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Comparative phylogeography of the *Veronica alpina* complex in Europe and North America

DIRK C. ALBACH,*‡ PETER SCHÖNSWETTER†§ and ANDREAS TRIBSCH*§¶
*Department of Systematic and Evolutionary Botany, Faculty Centre of Botany, University of Vienna, Rennweg 14, A-1030 Vienna, Austria, †Department of Biogeography, Faculty Centre of Botany, University of Vienna, Rennweg 14, A-1030 Vienna, Austria, ‡Institut für Spezielle Botanik, Johannes Gutenberg-Universität Mainz, Bentzelweg 9b, D-55099 Mainz, Germany, §National Centre for Biosystematics (NCB), Natural History Museums, University of Oslo, PO Box 1172 Blindern, N-0318 Oslo, Norway, ¶Department of Organismic Biology, University of Salzburg, Hellbrunnerstrasse 34, A-5020 Salzburg, Austria

Abstract

The *Veronica alpina* complex comprises eight species of alpine habitats over a wide range of mountain systems in the Northern Hemisphere. The occurrence of sympatric species in the European and North American mountain systems allowed us not only to investigate the effect of the ice ages on intraspecific phylogeographical patterns and genetic diversity in different continents of the Northern Hemisphere, but also to compare these patterns in closely related species. Plastid DNA *trnL-F* sequences and AFLP (amplified fragment length polymorphism) fingerprints were used to infer the phylogenetic history of the group and phylogeographical patterns within species. Hybrid origin of tetraploid eastern North American *V. wormskjoldii* from western North American *V. nutans* (= *V. wormskjoldii s.l.*) and Eurasian *V. alpina* is suggested. A number of phylogeographical groups have been found both in *V. alpina* from Europe and in *V. nutans* from western North America. Phylogeographical substructuring in the Alps is inferred for *V. alpina* but not for *V. bellidioides*, which is moreover characterized by an overall very low genetic diversity. Western North American *V. cusickii* is much more genetically diverse than its sympatric relative, *V. nutans*, an effect that is likely due to differences in the breeding system. Populations of *V. nutans* are differentiated into three groups, those from the Cascades and from the southern and the northern Rocky Mountains. Genetic diversity seems to be higher in the North American *V. nutans* than in the morphologically and ecologically similar European *V. alpina*. A possible scenario to explain this pattern is suggested.

Keywords: AFLP, Alps, cpDNA, hybridization, phylogeography, *Veronica*

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Introduction

A major goal of biogeography is to explain the distribution of extant taxa. The field of phylogeography approaches this goal by inferring the historical processes that led to the extant distribution patterns of biota. Phylogeographical analyses of taxa with similar geographical distribution have helped enormously to understand the effect of Pleistocene climate changes on the genetic structure of lowland species in Europe (Hewitt 1996, 2000; Taberlet *et al*. 1998), alpine species in the European Alps (Tribsch & Schönswetter 2003), arctic species (Abbott & Brochmann 2003), species in the American Pacific Northwest (Soltis *et al*. 1997; Brunsfeld *et al*. 2001) or northeastern North America (e.g. Griffin & Barrett 2004; Boys *et al*. 2005). These studies have revealed common patterns of genealogical relationships and contributed to pinpointing glacial refugia of general importance, but many differences among various taxa have also been found (Taberlet *et al*. 1998; Vargas 2003).

Few studies compared taxa that are found in more than one continent and those that did concentrated on the Arctic region (Abbot & Brochmann 2003). We are not aware of any study comparing phylogeographical history and genetic diversity patterns in closely related taxa of the European

Correspondence: Dirk C. Albach, Fax: +49 61313923524; E-mail: albach@uni-mainz.de

alpine regions and the North American Cordilleras in ecologically corresponding habitats. The histories of these taxa and their distributions, however, likely differ because more or less continuous mountain chains in a north–south direction in North America suggest a more limited effect of shifting latitudinal climate changes, whereas European mountain chains are primarily east–west orientated, which implies a more severe effect of climate changes across a latitudinal gradient.

One group that allows such an intercontinental comparison is the *Veronica alpina* complex (Plantaginaceae/Scrophulariaceae *sensu lato*) comprising about eight closely related species distributed throughout the Northern Hemisphere. In anticipation of our results and following Löve (1969), we recognize the western North American plants as *V. nutans* despite the fact that it is almost always treated under *Veronica wormskjoldii*. *Veronica nutans* and its geographically vicariant species, *V. alpina*, are widespread in the western North American and European mountain systems, respectively (Fig. 1). They are morphologically and ecologically similar and were treated as conspecific in earlier times (Römpp 1928; Fernald 1939). The *V. alpina* complex offers the additional chance to compare phylogeographical patterns among closely related sympatric species. This allows us to test whether certain characteristics (e.g. breeding system) distinguishing closely related species led to different responses despite the same climatic history, without other confounding characteristics acquired during long divergence times.

Within the species complex, two species pairs with overlapping distribution areas exist. In Europe, *V. alpina* and *Veronica bellidioides* differ mainly in the wider distribution of the former (Fig. 1), and predominantly higher ploidy level and strictly calcifuge habit of the latter. *Veronica nutans* and *Veronica cusickii* in the Pacific Northwest of North America differ in the more restricted distribution and distinctly larger flowers of *V. cusickii* (Fig. 1). The remaining taxa of the *V. alpina* complex are distributed mostly allopatrically (Fig. 1) in eastern North America and Greenland (*Veronica wormskjoldii sensu stricto*), California (*Veronica*

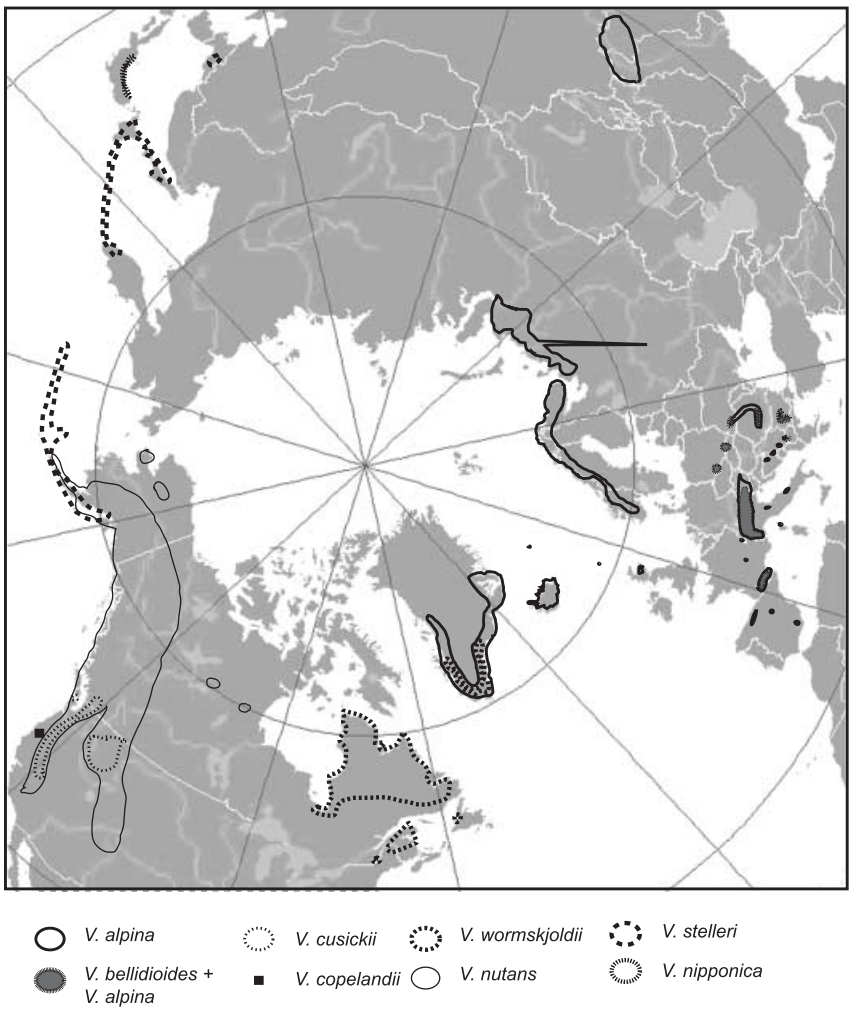

Fig. 1 Distribution of the eight species of the *Veronica alpina* complex.

copelandii), Honshu (*Veronica nipponica*), and between Hokkaido and Alaska (*Veronica stelleri*).

In this study, we use a phylogenetic and phylogeographical approach based on DNA sequence variation in the chloroplast genome and amplified fragment length polymorphisms (AFLP) to address the following questions: (i) What is the phylogenetic history of the *V. alpina* complex? (ii) What are the intraspecific phylogeographical patterns of the species in the complex? (iii) Are there similarities in the phylogeographical patterns among sympatric species? (iv) Are there differences in the patterns in North America and Europe that can be related to different Quaternary histories of the continents?

Materials and methods

Sampling

Samples were obtained almost exclusively from silica-gel-dried field collections. In a few cases, recently collected herbarium specimens (*Veronica alpina* from Pakistan, *Veronica stelleri* from Kuriles, *Veronica wormskjoldii* from Newfoundland) were used. For the analysis of plastid DNA sequence data, 30 individuals were selected, representing all previously recognized taxa in the *V. alpina* complex and a variety of species from the same subgenus, subgenus *Veronica*, as identified in previous broad-scale analyses of the genus (Albach & Chase 2001; Albach *et al*. 2004a, b; Appendix I). GenBank accession numbers are given in Appendix I. For the AFLP analysis, we sampled 244 individuals from 115 populations across the geographical range of the species (Figs 2 and 3). *Veronica urticifolia* and *Veronica baumgartenii* were included in the AFLP study and used as outgroups based on the results of the sequence analysis. Sampling locations for all individuals used for the AFLP fingerprinting are given in Appendix II.

DNA extraction, sequencing and AFLP fingerprinting

Total genomic DNA was extracted from silica-gel-dried leaves or herbarium specimens according to the 2× CTAB procedure of Doyle & Doyle (1987) and then washed twice with 70% ethanol. DNA pellets were dried and resuspended in TE-buffer.

The *trnL* intron, 3′ exon, and *trnL-trnF* spacer (hereafter *trnL-F*) were amplified with primers c and f from Taberlet *et al*. (1991) following the protocol of Albach *et al*. (2004a). Polymerase chain reaction (PCR) products were run on a 1.0% TBE-agarose gel, cut out of the gel, and cleaned using the QIAquick PCR purification and gel extraction kit (QIAGEN) following the manufacturer's protocols. Sequencing reactions (10 µL) were carried out using 1 µL of the BigDye Terminator Cycle Sequencing mix (Applied Biosystems). Reactions were run on an ABI PRISM 377 automated sequencer (Applied Biosystems), and both strands were sequenced. Sequences were assembled and edited using Sequence Navigator (Applied Biosystems). Assembled sequences were manually aligned prior to analysis. The aligned sequence matrix is available from the first author by request.

AFLP profiles were generated following established procedures (Vos *et al*. 1995) and according to the PE Applied Biosystems (1996) protocol with minor modifications (Tremetsberger *et al*. 2003). Genomic DNA (*c*. 500 ng) was digested with *Mse*I (New England BioLabs) and *Eco*RI (Promega) and ligated (T4 DNA-Ligase; Promega) to double-stranded adapters in a thermal cycler for 2 h at 37 °C. Preselective amplification (5-µL reactions) was performed

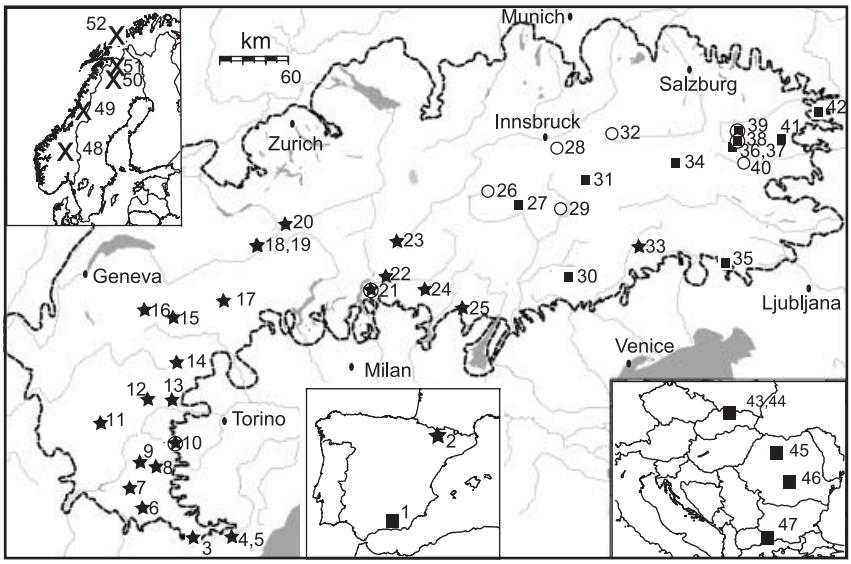

Fig. 2 Geographical distribution of samples from *Veronica alpina* in Europe used in the AFLP analyses grouped according to results of the STRUCTURE analysis. Open circles, B-clade; stars, Western group; squares, Eastern group; crosses, Scandinavian populations. See Appendix II for details.

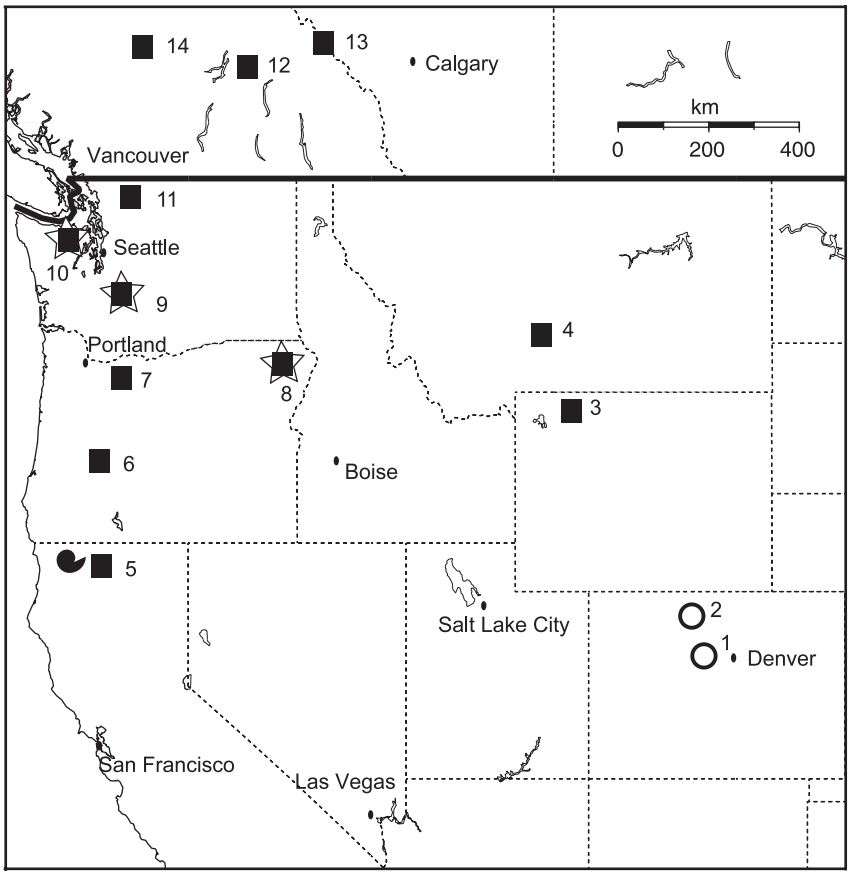

Fig. 3 Geographical distribution of samples from western North American species used in the analyses. Spiral, *Veronica copelandii*; stars, *Veronica cusickii*; squares, northern populations of *Veronica nutans*; circles, southern populations of *V. nutans*. See Appendix II for details.

using primer pairs with a single selective nucleotide, *Mse*I-C and *Eco*RI-A. Selective amplifications were performed with the primer combinations *Mse*I-CAT/*Eco*RI-ACA (5-Fam), *Mse*I-CAA/*Eco*RI-AGG (HEX) and *Mse*I-CAT/*Eco*RI-ACC (NED). Fluorescence labelled selective amplification products were combined and run on a 5% denaturing polyacrylamide gel with an internal size standard [GeneScan 500 (ROX); PE Applied Biosystems] on an automated DNA sequencer (ABI PRISM 377). The generated AFLP profiles were analysed using the ABI PRISM GENESCAN 2.1 Analysis Software (PE Applied Biosystems) and GENOGRAPHER 1.6 (Benham 1998). Fragments were scored as present or absent from 50 to 500 bp and used to construct a presence/absence matrix.

Data analysis

The aligned *trnL-F* sequence matrix was analysed in PAUP 4.0b (Swofford 2002) using parsimony in three heuristic searches (10 replicates) starting from a random tree and tree-bisection–reconnection (TBR) branch-swapping. Support for the branches was estimated using the bootstrap procedure, using the same options as above and 500 replicates.

The AFLP matrix was analysed using neighbour joining based on Nei & Li (1979) distances as implemented in PAUP 4.0b (Swofford 2002). Additionally, 10 runs of parsimony analyses were conducted in PAUP 4.0b (Swofford 2002) using equal weights, starting from random, TBR and a tree limit of 2000 trees. Support for the branches was estimated using the bootstrap procedure, using the same options as above and 1000 replicates. A principal coordinate analysis (PCoA) based on Dice genetic distances (equivalent to Nei–Li distances) was conducted using R4.0 (Casgrain & Legendre 2001). Average gene diversity over loci and analyses of molecular variance (AMOVA) were computed with ARLEQUIN 2.0 (Schneider *et al*. 2000). Average gene diversity over loci was measured for *V. alpina*, *V. bellidioides*, *V. copelandii*, *V. cusickii*, and *V. nutans* as a whole ('species-wide') and for some phylogroups (Table 1). Additionally, the number of fixed private (f_{fp}) and polymorphic private (f_{pp}) fragments was estimated for species and major phylogeographical groups. AFLP fragments were regarded as fixed when they occurred in all investigated individuals of a respective group, and as private fragments when they were restricted to a group. Only markers present in more than one sample have been considered as private marker to reduce the possible impact of genotyping errors (Bonin *et al*. 2004).

In order to detect groupings among the 107 *V. alpina* samples with an independent approach, we applied a model-based genetic structure analysis implemented in the

Table 1 Number of sampled individuals (N_i); number of populations (N_p); number of AFLP fragments scored; average gene diversity over loci for the species and for major intraspecific phylogeographical groups; number of fixed private fragments (f_{fp}); and number of polymorphic private fragments (f_{pp}) of the investigated species of the *Veronica alpina* group

Species	N_i	N_p	Number of fragments	Average gene diversity over loci	f_{fp}	f_{pp}
V. alpine (main clade/B-clade)	107 (89/18)	53 (48/9)	134.6 ± 6.2	0.069 ± 0.034 (0.057 ± 0.028/ 0.055 ± 0.028)	1 (0/1)	116 (55/23)
V. bellidioides (Bulgaria/ Alps and Pyrenees)	68 (6/62)	30 (3/27)	207.1 ± 3.6	0.032 ± 0.016 (0.019 ± 0.011/ 0.014 ± 0.007)	27 (16/22)	64 (6/11)
V. copelandii	3	1	145.3 ± 3.5	0.024 ± 0.018	7	1
V. cusickii (Rocky Mountains/Cascades)	11 (4/7)	3 (1/2)	148.1 ± 8.5	0.081 ± 0.042 (0.056 ± 0.037/ 0.066 ± 0.037)	0 (3/0)	28 (10/9)
V. nutans (Cascades/northern Rocky Mountains/southern Rocky Mountains)	1 (23/12/6)	14 (8/5/2)	146.3 ± 10.3	0.091 ± 0.044 (0.061 ± 0.030/ 0.055 ± 0.029/ 0.035 ± 0.021)	0 (2/1/7)	59 (18/6/13)
V. nipponica	1	1	141		4	n.a.
V. stelleri	4	4	141.0 ± 21.9		1	7
V. wormskjoldii	3	3	151.0 ± 13.5		—	—

program STRUCTURE (Pritchard *et al*. 2000). This program aims at delineating clusters of individuals on the basis of their genotypes at multiple loci using a Bayesian approach. The model assumes the presence of Hardy–Weinberg equilibrium by introducing population structure and tries to find population groupings that are not in disequilibrium (Pritchard *et al*. 2000; Evanno *et al*. 2005). As suggested in the manual (Pritchard & Wen 2004), the 'No Admixture' model was used because AFLP are dominant markers, and independence of allele frequencies among populations was assumed (Semerikov & Lascoux 2003). Assuming Hardy–Weinberg equilibrium, the number of 'populations' (clusters), K, was estimated by a Markov chain Monte Carlo (MCMC) algorithm implemented in the program. The burn-in period was set to 5×10^4, the number of MCMC replicates after burn-in to 10^5. K was set from 2 to 10. In order to test stability of the results, all runs were replicated three times and runs from $K = 3$ to $K = 7$ six times. Ideally, a comparison of the replicates and the probability of data $\Pr(X \mid K)$ for each value of K allows the estimation of the point of inflection and the more likely numbers of clusters (Evanno *et al*. 2005).

Results

DNA sequences

The aligned data matrix of the *trnL-F* region for 30 individuals comprised 918 characters, 78 of which were variable and 34 potentially parsimony-informative. Multiple heuristic searches found four most parsimonious trees of 84 steps (Fig. 4; CI = RI = 0.94). The strict consensus of the four trees retrieves the *Veronica alpina* complex in three clades, which form a polytomy with *V. urticifolia* and *V. baumgartenii* (59% BS = bootstrap support). The first clade includes all samples of *V. alpina* and *V. wormskjoldii* from eastern North America (63% BS), whereas the second clade includes the western North American and East Asian species (81% BS) and the third is comprised of *V. bellidioides*.

AFLP fingerprinting

Altogether, 898 fragments were scored with 22 present in all 240 individuals. The number of AFLP fragments per taxon is not significantly different for all taxa (Table 1) except for the tetraploid *V. bellidioides*, which has approximately 30% more fragments than the other taxa. AFLP from herbarium specimen did not prove to be problematic and individuals were found at expected places without abnormal branch lengths or distances. However, the position of the single accession of *V. alpina* from Pakistan should be considered carefully.

Neighbour-joining and parsimony analyses retrieved similar optimal phylogenetic trees. The parsimony analyses with 746 potentially parsimony-informative characters found most parsimonious trees with 4029 steps (CI = 0.22; RI = 0.86; result not shown). However, not all most parsimonious trees were found because all searches reached the tree limit of 2000 most parsimonious trees. Neighbour-joining (Fig. 5) and principal coordinate analyses (Fig. 6) distinguished three main groups — *V. alpina*, *V. bellidioides*, and the circum-

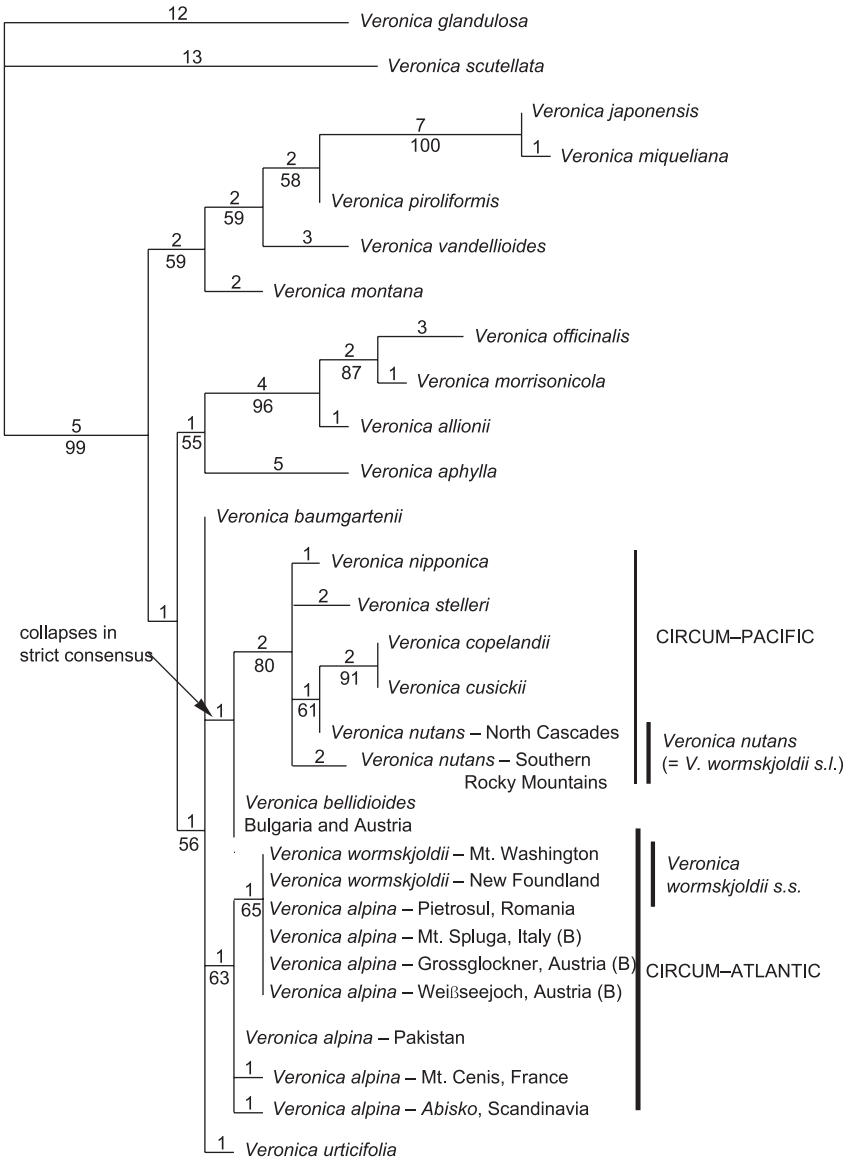

Fig. 4 One of four most parsimonious trees of the analysis of the *trnL-F* sequence data set. Numbers above the branches indicate branch lengths, those below the branches bootstrap support values. (B) indicates samples from the *Veronica alpina* B-clade.

Pacific group. *V. wormskjoldii* is either intermediate between *V. alpina* and the circum-Pacific group (principal coordinate analysis, Fig. 6), or sister to the circum-Pacific group (Fig. 5). Both the monophyly of *V. bellidioides* (98–100% BS) and that of *V. alpina* are strongly supported (99–100% BS) by the neighbour-joining and parsimony analyses. Monophyly of the circum-Pacific group is moderately (88% BS) supported by the bootstrap analysis using neighbour joining but not using parsimony.

Within the three main groups, several subgroups were found. Within the Pacific group, all species are well supported (93–100% BS) as monophyletic with the exception of *V. nutans*, which consists of two well-supported phylogroups (100% BS) comprising the samples from Colorado and those from the rest of the distribution area (Fig. 5A). Within *V. bellidioides*, we found two highly distinct sister groups with 100% BS in all analyses (Fig. 5B), one including the six Bulgarian samples and the other the 62 samples from the Alps and Pyrenees. Resolution within both groups is very low. Within *V. alpina*, we also found two unequal sister groups [100% BS for the smaller and 84% (parsimony) or 94% (neighbour joining) for the larger group], although there is no clear geographical distinction. The smaller group of samples from the Alps is here called 'B-clade', whereas the larger group is called the 'main clade'.

In the STRUCTURE analysis including the 107 individuals from *V. alpina* only, identical groupings among the replicated runs were obtained with $K = 2$–4. At $K = 2$ the B-clade and the remaining samples were indicated as the major split in the species, while a 'Western group' and an 'Eastern group'

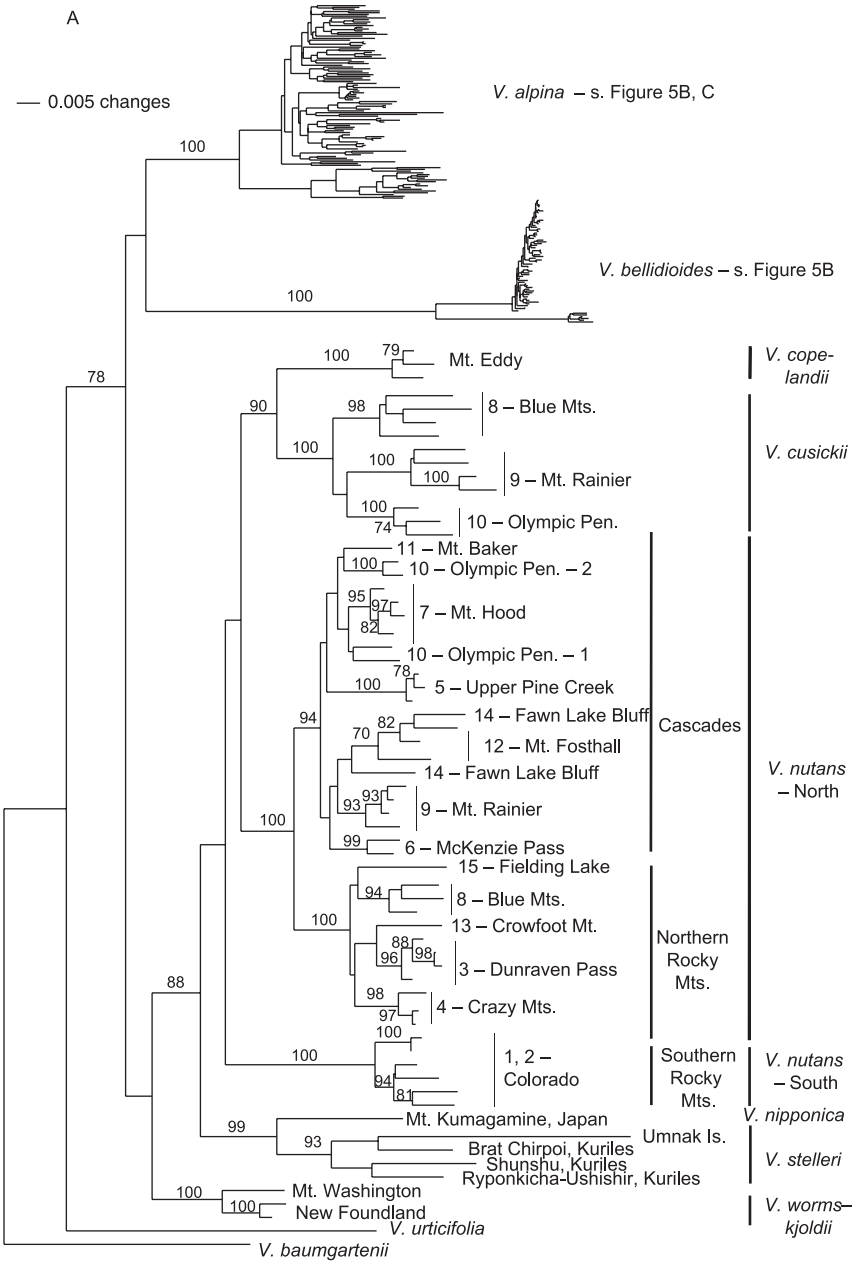

Fig. 5 Tree derived from neighbour-joining analysis of AFLP fingerprints. Numbers above the branches are bootstrap support values. (A) outgroup and North American species; (B) *Veronica bellidioides* and *Veronica alpina* B-clade; (C) *V. alpina* main clade. The three groups obtained with STRUCTURE within the main clade are indicated with double vertical lines.

were separated at $K = 3$, and the Scandinavian samples were obtained as a fourth group at $K = 4$ (see below for details). Only very few samples were admixed. Groupings obtained with $K > 4$ were not repeatable between different runs, although the estimated probability of the data slightly improved with higher K. Moreover, from $K > 4$ the variation of the probability among the replicates increased suggesting instability among the replicates and empty groups were obtained frequently, a phenomenon that happens when K is higher than the number of 'real' groups. Thus, the point of inflection was clearly at $K = 4$. The geographical distribution of the four groups as defined by STRUCTURE is given in Fig. 2. These groups are the 10 Scandinavian samples (populations 48–52; no private marker, 99% BS in the neighbour-joining analysis; Fig. 5C), the second comprises samples from the Western Alps and Pyrenees ('Western group'; populations 2–25, 33; seven private markers albeit without BS support of more than 70% in the neighbour-joining analysis), and a third group ('Eastern group') that is not monophyletic in phylogenetic analyses including samples from the Carpathians and Bulgaria (populations 43–47; three private markers, 94% BS for 43–46), the Eastern Alps (populations 27, 30, 31, 34–39, 41, 42; 11 private markers) and the Sierra Nevada

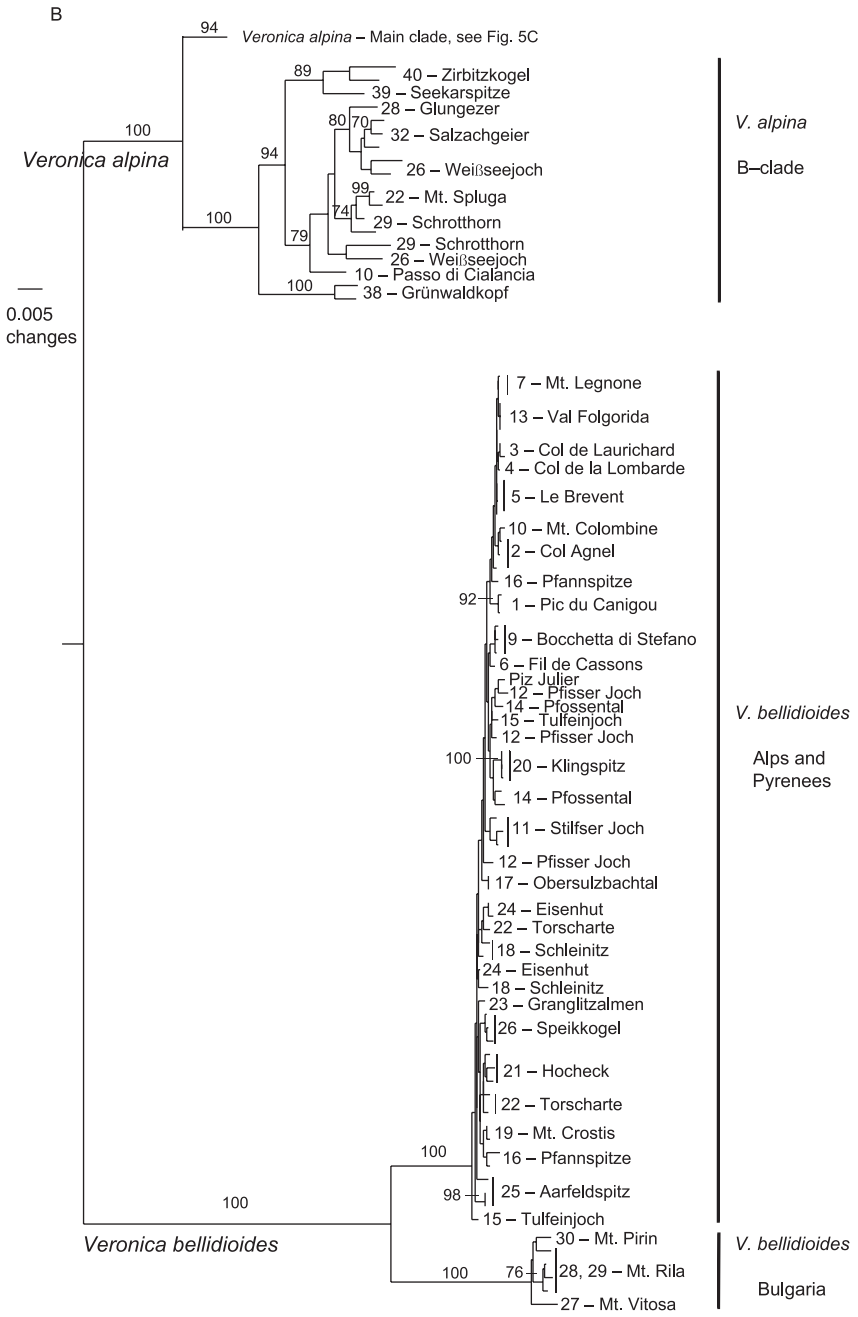

Fig. 5 *Continued*

(population 1; one private marker, 100% BS). The sample from Pakistan was the only sample that could not be grouped consistently in the STRUCTURE analysis and was either sister to the rest of the main clade (parsimony analysis; not shown) or sister to the Bulgarian and then to the Eastern European samples (neighbour joining; Fig. 5C).

Species-wide intraspecific diversity measured as average gene diversity over loci as well as the partitioning of the overall genetic variation in the AMOVA differs strongly among the species (Tables 1 and 2). However, sampling in V. *nipponica*, *V. stelleri*, and *V. wormskjoldii* is insufficient to allow any inference. Average gene diversity over loci is low in *V. bellidioides* as compared to *V. alpina* (0.032 vs. 0.069; Table 1), especially when considering the Bulgarian samples as separate (Table 1). Analyses of molecular variance (Table 2) show the high degree of distinction between the Bulgarian and the Alpine plus Pyrenean samples of *V. bellidioides*. Species-wide genetic diversity is highest in *V. cusickii* despite the small sample size in this species (0.081; Table 1) and intrapopulation variation accounts for a higher proportion of the overall genetic variation than in any other species (48.6%; Table 2). Genetic

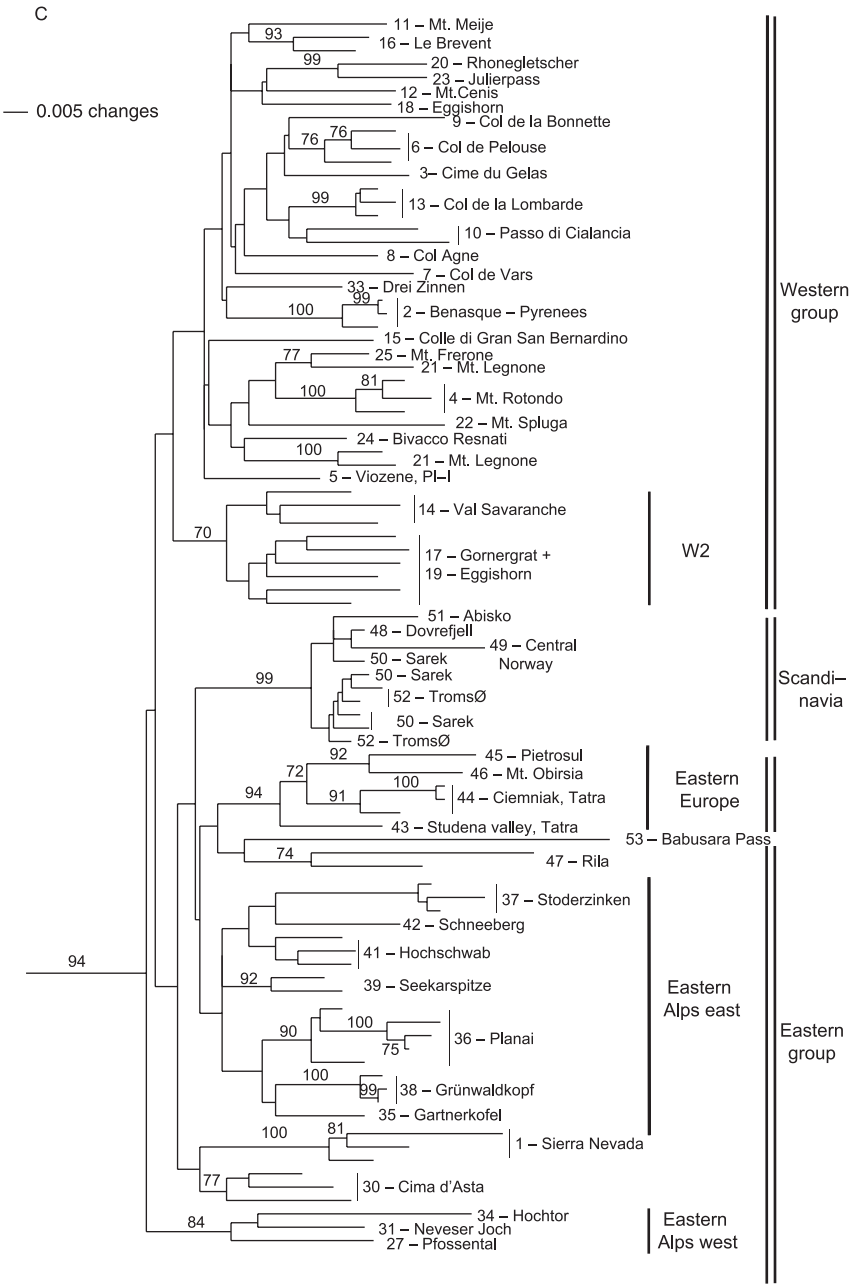

Fig. 5 *Continued*

Table 2 Analyses of molecular variance (AMOVAS) of four species of the *Veronica alpina* group. Populations were either grouped according to the results of the phylogenetic analyses (*Veronica bellidioides*, *Veronica nutans*, *Veronica cusickii*; Fig. 5) or the STRUCTURE analysis (*V. alpina*). The following major phylogeographical groups were differentiated: *V. alpina*: B-clade, Western group, Eastern group + Pakistan; *V. bellidioides*: Bulgaria, Alps-Pyrenees; *V. nutans*: southern Rocky Mountains, northern Rocky Mountains, Cascades; *V. cusickii*: Rocky Mountains, Cascades

	V. alpina	*V. bellidioides*	*V. nutans*	*V. cusickii*
Among major phylogeographical groups	39.8%	88.5%	47.4%	9.3%
Among populations within major phylogeographical groups	36.3%	6.7%	29.8%	42.1%
Within populations	23.9%	4.7%	22.8%	48.6%

All P values were < 0.001.

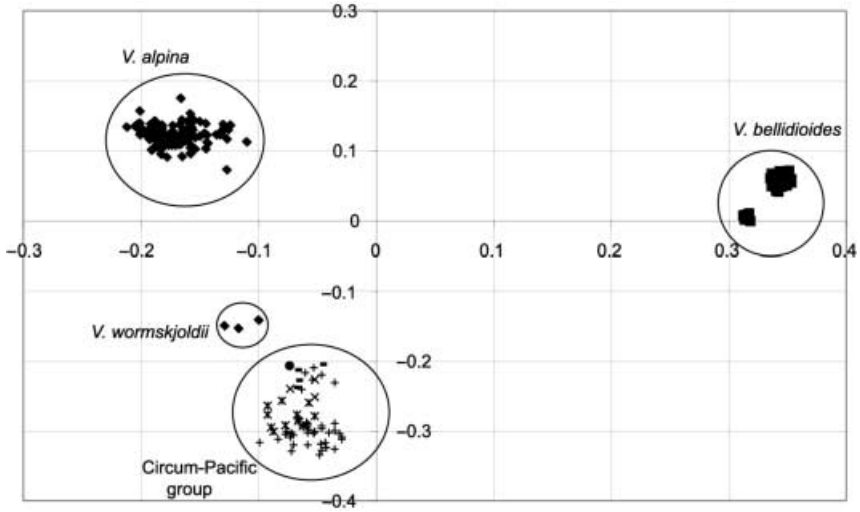

Fig. 6 Graph showing the results of the principal coordinate analysis of AFLP data with the *x*-axis explaining 25.7% and the *y*-axis 15.3% of the total variation. Symbols in circum-Pacific group: ●, *Veronica nipponica*; –, *V. stelleri*; ×, *V. copelandii*; ✳, *V. cusickii*; +, *V. nutans*.

diversity of *V. nutans* is in the range of that of *V. alpina* or slightly higher (Table 1).

Discussion

Species phylogeny of the Veronica alpina *complex*

The results presented here suggest a major division in the *V. alpina* complex between the Eurasian and the amphi-Pacific species (Figs 4 and 5). Such a diversification pattern seems typical for many circumpolar organisms (Abbott *et al*. 2000; Brochmann *et al*. 2003; Holderegger & Abbott 2003). An origin of the North American species from Eurasian ancestors is most likely based on the fact that all relatives are Eurasian. However, it is unclear whether the ancestors entered North America from Asia or from Europe. An argument in favour of a European origin is that all relatives seem to be restricted to Europe, such as *Veronica alpina* (with the exception of the isolated populations in Pakistan), *V. bellidioides*, *V. baumgartenii* and *V. urticifolia*. However, comparing cpDNA divergence in the group with that for plants in general (Hewitt 2000) reveals that the species complex is of Pleistocene origin. It is thus too young to have been able to disperse via a North Atlantic land bridge, which played a role for plant migration until at most the Miocene (Tiffney & Manchester 2001). Therefore, the group must have spread across the Atlantic if it arrived from the east. On the other side, an Asian origin seems to be common for North American alpine species (Weber 2003; Schneeweiss *et al*. 2004) and is a possible alternative here. Detailed phylogeographical analysis of populations in Alaska, Greenland or other parts of the Arctic may uncover genotypes that may help to answer this question.

The results support the recognition of eight species, *V. alpina*, *V. bellidioides*, *V. copelandii*, *V. cusickii*, *V. nipponica*, *V. nutans*, *V. stelleri* and *V. wormskjoldii*, as morphologically, geographically and genetically distinct units. An exception to the criterion of morphological distinctness is presented by *V. nutans* and *V. wormskjoldii* but outweighed by the sharp genetic separation. Furthermore, *V. wormskjoldii* is tetraploid (e.g. Böcher & Larsen 1950), whereas *V. nutans* is diploid (e.g. Mulligan in Löve 1970). Based on the intermediate position of eastern North American *V. wormskjoldii* between western North American *V. nutans* and European *V. alpina* in the AFLP analyses (PCoA, Fig. 6) and the grouping with *V. alpina* in the analysis of cpDNA sequence data (Fig. 4), we hypothesize that the tetraploid *V. wormskjoldii* originated from hybridization of diploid *V. nutans* and *V. alpina* with the latter being the maternal parent. A further inspection of AFLP band sharing supports the hypothesis. *V. wormskjoldii* shares 85% of its bands with *V. nutans* (including nine bands exclusive to these two species) and 80% of them with *V. alpina* (including six private bands) compared with 40–60% sharing of bands between other species. Such a scenario would necessarily involve a former more easterly distribution of *V. nutans* in the North American Arctic with subsequent extinction in this area.

An exception to the criterion of genetic distinctness is again *V. nutans*, which is paraphyletic with respect to *V. copelandii* and *V. cusickii* — albeit without bootstrap support of more than 50% (Fig. 5A). However, much larger flowers, a differently coloured corolla and differences in the fruit morphology support the further recognition of the latter two species, whereas no character could be found to morphologically distinguish the two groups of *V. nutans* (southern vs. northern Rocky Mountains).

The two tetraploid species, *V. wormskjoldii* and *V. bellidioides*, appear to have originated at very different time horizons. In *V. wormskjoldii*, the number of AFLP markers is not significantly different from its proposed diploid ancestors and no private fragment was scored. This suggests a recent

origin from genetically and genomically similar parents. *V. bellidioides*, however, has significantly more fragments per individual than all other members of the *V. alpina* complex and a large number of private fragments (Table 1). This may indicate either a more ancient origin or an origin from divergent diploid ancestors. However, our analysis does not suggest possible ancestors.

Phylogeographical pattern in Europe

Veronica alpina is widely distributed in Europe (Figs 1 and 2). Populations occur disjunctly in mountain regions that are well separated by lowland habitats uninhabitable by *V. alpina*. To our knowledge, fossil records of *V. alpina* are lacking. Its close habitat association with palaeoecological key species like *Salix herbacea*, however, allows assuming a more continuous distribution of *V. alpina* in large parts of Northern and Central Europe during cold periods of the Pleistocene. *S. herbacea* is well documented in the fossil record and had been fairly widespread in periglacial areas during cold periods (Lang 1994).

Despite the probably wider distribution in Pleistocene times that suggests mixing of populations and their gene pools, four clearly distinct genetic groups were retrieved that are supported by all analyses (Figs 2 and 5), i.e. the B-clade, a western Alpine-Pyrenean group (populations 2–25, 33), an eastern Alpine-Carpathian group including the sample from the Sierra Nevada (populations 1, 27, 30, 31, 34–39, 41–47) and the Scandinavian samples nested within the previous group in phylogenetic analyses (Fig. 5C). With the exception of the Scandinavian group, all groups show many private markers. A genetically clearly distinct group that was hitherto unexpected is the enigmatic B-clade that is sister to the remaining samples of *V. alpina* (Figs 1 and 2). These samples are morphologically not distinct and sometimes even occur in mixed populations with samples from the main group of *V. alpina* (Fig. 2). There is also some evidence that B-clade individuals occur in the Southern Carpathians, because one sample clustered with the B-clade samples in an initial analysis. This sample, however, was excluded from the final analysis because of poor profile quality. We excluded the possibility that the B-clade is a technical artefact by processing all samples in parallel, and samples of the B-clade have been included in different rounds of extraction, amplification and on different gels. They neither differ in number nor clarity of the AFLP fragments and are reproducible with all primer combinations. Furthermore, the results have been reproduced with a subset in a second laboratory (D. C. Albach & M. Fay, unpublished).

Although the B-clade is genetically clearly distinct and characterized by many private markers, the samples do not form a geographically coherent group. We cannot offer a sound interpretation of the pattern found, but it is likely that this group emerged in geographical isolation from other populations. How the integrity is maintained in mixed populations (e.g. populations 10, 22, 38, 39; Fig. 2) is unclear. The small size of the flowers suggests that inbreeding prevails in *V. alpina*. As no genetically intermediate individuals between the main clade and the B-clade were detected, it seems even likely that strong (pre- or postzygotic) crossing barriers exist. This does not exclude the possibility that we would have found such intermediates with a higher sample size. A genetically heterogeneous population was previously found in *Comastoma tenellum* (Schönswetter *et al*. 2004) in which selfing also likely prevents the mixing of the different genotypes.

The major split in the main clade of *V. alpina* results in a west–east differentiation in the Alps (Fig. 5C), approximately following the Etsch valley. Generally, differentiation of western and eastern phylogeographical groups seems to prevail in alpine plants of the European Alps (e.g. Comes & Kadereit 2003; Schönswetter 2005), and weakly supported subdivisions in the Alps generally agree with results from previous phylogeographical studies in these areas (Schönswetter 2005). Individuals of *V. alpina* from the Pyrenees are nested within samples from the Western Alps (Fig. 5C). This pattern was already found in *Phyteuma globulariifolium* (Schönswetter *et al*. 2002), whereas other studies found highly divergent genotypes in the Pyrenees originating before the diversification in the Alps took place (Konnert & Bergmann 1995; Zhang *et al*. 2001; Kropf *et al*. 2002, 2003). The samples from southern Spain form a distinct group within the samples from the 'Eastern group' but none of the relationships is supported by any of the bootstrap analyses or the STRUCTURE results. It is noteworthy that none of the analyses identified a connection between the samples from the Sierra Nevada (southern Spain) and the Pyrenees (northern Spain), a connection found in several other alpine plants in Mediterranean Europe (Vargas 2003).

The Scandinavian samples are nested as a group within the Eastern Alpine and Carpathians samples almost without having private markers. Moreover, this group is genetically depauperate (average gene diversity over loci 0.016 ± 0.009; see also Fig. 5C). In spite of our limited sampling in Northern Europe, the results suggest postglacial colonization of Northern Europe out of a refugium in the Eastern Alps or Carpathians, although palaeoenvironmental data suggest that potential habitats for *V. alpina* were available in the lowlands south of the Scandinavian ice shield (see above). Colonization of Scandinavia from the Carpathians (Després *et al*. 2003) or Eastern Alpine source populations (Schönswetter *et al*. 2003) has previously been demonstrated.

Veronica alpina and *V. bellidioides* co-occur over most of the distribution area of the latter, and both taxa grow in alpine habitats at similar elevations. It would thus be

plausible to assume that they also share the same history. Both species show marked intraspecific differences in nuclear markers (Fig. 5) but little variation in plastid DNA sequences (Fig. 4). Our analyses demonstrate that the history of the two species must have been different. A deep split between the Bulgarian populations and the Alpine and Pyrenean accessions of *V. bellidioides* (Fig. 5B; Table 2) combined with the presence of several private markers in both groups (Table 1) suggests old vicariance. The reduced level of genetic diversity could be a result of historical bottlenecks in isolated refugia and/or bottlenecks during early phases of postglacial colonization. Together with the high number of fixed markers, the low genetic diversity (Table 1) in *V. bellidioides* indicates that the species seems to have been able to disperse from a bottlenecked population without an increase in genetic variation. Amsellem *et al*. (2000) argued that successful colonizers need both ideally be able to reproduce asexually (or at least without an obligate partner) and have a high ploidy level. Both requirements are met in *V. bellidioides*, a self-compatible tetraploid. Within the group of samples from the Alps and Pyrenees, the neighbour-joining tree reveals some underlying pattern (Fig. 5B). More western populations in the Alps and those from the Pyrenees seem to be derived from relatively more variable refugial populations of the Eastern Alps but, because of the low overall genetic variation in *V. bellidioides*, support for this pattern is weak.

Therefore, genetic data for *V. bellidioides* do not offer evidence for more than one refugium in the Alps, possibly in the Eastern Alps (Fig. 5B). In contrast, higher genetic diversity and a stronger phylogeographical structure in *V. alpina* (Tables 1 and 2) suggest at least three once isolated sources from which the current gene pool in the Alps was generated. These different patterns may be explained by slightly different ecological requirements. Guisan & Theurillat (2000) showed by ecological simulations that, despite occurring in proximate habitats, *V. alpina* is favoured by cooler temperatures allowing it to expand its distribution area during glacials, whereas *V. bellidioides* benefits from warmer climates and may have been restricted to smaller and fewer populations during the cold stages of the Pleistocene.

Phylogeographical patterns in North America

Veronica nutans is widespread across western North America. The analyses revealed three separate groups within this species. Populations from the southern Rocky Mountains (Colorado) are strongly differentiated from accessions from the northern Rocky Mountains and the Cascades that formed highly supported sister groups (Fig. 5A). Thus, there seems to have been a vicariant event that has caused a phylogeographical break between the southern and northern Rocky Mountains across the Great Divide Basin in eastern south-central Wyoming (Wyoming Basin). The corridor is only 150 km wide but marked by lower elevation and, probably more importantly, lower precipitation (< 350 mm/year). The Wyoming Basin has previously been shown to act as a barrier to gene flow for other alpine organisms (Noonan 2001; DeChaine & Martin 2004, 2005a, b).

Veronica nutans populations that inhabit the northern Rocky Mountains and the Cascades are strongly supported as monophyletic (Fig. 5A). The division between these two mountain ranges represents the most basal split in this group, which coincides with results from other phylogeographical analyses (Brunsfeld *et al*. 2001; Carstens *et al*. 2005). This pattern indicates that populations in the two mountain ranges have been separated for a long time, in line with the 'ancient vicariance hypothesis' *sensu* Brunsfeld *et al*. (2001).

The two groups of *V. nutans* ('Cascades' and 'northern Rocky Mountains') meet today in British Columbia but this seems to be a secondary contact zone because samples from the two groups in British Columbia are not closely related (Fig. 5A — populations 12–14). The sample from Alaska is nested within samples from the northern Rocky Mountains, suggesting that *V. nutans* has colonized Alaska from the south after the last glaciation. Additional sampling, however, would be necessary to exclude the possibility that *V. nutans* survived the last glaciation in the Beringian refugium as many other species (Abbott & Brochmann 2003).

Veronica cusickii occurs in the Cascades and a small part of the Rocky Mountains in Idaho and adjacent Oregon and Montana. In spite of the more restricted occurrence in the Rocky Mountains and the low number of sampled populations, *V. cusickii* appears to show the same separation between samples from the Cascades and the Rocky Mountains as observed in *V. nutans*. *V. nutans* and *V. cusickii* differ considerably in intrapopulation diversity with *V. cusickii* showing higher average gene diversity within populations (0.040 vs. 0.026). *V. cusickii* has flowers that are approximately three times larger than those of *V. nutans* and its stamens are far spreading. Thus, despite being self-compatible (Pojar 1974), *V. cusickii* most likely has a much higher outcrossing rate than *V. nutans*. Outcrossers are well-known to harbour more intrapopulation diversity than selfers (Hamrick & Godt 1997), which probably explains the higher proportion of genetic variation present within populations (Table 2).

Sampling in *V. wormskjoldii* is insufficient to allow inference of its geographical origin and Pleistocene refugia. The presumed diploid parental species (*V. alpina*, *V. nutans*; see above) currently do not have overlapping distribution areas. Information from pollen and macrofossils (Ritchie 1992; Delcourt & Delcourt 1993; Jackson *et al*. 2000) and DNA-based analyses of other alpine species (Tremblay & Schoen 1999) allow hypothesizing that *V. wormskjoldii* had a wider distribution south and southeast of the Laurentian ice sheet.

Moreover, potential refugia for arctic plants were present in Newfoundland and the northeastern USA (Brochmann *et al*. 2003). After the Pleistocene, *V. wormskjoldii* likely became extinct in the south except for a few scattered occurrences in mountain areas that have long been hypothesized to be Pleistocene refugia (e.g. Fernald 1925; Spear 2000).

Phylogeographical comparison — North America and Europe

The present study offers the opportunity to compare phylogeographical and genetical diversity patterns of closely related plant species in North America and Europe. Such an intercontinental comparison based on a limited sampling of populations and individuals per population is difficult, but allows some cautious conclusions. Overall diversity is higher in western North America as estimated by cpDNA diversity (Fig. 4) and average gene diversity based on AFLP data (Table 1), most pronounced in the genetically depauperate *V. bellidioides* in Europe and the genetically most diverse *V. cusickii* in North America. Also, in a direct comparison of the morphologically almost indistinguishable *V. nutans* and *V. alpina*, the North American species is slightly more variable (Table 1) and shows a stronger phylogeographical structure as indicated by higher bootstrap support for intraspecific nodes (Fig. 5). These results, however, should be taken with caution, because three main factors might have a strong influence: (i) the differences in the sampling strategy of the two species in our study; (ii) differences in the actual ecological factors limiting the present distributions; and (iii) differences in historic distributions and climate history. These factors are almost impossible to disentangle. We regard historic differences as a likely explanation for such a pattern. DeChaine & Martin (2005a) suggested that high genetic variation is caused by repeated cycles of isolation on mountain tops in warm interglacials and reconnection during the glacials. Consequently, our favoured hypothesis is that *V. nutans* retained higher genetic diversity than *V. alpina* by a less dramatic contraction and extinction period on many isolated mountain tops during the interglacial periods and limitation of gene flow among refugia in glacial periods. Furthermore, genetic diversity was retained in three major mountain areas (Cascades, northern and southern Rocky Mountains) with the southern Rocky Mountains experiencing the strongest contraction and isolation in interglacials and therefore having less genetic diversity (Table 1). Such a scenario is supported by the distribution of alpine habitats in the Rocky Mountains in glacials and interglacials (DeChaine & Martin 2005b). In contrast, *V. alpina* may have been widespread at lower elevations north, east and west of the Alps during glacial times (cf. Lang 1994) allowing for gene flow of *V. alpina* across Europe (e.g. from the Alps to the Pyrenees). Outside the Alps, population contraction appears to have been more severe, and even population sizes in the glacials might have been rather small for alpine plants (Schönswetter *et al*. 2005). Therefore, the Alps may have been the only major mountain area harbouring genetic diversity during interglacials for *V. alpina* rather than three for *V. nutans* in North America. This would correspond to a source-sink model of gene flow across Europe in *V. alpina* but three mostly separated subgroups with more or less equal retention of genetic diversity in North American *V. nutans*.

Limited gene flow among refugia at lower elevations in North America during the interglacials is apparent from the separation of *V. nutans* by the arid intermountain region and the Wyoming Basin (see above) and the stronger separation of the major phylogeographical groups in the AMOVA (Table 2). However, more detailed analyses are necessary to test this hypothesis. One inference of this hypothesis is that genetic differentiation should be higher and intrapopulation diversity lower in the southern than in the northern Rocky Mountains because possible refugia were more numerous and smaller in the south, and potential for gene flow in glacials was higher in the north (DeChaine & Martin 2005b). This is only partially supported by our data (Table 1), which may be an artifact of our limited sampling, especially in the southern Rocky Mountains. It is more clearly supported by data for two alpine butterflies (DeChaine & Martin 2005b). A second inference is that the genetic diversity of all populations in Southern Europe and the Carpathians (and not only in Scandinavia) should be smaller and be derived from the Alps. At least the latter is supported by our analysis (Fig. 5C). However, a more complete sampling in both Europe and North America would be necessary to rigorously test this hypothesis.

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Appendix I

Species name, origin, voucher information and GenBank accession number of accessions of *Veronica* used in the *trnL-F* data set

V. abyssinica — cult. BG Bonn, ex Rwanda (E. Fischer 8060, WU — AF513350)
V. allionii — cult. RBG Kew, ex Italy (Chase s. n., K — AF513348)
V. alpina — Grossglockner road, Austria (Albach 207, WU — AY847154)
V. alpina — Weißseejoch, Austria (Schönswetter & Tribsch 9741, WU — AY847156)
V. alpina — Mont Cenis, France (Albach 184, WU — AF486387)
V. alpina — Monte Spluga, Italy (Schönswetter & Tribsch 9484, WU — AY847155)
V. alpina — Babusara Pass, Pakistan (Dickoré 12864, GOET — AY847150)
V. alpina — Pietrosul, Romania (Comes s. n., WU — AY847153)
V. alpina — Abisko NP, Sweden (Benson 1999.08, UPS — AF511483/4)
V. aphylla — Passo di Rolle, Italy (Zhang s. n., WU — AF513349)
V. baumgartenii — Mt. Midjur, Bulgaria (Albach 542, WU — AY780808)
V. bellidioides — cult. RBG Kew, ex Austria (Albach 118, K — AF513345)
V. bellidioides — Mt. Rila, Bulgaria (Albach 563, WU — DQ232750)
V. copelandii — Mt. Eddy, California, USA (Janeway 6557, WU — AF513344)
V. cusickii — Blue Mts., Oregon, USA (Albach 288, WU — AY486443)
V. glandulosa — cult. BG Bonn, ex Kenya (E. Fischer 713/98, WU — AF486394)
V. japonensis — Tanzawa Mts. NP, Japan (Stuessy *et al*. 17270, WU — AF486392)
V. miqueliana — near Nango, Japan (Stuessy *et al*. 17225, WU — AY486444)
V. montana — Oberkassel, Germany (Albach 151, WU — AF486388)
V. morrisonicola — cult. RBG Kew, ex Taiwan (Kirkham & Flanagan 1060, K — AF513347)
V. nipponica — Mt. Kumagamine, Japan (Horii 20401, WU — AY776286)
V. nutans — Mt. Baker, Washington, USA (Olmstead 99–180, WTU — AF511481/2)
V. nutans — near Caribou, Colorado, USA (Lederer s. n., WU — DQ232749)
V. officinalis — cult. RBG Kew, ex UK (Albach 114, K — AF486391)
V. piroliformis — Yunnan, China (Dickoré 14146, GOET — AF486390)
V. stelleri — Brat Chirpoi, Kuriles, Russia (Gage 4627, WTU — AY847149)
V. urticifolia — cult. BG Bonn, ex Austria (Albach 73, WU — AF486389)
V. vandellioides — Sichuan, China (Dickoré 8417, GOET — AY776287)
V. wormskjoldii — Newfoundland, Canada (Brouillet 99–149, MT — AY847152)
V. wormskjoldii — Mt. Washington, New Hampshire, USA (Albach 217, WU — AY847151)

Appendix II

Geographical origin of samples and number of investigated individuals per population (N_i) of the *Veronica alpina* group used in the AFLP analysis. Longitudes are east if not otherwise indicated

Population number	Population name	N_i	Location	Coordinates (Long/Lat)
V. alpina				
1	Sierra Nevada	3	Spain — Sierra Nevada	W3.2/37.1
2	Benasque	3	Spain — Pyrenees	0.3/42.4
3	Cime du Gelas	1	France	7.22/44.07
4	Mt. Rotondo	3	Italy	7.47/44.08
5	Viozene	1	Italy	7.47/44.08
6	Col de Pelouse	3	France	6.51/44.21
7	Col de Vars	1	France	6.42/44.32
8	Col Agnel	1	France	6.59/44.41
9	Col de la Bonnette	1	France	6.48/44.44
10	Passo di Cialancia	3	Italy	7.07/44.52
11	Mt. Meije	1	France	6.23/45.02
12	Mt. Cenis	1	France	6.56/45.13
13	Col de la Lombarde	3	Italy	7.09/45.13
14	Val Savaranche	3	Italy	7.12/45.32
15	Colle di Gran San Bernardino	1	Italy	7.09/45.52
16	Le Brevent	2	France	6.51/45.56
17	Gornergrat	3	Switzerland	7.48/45.59
18	Eggishorn-1	1	Switzerland	8.06/46.26

Appendix II *Continued*

Population number	Population name	N_i	Location	Coordinates (Long/Lat)
19	Eggishorn-2	3	Switzerland	8.06/46.26
20	Rhonegletscher	1	Switzerland	8.2/46.4
21	Mt. Legnone	3	Italy	9.25/46.06
22	Mt. Spluga	3	Italy	9.34/46.11
23	Julierpass	1	Switzerland	9.43/46.28
24	Bivacco Resnati	1	Italy	10.00/46.05
25	Mt. Frerone	1	Italy	10.26/45.57
26	Weißseejoch	3	Austria	10.42/46.52
27	Pfossental	1	Italy	11.02/46.45
28	Glungezer	1	Austria	11.31/47.12
29	Schrotthorn	3	Italy	11.33/46.44
30	Cima d'Asta	3	Italy	11.36/46.11
31	Neveser Joch	1	Italy	11.49/46.57
32	Salzachgeier	3	Austria	12.07/47.18
33	Drei Zinnen	1	Italy	12.3/46.6
34	Hochtor	1	Austria	12.51/47.05
35	Gartnerkofel	1	Austria	13.4/46.3
36	Planai	5	Austria	13.5/47.2
37	Stoderzinken	3	Austria	13.5/47.2
38	Grünwaldkopf	5	Austria	13.32/47.15
39	Seekarspitze	3	Austria	13.33/47.17
40	Zirbitzkogel	2	Austria	14.34/47.04
41	Hochschwab	3	Austria	15.1/47.2
42	Schneeberg	1	Austria	15.5/47.5
43	Studena valley, Tatra	1	Slovakia	20.1/49.1
44	Ciemniak, Tatra	3	Poland	19.54/49.14
45	Pietrosul	1	Romania	24.2/47.2
46	Mt. Obirsia	1	Romania	25.3/45.2
47	Rila	2	Bulgaria	23.22/41.48
48	Dovrefjell	1	Norway	10/62
49	Central Norway	1	Norway	14/65
50	Sarek	4	Sweden	18/67
51	Abisko	1	Sweden	19/68
52	Tromsø	3	Norway	19/70
53	Babusara Pass	1	Pakistan	74.1/35.5
V. baumgartenii				
	Midjur	1	Bulgaria	22.40/43.24
V. bellidioides				
1	Pic du Canigou	2	France — Pyrenees	2.26/42.31
2	Col Agnel	3	Italy	6.59/44.41
3	Col de Laurichard	2	France — Alps	6.24/45.06
4	Col de la Lombarde	1	Italy	7.09/45.13
5	Le Brevent	3	France — Alps	6.51/45.56
6	Fil de Cassons	1	Switzerland	9.16/46.53
7	Mt. Legnone	2	Italy	9.25/46.06
8	Alpe Suvretta	1	Switzerland	9.46/46.30
9	Bocchetta di Stefano	3	Italy	9.57/46.7
10	Mt. Colombine	1	Italy	10.22/45.51
11	Stilfser Joch	3	Italy	10.27/46.31
12	Pfisser Joch	3	Austria	10.35/47.05
13	Val Folgorida	3	Italy	10.37/46.10
14	Pfossental	3	Italy	11.02/46.45
15	Tulfeinjoch	2	Austria	11.32/47.13
16	Pfannspitze	3	Italy	11.43/46.41
17	Obersulzbachtal	2	Austria	12.18/47.08
18	Schleinitz	3	Austria	12.45/46.54
19	Mt. Crostis	2	Italy	12.54/46.34

Appendix II *Continued*

Population number	Population name	N_i	Location	Coordinates (Long/Lat)
20	Klingspitz	3	Austria	12.58/47.21
21	Hocheck	3	Austria	13.24/46.53
22	Torscharte	3	Austria	13.32/47.01
23	Granglitzalmen	1	Austria	13.47/47.13
24	Eisenhut	3	Austria	13.56/46.57
25	Aarfeldspitz	3	Austria	14.06/47.16
26	Speikkogel	3	Austria	15.03/47.14
27	Vitosa	1	Bulgaria	23.17/42.35
28	Rila-2	1	Bulgaria	23.35/42.15
29	Rila-1	3	Bulgaria	23.34/42.14
30	Pirin	1	Bulgaria	23.25/41.45
V. copelandii				
	Mt. Eddy	3	USA — California	W122.29/41.19
V. cusickii				
8	Blue Mts.	4	USA — Oregon	W117.25/45.17
9	Mt. Rainier	4	USA — Washington	W121.31/46.53
10	Hurricane Ridge, Olympic Peninsula	3	USA — Washington	W123.29/47.59
V. nipponica				
	Hokkaido-Kumagamine	1	Japan	139/37
V. nutans				
1	near Caribou	3	USA — Colorado	W105.2/39.4
2	Arapaho Pass	3	USA — Colorado	W106.1/40.1
3	Dunraven Pass	4	USA — Wyoming	W110.27/44.47
4	Crazy Mts.	3	USA — Montana	W110.2/46.1
5	Upper Pine Creek	3	USA — California	W121.09/40.32
6	McKenzie Pass	2	USA — Oregon	W121.15/44.25
7	Mt. Hood	5	USA — Oregon	W121.40/45.20
8	Blue Mts.	3	USA — Oregon	W117.25/45.17
9	Mt. Rainier	4	USA — Washington	W121.31/46.53
10	Hurricane Ridge, Olympic Peninsula	3	USA — Washington	W123.29/47.59
11	Mt. Baker	1	USA — Washington	W121.3/48.3
12	Mt. Fosthall	3	Canada — British Columbia	W118.15/50.29
13	Crowfoot Mts.	1	Canada — British Columbia	W119.09/51.01
14	Fawn Lake Bluff	2	Canada — British Columbia	W121.1/51.3
15	Fielding Lake	1	USA — Alaska	W145.2/63.1
V. stelleri				
	Brat Chirpoi	1	Russia — Kuriles	150.48/46.28
	Ryponkicha	1	Russia — Kuriles	152.50/47.32
	Shumshu	1	Russia — Kuriles	156.29/50.49
	Umnak Island	1	USA — Alaska	W167.48/53.28
V. urticifolia				
	cult. BG Bonn	1	unknown	
V. wormskjoldii				
1	Lewis Hills-1	1	Canada — Newfoundland	W58.28/48.49
2	Lewis Hills-2	1	Canada — Newfoundland	W58.31/48.51
3	Mt. Washington	1	USA — New Hampshire	W71.17/44.16