775	0.1698 ± 0.0080	0.0077 ± 0.0000	0.0435	<0.0001	2.10	3	131.5
11 Urals	4	0.1918	0.1675 ± 0.0027	0.0243 ± 0.0000	0.1264	<0.0001	2.49	3	755.4

n, number of populations or regions; H_t, total gene diversity; H_w, mean gene diversity within populations (or regions); H_b, average gene diversity among populations (or regions); F_{ST}, population differentiation with *P*-values based on 10,000 random permutations for an F_{ST} larger than the observed value (calculations with AFLP-SURV v. 1.0; Vekemans, 2002); R_{reg}, a measure of the number of rare fragments per plant within a region (rarity 1, calculated with AFLPdat; Ehrich, 2006); N_{pf}, number of private fragments (occur only in individuals within the given region); Populations 4–31 include regions 3–9, for population numbering see Table 1; *KM*, mean distance among populations; †only one population was found in this region and all 7 individuals of the population were sampled; ‡values have to be interpreted with caution since only two plants were sampled in each of the two populations, otherwise 10 individuals per population were sampled.

Table 3 Analysis of molecular variance (AMOVA) for all 40 investigated *Veratrum album* populations: (a) analysis includes all populations and (b) analysis with European populations that did not separate into regions in the principal coordinates analysis (Fig. 2a).

Source of variation	d.f.	SSD	Variance components		
			Absolute	Rel. to total	*P*-value
(a) All populations					
Among regions	10	6124.91	14.33†	15.78%	<0.001
Among populations within regions	29	3772.98	6.15†	6.77%	<0.001
Within populations	341	23986.01	70.34‡	77.45%	<0.001
(b) European core populations (4–31)					
Among regions	6	2909.64	9.24†	10.61%	<0.001
Among populations within regions	21	2443.31	4.28†	4.92%	<0.001
Within populations	252	18532.30	73.54‡	84.47%	<0.001

SSD, sum of squared deviations; for the grouping of populations into predefined regions, see Table 1; significance tests of variance components were based on 100,000 random permutations with *P*-values for variance components, †larger or ‡smaller than the observed value.

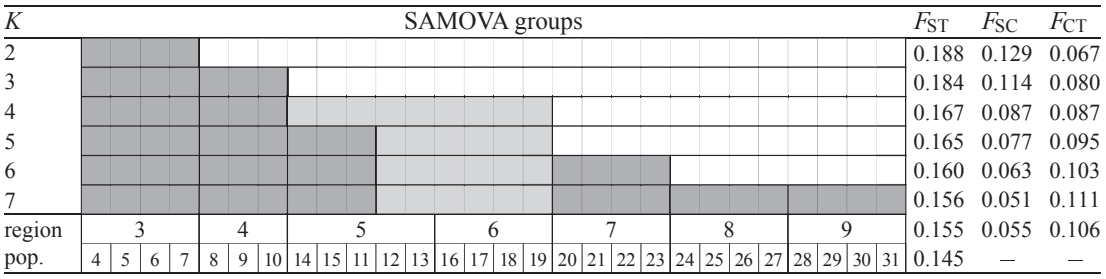

Figure 5 Spatial analysis of molecular variance (SAMOVA) for the 28 European *Veratrum album* core populations. *K* is the *a priori* number of groups. Numbers for predefined regions and populations are shown at the bottom (see also Table 1). To the right are indices based on hierarchical analyses of molecular variance for the various population groups: F_{ST}, among-population differentiation; F_{SC}, differentiation among populations within groups; F_{CT}, differentiation among groups (all $P < 0.001$, based on 1000 permutations). Grey shading, groups that separated from the remaining populations; light grey shading, groups that did not agree with predefined mountain regions.

Genetic differentiation

Genetic differentiation among geographical regions ($F_{ST} = 0.15$) or among all populations ($F_{ST} = 0.13$) was moderate and low among only European core populations ($F_{ST} = 0.07$, Table 2). Genetic differentiation of populations within regions was very low, although most F_{ST}-values were significant (Table 2). These results were supported by AMOVA: the among-region as well as the among-population-within-region variance component was significant, confirming differentiation at both levels (Table 3). But as indicated by F_{ST}-values, among-region variation (11–16%) was more than twice as large as that among populations within regions (4–6%) attributing most of the among-population differentiation to differences among the 11 geographical regions. Most of the molecular variation, however, was found within populations (77–85%). The AMOVA derived overall F_{ST} was 0.22 (Appendix S3). SAMOVA analyses on European core populations uncovered a west to east stepwise decrease in the differentiation of population groups, mostly coinciding with the predefined mountainous regions (Fig. 5).

Genetic diversity

Nei's gene diversity varied from 0.13 to 0.22 among populations (H_j, Appendix S2) and mean gene diversity of populations was 0.18 (H_w, Table 2). Regions harboured similar levels of gene diversity (H_t) despite large differences in their geographic extent (Table 2). Gene diversity of populations, however, was lower within westernmost and easternmost regions than within the European core region ($n = 12$ populations in region 1, 2, 10 and 11 vs. $n = 28$ populations in region 3–9; Mann–Whitney *U*-test: $Z = -3.87$, $P < 0.001$), which was mirrored by a quadratic model ($F_{2,37} = 4.6$, $P = 0.017$, $R^2_{adj} = 0.16$) illustrating the relationship between population genetic diversity and distance to 'Asia' (Fig. 6).

Measures of genetic diversity (H_j, P_{pl}) were highly intercorrelated, positively correlated with *V. album* shoot density, and negatively correlated with soil-P but only H_j showed a negative correlation with longitude (Table 4). Stepwise multiple linear regression supported these findings, identifying shoot density and soil-P as best predictors for within-population genetic diversity (Table 5). These two factors

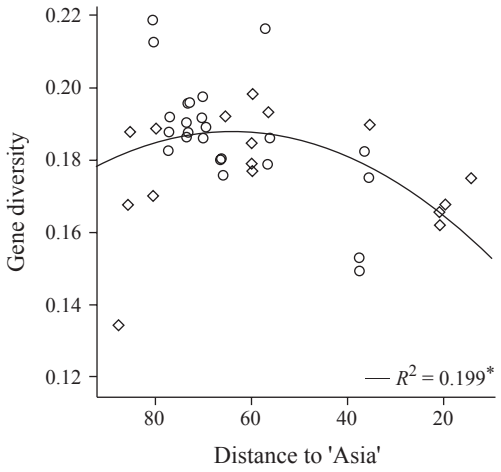

Figure 6 Relationship between genetic diversity and distances to 'Asia' of 40 investigated *Veratrum album* populations explained by a quadratic model ($F_{2,37} = 4.6$, $P = 0.017$). Circles and diamonds indicate populations in managed habitats or in habitats with no obvious management, respectively. Distance to 'Asia' measured in longitudes (degrees) to 80° E.

explained about 40% of the variation in genetic diversity. Genetic diversity was higher in managed (mean: 0.187, SE: ± 0.003) than in habitats with no obvious management (0.177 ± 0.004, $t_{38} = 1.95$; $P = 0.058$). The same was found for *V. album* shoot density (managed: 3.4 ± 0.6 plants m^{-2}, unmanaged: 1.6 ± 0.4 plants m^{-2}; $t_{38} = 2.28$, $P = 0.029$) and soil-pH (managed: 5.2 ± 0.1, unmanaged: 4.8 ± 0.2; $t_{37} = 2.03$, $P = 0.050$) while soil-N tended to be lower in managed (5.5 ± 0.5 g kg^{-1} dry soil) than unmanaged habitats (7.9 ± 1.1 g kg^{-1}; $t_{20} = 1.96$, $P = 0.068$, d.f. corrected for unequal variances). Soil-P did not differ with respect to management (average: 1.1 ± 0.1 g kg^{-1}). Additionally, managed habitats predominated in the centre of the investigated area, coinciding with higher within-population diversity (Fig. 6). Measures of genetic diversity, soil parameters, and *V. album* shoot density did not differ between the two groups of 26 large and 14 smaller population areas (see Appendix S2). The number of rare fragments, however, was lower in large area (mostly managed) populations (2.17 ± 0.05) than in those covering smaller areas (2.50 ± 0.09; $t_{38} = 3.37$, $P = 0.002$).

Table 4 Spearman's rank correlations between various geographic, ecological and genetic parameters of the 40 investigated *Veratrum album* populations.

	long.	lat.	elev.	dens.	*N*	*P*	pH	P_{pl}	H_j
lat.	**0.54*****								
elev.	−0.15	**−0.62*****							
dens.	−0.09	0.13	0.09						
N	−0.01	−0.16	0.12	−0.07					
P	0.25	−0.01	0.07	−0.14	**0.66*****				
pH	−0.21	**−0.38***	0.20	0.16	**−0.47****	−0.14			
P_{pl}	−0.10	0.14	0.04	**0.61*****	−0.16	**−0.36***	0.07		
H_j	**−0.31***	−0.00	0.04	**0.53*****	−0.20	**−0.36***	0.13	**0.90*****	
R_{pop}	−0.04	−0.05	−0.11	0.06	0.23	0.04	−0.05	0.19	0.29

long., longitude; lat., latitude; elev., elevation; dens., *V. album* shoot density; N, soil nitrogen; P, soil phosphorus; pH, soil pH–H_2O; P_{pl}, proportion of polymorphic loci; H_j, gene diversity; R_{pop}, number of rare fragments per plant within a population (rarity 1); coefficients in bold are significant, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Table 5 Multiple regression models predicting genetic diversity of the 40 *Veratrum album* populations with population and site parameters.

				ANOVA				
Variable	β	*P*	Source	d.f.	MS	*F*-ratio	*P*	R^2_{adj}
Gene diversity (H_j)								
Density	0.550	0.000	Regression	2	0.002	13.516	<0.001	0.397
Soil-P	−0.294	0.026	Residual	36	0.000			

MS, mean squares; H_j as response variable; soil phosphorus (soil-P, cube root transformed) and *V. album* shoot density (density, ln-transformed) as predictor variables. β, standardized partial regression coefficient.

DISCUSSION

Ancient east–west range expansion

Distributional, taxonomic and phylogenetic evidence strongly indicates an Asian origin of *V. album* (Liao *et al.*, 2007). Our analyses provide further support that *V. album* evolved in Asia and subsequently migrated to its western distribution border in Europe: fragment frequency clines together with genetic clustering and PCoA revealed an east–west direction in the rather strong longitudinal genetic population structure (Figs 1 & 2). We estimated that about 35% of the variation can be attributed to this east–west pattern, a possible imprint of ancient migration into Europe.

Geographic distance accounted for 55% of the variation in the genetic differentiation among populations, which increased to 74% by excluding the three westernmost populations (Fig. 3). A stepwise increase in genetic drift and a concomitant decrease in genetic diversity caused by serial founder events during colonization can lead to such a range-wide isolation by distance pattern (Ramachandran *et al.*, 2005). However, it might also suggest equilibrium between gene flow and drift due to the presence of suitable habitats without significant barriers to dispersal and sufficient time of occupation (Hutchison & Templeton, 1999). At the time of colonization, possibly during past cold periods, environmental conditions might indeed have been more favourable for *V. album* to establish and maintain gene flow among new and established populations. However, since time is needed to reach equilibrium between gene flow and drift, we expect it to be more pronounced towards the assumed origin than in more recently colonized areas where founder events still may predominate. Accordingly, isolation by distance within European core regions tended to decrease towards the western edge of the distribution range (Fig. 4) while differentiation of geographic adjacent regions increased (Fig. 5), supporting east–west range expansion. An indication that drift only recently became more influential than gene flow might be low F_{ST}-values and fairly constant scatter over all geographic distances (Fig. 3), moderate among-region differentiation (Tables 2 & 3), and a nearly perfect match of the genetic and geographic pattern (Fig. 1). Indeed, the significant overall F_{ST}-value was mainly a result of differentiation among regions, while populations within regions showed little or no differentiation (Tables 2 & 3). Thus today, geographic regions seem to be more isolated than at the time when *V. album* extended its range, suggesting post-glacial vicariance. More interconnected ranges during glacials and only recent isolation due to climate warming in the Holocene have been suggested for other species too (Despres *et al.*, 2002; Schönswetter *et al.*, 2006; Alsos *et al.*, 2009) and may apply to most species adapted to conditions prevailing during glacials (Birks & Willis, 2008). Since glacials constitute about 80% of the Quaternary, these species might have had the opportunity to expand their ranges and maintain high gene flow over long periods. This may have preserved genetic imprints of ancient range expansion and low differentiation among populations. Along the same line of argumentation, Kropf *et al.* (2003) found reduced intraspecific divergence in an upper elevation species compared to a species with lower elevation occurrence. Hence, low genetic differentiation does not necessary imply current high levels of gene flow as historical events can leave imprints in the population genetic structure that last for many generations (Ibrahim *et al.*, 1996).

Quaternary range dynamics and vicariance

Our analyses further offer insight on how Quaternary range dynamics possibly have shaped the genetic pattern, especially among European populations. Besides post-glacial vicariance into mountainous regions, highly supported population clusters (Fig. 1) suggest that during glacials *V. album* occurred in several areas along the edge of the Alpine ice sheets. Such peripheral areas have been suggested at the eastern, southern, and south-western borders of the Alps (Schönswetter *et al.*, 2005). Our data indicate that *V. album* recolonized the Alps from both the west and east. We found a clear separation of eastern Alpine populations (20–23), also demonstrating a major split in the European gene pool (Fig. 1, Appendix S3). This strongly agrees with findings for other plant species (Scotti *et al.*, 2000; Schönswetter *et al.*, 2002) and suggests no east–west migration after the last glaciation (Parisod, 2008), again indicating that interglacials have been main periods of vicariance for *V. album*.

Some of the small-scale patterns (Fig. 1, Appendix S3) allow further insight although additional local-scale sampling would be needed for more rigorous testing. For example, populations 12 and 13 located in the south-western Alps were most similar to central Alpine populations (16–19), which supports south-west recolonization of the central Alps. The strongly supported cluster of the most northern population from the south-western Alps (11) and populations from the Jura (14, 15) may suggest that in addition to a south-west recolonization route along the southern edge of the Alps (transalpine southern pathway), *V. album* may also have followed a more northern migration route (Rhodanian pathway; cf. Parisod, 2008). Furthermore, Carpathian populations (28, 31) show similarity to Tatra populations (24–27) suggesting colonization of the Tatra from the Carpathians. It should be noted, however, that molecular data cannot precisely localize areas of glacial survival without ancillary evidence, e.g. fossil records (Taberlet *et al.*, 1998; Alsos *et al.*, 2009). Such evidence is unfortunately limited for many species, including *V. album*. Pre- and early-Holocene occurrences of *V. album* north of the Alps as well as in inner and southern Alpine valleys (Burga & Perret, 1998; van der Knaap, 2007; Appendix S1) may indicate a larger distribution during glaciations. Furthermore, at our western-most sampling site in Portugal's Serra da Estrela, pollen data suggest a vegetation type in the Younger Dryas (*c.* 12,000 years ago) with occurrence maxima of *V. album* and other herbs (van der Knaap & van Leeuwen, 1997). However, our

molecular data do not indicate the Iberian Peninsula as a source for recolonization.

Effects of contemporary processes

Based on our hypothesis of east–west migration into Europe and the pattern inferred from the genetic population structure, we expected genetic diversity to decrease from east to west. Our results did not support this prediction. Instead, we found that populations had higher genetic diversity in central Europe and lower levels towards the east and west (Fig. 6). Furthermore, stepwise linear regression determined high shoot density and low soil-P as main determinants of within-population genetic diversity. These two factors explained nearly 50% of the variation in genetic diversity (Table 5) and suggest that within-population genetic diversity of *V. album* may be influenced to a large extent by local habitat conditions. In general, population size is positively correlated with genetic diversity (Frankham, 1996; Leimu *et al.*, 2006). Our density measure may be a surrogate for population size, also because the area covered by our study populations did not affect genetic diversity. Additionally, we found higher genetic diversity and higher shoot density in managed habitats suggesting that *V. album* invaded man-made habitats with a genetically diverse set of genotypes (cf. Kleijn & Steinger, 2002) and not by a few 'general purpose' genotypes (cf. Baker, 1974). This result provides evidence that land use may have strongly influenced patterns of genetic diversity in this species. The observed negative correlation between soil-P and genetic diversity (Tables 4 & 5) may indicate a positive influence of soil nutrients on clonal growth of *V. album*. However, experimental studies are needed to shed light on the hypothesized causality of this relationship and to pinpoint underlying mechanisms. We note that managed habitats predominate in the southern and central Alps and other non-measured factors related to this region cannot be excluded from having caused observed higher levels of genetic diversity.

Quaternary glaciations, for example, may have influenced patterns of genetic diversity in *V. album*, as has been found for many other European species (Hewitt, 1996; Comes & Kadereit, 1998; Taberlet *et al.*, 1998). Glacial survival of *V. album* in several areas along the periphery of the Alpine ice sheets may have maintained high levels of genetic diversity in the Alps. Higher levels of genetic diversity can also arise through admixture of divergent gene pools during post-glacial recolonization from different refugia (Petit *et al.*, 2003; Walter & Epperson, 2005). However, this effect is probably limited in *V. album* as our analyses evidenced limited post-glacial gene flow among eastern and western alpine populations or in general among mountainous regions. Hence, our study shows that patterns of genetic diversity expected from migration might not only be obscured by admixture but also by contemporary processes that have increased levels of genetic diversity.

Large population sizes, a predominantly outcrossing mating system, longevity and the potential for long-distance dispersal may additionally maintain within-population genetic diversity and prevent pronounced among-population differentiation (Loveless & Hamrick, 1984). Indeed, *V. album* is a very long-lived and predominantly outcrossing species (Kleijn & Steinger, 2002; Hesse *et al.*, 2008) and the broadly winged seeds are well adapted to wind dispersal. Flowering stems of *V. album* persist into winter still carrying seeds, which, once detached from the plant, can be carried away by wind on the frozen snow cover possibly allowing frequent long-distance dispersal (Matlack, 1989; Greene & Johnson, 1997). Especially during cold periods in the past such long-distance dispersal events may have facilitated fast range expansion, maintained high levels of gene flow, and thus slowed down the process of genetic divergence and drift.

Conclusion and further implications

Our results show that historical migration from an assumed Asian origin is still evident in the current genetic pattern of *V. album*. Climate warming in the Holocene, however, increased isolation of populations among different mountain ranges. Post-glacial vicariance thus may have significantly shaped the current genetic structure in this species while occurrence during the last glaciation in several areas along the periphery of the Alpine ice sheet may have maintained higher genetic diversity in the Alps compared to easternmost and westernmost populations. However, genetic diversity seemed to be influenced by contemporary processes, for example land use. Hence, our results highlight the importance of examining both historical and contemporary processes as drivers of present-day phylogeographic patterns. The differential influence of these processes on genetic structure and diversity, furthermore, underlines the need for a synthetic approach for understanding their relative importance. Biogeographic processes older than Quaternary temperature fluctuations so far have received little attention in phylogeographic studies of European plants, but their imprint may have persisted in the species' genetic pattern (Hampe & Petit, 2007). This may especially apply to species adapted to conditions that prevailed during glaciations, which potentially have maintained more interconnected ranges through most of the Quaternary. Integrating contemporary processes may be especially important for widespread species, such as weeds, which are highly influenced by human activities. We hope that our study will motivate researchers to apply a more synthetic view on this issue by considering historical and contemporary effects simultaneously.

Our results have important implications for ongoing biological control programmes against this widespread weed (Schaffner *et al.*, 2001; Gvritishvili *et al.*, 2006). The pronounced genetic differentiation among the study regions and the finding that certain land uses may not only increase plant density but also genetic diversity implies a need for careful selection of biological control agents that are adapted to their target populations. High within-population genetic diversity may, however, hamper the establishment, spread and thus, population impact of highly specific biological control agents, such as genotype-specific plant pathogens.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Distribution map and pollen records of *Veratrum album* L.

Appendix S2 Ecological and genetic population parameters.

Appendix S3 Individual-based STRUCTURE and population based SAMOVA analyses.

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BIOSKETCHES

Urs A. Treier is an evolutionary ecologist interested in questions related to weed and invasion biology. This study is part of his PhD investigating biogeographic and phylogenetic aspects of weeds and plant invaders.

Heinz Müller-Schärer is Professor of Ecology and Evolution at the University of Fribourg Switzerland with research interests in ecological and evolutionary aspects of plant–antagonist interactions, especially in the context of plant invasion and biological control.

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