RESEARCH ARTICLE

Altai Mountains – cradle of hybrids and introgressants: A case study in *Veronica* subg. *Pseudolysimachium* (Plantaginaceae)

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Abstract Mountains form a diverse mosaic of microhabitats over small distances created by changes in climate, soil, and water availability. A key to adaptation of plants to such microhabitats is genetic variation; however, natural accumulation of genetic variation through mutation is slow and often not sufficient alone. Adaptive introgression via hybridization is an alternative to generate genetic variation. Here, we investigate hybridization and discuss its adaptive role in *Veronica* subg. *Pseudolysimachium* at their Altai Mountains distribution. To support our hypotheses of frequent hybridization, we genotyped thousands of SNPs for 233 individuals from 10 species and 7 putative hybrids previously described based on morphology. We employed Bayesian and likelihood statistical models and supported our results by morphometric analysis and genomic *in situ* hybridization (GISH). The results suggest that almost all the individuals of the putative hybrids are of F1 type. The GISH investigation in one case strongly supports homoploid hybridization (origin of *V.* ×*schmakovii* from *V. longifolia* and *V. porphyriana*. Divergence times of Altai *Veronica* species are estimated to be within 1–2 million years ago with high probability of gene flow over that time. Our results also demonstrate that the direction of gene flow is mainly from the locally endemic *V. porphyriana*. We hypothesize that the large Siberian plains and topographically diverse foreland of the Altai Mountains provide an ideal setting for hybridization with the potential for adaptive introgression of alleles conferring tolerance to cooler climates, to the lowland species migrating into the Altai Mountains.

Keywords adaptive introgression; Altai Mountains; hybridization; species complex; *Veronica*

Supporting Information may be found online in the Supporting Information section at the end of the article.

■ INTRODUCTION

The exceptional diversity of mountains has been a topic of intense study since the early work of Humboldt, Darwin, and Wallace. Recently, the works of Fjeldså & al. (2012), Badgley & al. (2017), Antonelli & al. (2018), and Rahbek & al. (2019) proposed that high diversity in mountains is due to the instability of mountain systems and significant changes in their landscape/topology in response to different forces over a short geological time period. In addition, they suggested that mountain substrates, life forms, and climate interact at a range of spatial scales, leading to establish diverse, distinct, and challenging microhabitats. These studies, together with the biogeographical importance of mountains, led to different characterizations of mountains, i.e., mountains as cradles, as innovation hubs, as elevators, corridors, barriers, reservoirs, refugia, museums, sinks and graves. From this diversity of terms, it becomes obvious that mountains influenced species idiosyncratically through different evolutionary processes (Rahbek & al., 2019). However, "what are the processes leading to this increased biodiversity in mountains?" continues to puzzle researchers (Stein & al., 2014). Studies of diverse lineages (species and populations) adapted to mountains are necessary to disentangle taxon-specific and general factors allowing them to exploit new, challenging habitats (Wiens & al., 2006).

High genetic diversity is a prerequisite to deal with rapidly changing and challenging habitats (Dobzhansky, 1937; Mayr, 1942) despite the fact that many island and likely mountain radiations seem to have been founded by few colonizers (Silvertown, 2004; Hughes & Atchison, 2015). Since mutations (the ultimate source of genetic variation) occurring at a slow rate are unlikely to offer enough variability for populations, hybridization and adaptive introgression offer an alternative to populations facing environmental challenges (Dobzhansky, 1937; Hamilton & Miller, 2016; Vallejo-Marín & Hiscock, 2016). In recent years, hybridization has been recognized more and more

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as a creative force in evolution allowing adaptation and speciation (Arnold, 1996; Mallet, 2007; Abbott & al., 2013) and even the most enigmatic examples of radiations have been demonstrated to be caused by hybridization and introgression (Farrington & al., 2014). Hybridization allows species to rapidly adapt and cope with the stressful and harsh environment at their range edges. However, the important challenge is to understand hybridization's role as a process leading either towards adaptation and speciation or towards merger and loss of biodiversity (Hoffmann & Sgrò, 2011).

Different studies have previously demonstrated that interspecies hybridization has increased due to global climate change providing many cases of adaptive introgression. However, hybridization is also a potential threat to species subsuming rare species or collapsing multiple species into a single hybrid swarm (see details in Taylor & al., 2005; Oliveira & al., 2008; Todesco & al., 2016). The weakening of ecologically mediated reproductive barriers is central to this loss of biodiversity (Vonlanthen & al., 2012; Chunco, 2014). Owens & Samuk (2020) expanded on this and found that introgression can readily weaken the ecologically mediated reproductive barriers. They also confirmed that introgression facilitates homogenization of alleles involved in reproductive isolation. Therefore, the study of patterns of hybridization and introgression is not only important to understand evolution but also for conservation of biodiversity.

The Altai Mountains range is characterized by at least 2700 species and diverse habitats, from the alpine zone to highly scattered mountain forests, mountain steppes to lowland desert steppe and sparse riparian forests in the valleys (Kamelin, 2005). These mountains cover an area of 550,000 km^2 in central Asia from Russia, through Mongolia and Kazakhstan to China with an average altitude of 2000 m, receiving annual precipitation of 120 mm to 800 mm (Kamelin, 2005). The flora of the Altai Mountains lies at the crossroads of three different floras, i.e., Boreal Euro-Siberian elements, Steppe elements and Ancient Mediterranean elements (Kamelin, 2005). Besides these floristic elements, the Altai Mountains are also inhabited by at least 265 endemic vascular plant species (Olonova & al., 2010; Batlai & al., 2017). These characteristics, different vegetation types, high endemism and different floristic elements make it an attractive biogeographical system. Hybridization is a widespread phenomenon in the Altai Mountains, especially in many genera notorious for hybridization (see examples in Kamelin, 2005; Shmakov, 2005; Elisafenko, 2012; Rodionov & al., 2015; Nosov & al., 2017; Tzvelev & Probatova, 2019). However, a general assessment of hybridization in the Altai flora is still not available. Ebel (2012) provided some quantitative evaluation and found more than 50 interspecific hybrids from the northwestern part of the Altai-Sayan region, which comprises just about 2%–3% of the entire Altai flora.

Here, we focus on *Veronica* subg. *Pseudolysimachium* as a model to assess the importance of hybridization on biodiversity of the Altai Mountains. This subgenus has ~30 species distributed over Eurasia from Japan to the United Kingdom, with wide ecological amplitudes from dry semi-deserts to aquatic habitats, and some well-known ornamental species, e.g., *V. spicata* and *V. longifolia* (Albach & al., 2008; Kosachev & al., 2016). Hosting 10 different species of *V.* subg. *Pseudolysimachium* and numerous hybrids of intermediate morphology, the Altai Mountains are the most species-rich region for the subgenus (Kosachev, 2003, 2017; Kosachev & German, 2004; Kosachev & Ebel, 2010; Kosachev & al., 2013). Hypotheses of frequent hybridization in *V.* subg. *Pseudolysimachium*, in general, are so far based mostly on morphological studies (starting with Härle, 1932) and few or anonymous molecular markers (Bardy & al., 2011; Kosachev & al., 2016, 2019). The widespread species include *V. incana* (up to 2000 m above sea level [a.s.l.]), *V. longifolia* (300 m to 3200 m a.s.l.), *V. pinnata* (300 to 1500 m a.s.l.), *V. porphyriana* (300 to 3200 m a.s.l.), *V. spicata* (up to 500 m a.s.l.) and *V. spuria* (up to 400 m a.s.l.). Among these, *V. spicata* and *V. spuria* reach the eastern margin of their Western Eurasian steppe distribution, *V. pinnata* the northwestern margin of its semi-desert distribution, *V. longifolia* and *V. incana* the southern margin of their Euro-Siberian semi-aquatic and steppe distribution area, respectively, whereas *V. porphyriana* is restricted to Central Asian mountains (Kosachev, 2017).

Besides frequent hybridization, polyploidy is also prominent in the subgenus. Albach & al. (2008) reported that about 45% of the species in the subgenus are diploid and 55% are tetraploid, with eight species being purely diploid, two tetraploid and seven mixoploid. Frequent occurrence of hybridization and polyploidization in combination with large morphological variability (as well as interest in them as ornamental plants) led to the publication of more than 600 valid names for the ~30 taxa in this subgenus (Albach & al., 2017). Nevertheless, the importance of hybridization and polyploidization to speciation in the subgenus was perceived differently by different researchers, i.e., Graze (1935) considered hybridization as the likely explanation for morphological variation in the subgenus, whereas Fischer (1974) disagreed and considered intraspecific cytotype variation as the likely source. Therefore, here, we use *Veronica* subg. *Pseudolysimachium* in the Altai Mountains to test the hypothesis that mountains are a cradle of diversity due to hybridization of species co-occurring in a mosaic of microhabitats typical for mountainous regions. We sampled 233 individuals of the subgenus across its Altai Mountains' distribution and genotyped 7430 bi-allelic unlinked SNPs (single nucleotide polymorphisms) to support our hypotheses. We used different statistical toolkits to assess species delimitation in Altai *Veronica* and evidence of hybridization. In addition, we inferred demographic parameters including effective population size, divergence time and gene flow.

■ MATERIALS AND METHODS

Sampling. — Fresh leaf samples of 233 wild adult individuals were collected during field excursions between 2012 and 2018, complemented with a few well-preserved herbarium specimens (herbaria ALTB, KW, and OLD). The sampling strategy was based on four criteria; to represent all

taxonomic entities of *Veronica* subg. *Pseudolysimachium* from the Altai Mountains; to include more individuals from the species with a wider distribution in the Altai Mountains; to focus on populations growing side-by-side with putative hybrids; and to collect representatives of all putative hybrids (suppl. Fig. S1; Table 1, suppl. Table S1). Altogether, our sampling included 58 putative hybrid individuals (from 7 different morphotypes) and 172 individuals from the putative parent species based on morphology (10 species in total; detailed map in Fig. 1). During the excursions, we kept all samples in silica gel to dry, tagged with locality and georeferencing information, reference numbers and morphotypes (suppl. Table S1). All samples were stored in cold and dark environment after collection until total genomic DNA extraction. Vouchers for all specimens were deposited in ALTB, KW, and OLD. All samples were identified to species except three samples, included as unidentified (suppl. Table S1).

Genomic *in situ* hybridization (GISH). — Mitotic chromosome spreads of *Veronica incana* (*Albach S629*), *V. longifolia* (*Albach S625*), *V. pinnata* (*Albach S630*), *V. porphyriana* (*Albach S626*), *V. ×schmakovii* (*Pfanzelt 1181*) and *V. spicata* (*Albach S628*) were prepared from root tips as described by Mandáková & Lysak (2016a). Chromosome preparations were treated with 100 µg/ml RNase in 2× sodium saline citrate (SSC; 20× SSC: 3 M sodium chloride, 300 mM trisodium citrate, pH 7.0) for 60 min and with 0.1 mg/ml pepsin in 0.01 M HCl at 37°C for 5 min; then postfixed in 4% formaldehyde in 2× SSC for 10 min, washed in 2× SSC twice for 5 min, and dehydrated in an ethanol series (70%, 90%, and 100%, 2 min each). After chromosome preparations, we used the BAC clone T15P10 (AF167571) of *Arabidopsis thaliana* bearing 35S rRNA gene repeats for *in situ* localization of nucleolar organizer regions (NORs), and the *A. thaliana* clone pCT 4.2 (M65137), corresponding to a 500 bp 5S rRNA repeat, to localize 5S rDNA loci. For GISH in *V. ×schmakovii*, total genomic DNA (gDNA) of *V. longifolia* and *V. porphyriana* was extracted from healthy young leaves using the NucleoSpin Plant II kit (Macherey-Nagel, Düren, Germany). All DNA probes were labeled with biotin-dUTP or digoxigenin-dUTP by nick translation as described in Mandáková & Lysak (2016b). For *in situ* hybridization, selected labeled DNA probes were pooled, ethanol precipitated, dissolved in a 20 µl mixture of 50% formamide, 10% dextran sulfate, and 2× SSC, and pipetted onto each of the microscopic slides. The slides were heated to 80°C for 2 min and incubated at 37°C overnight. The hybridized probes were visualized by fluorescently labeled antibodies against biotin-dUTP and digoxigenin-dUTP as in Mandáková & Lysak (2016b). Chromosomes were counterstained with 4′,6-diamidino-2-phenylindole (DAPI, 2 µg/ml) in Vectashield antifade. Fluorescence signals were analyzed and

Table 1. Details of sampled morphotypes and their geographical distribution.

Serial no.	Species	Distribution	Latitude	Longitude	Number of individuals
1	*V. ×altaica*	Russia	50.9158	82.3274	12
2	*V. ×grisea*	Russia	50.6399	86.3131	9
3	*V. ×kolyvanensis*	Russia	51.7684	82.1381	6
4	*V. ×sapozhnikovii*	Mongolia			1
5	*V. ×schmakovii*	Russia	50.1567	88.2953	11
6	*V. ×sessiliflora*	Russia	50.3437	87.4315	13
7	*V. ×smirnovii*	Mongolia	46.3533	91.2095	6
8	*V. arenosa*	Mongolia			3
9a	*V. incana*	Russia	50.6461	86.3144	10
9b	*V. incana*	Russia	51.3924	82.2084	24
10	*V. longifolia*	Russia	53.3346	84.2004	27
11	*V. pinnata*	Russia	50.3501	87.4125	22
12	*V. porphyriana*	Russia	51.0431	85.6399	36
13	*V. reverdattoi*	Russia	50.4940	91.3311	1
14	*V. sajanensis*	Russia	56.1262	92.9057	2
15	*V. spicata*	Russia	50.3605	82.2448	37
16	*V. spuria*	Russia	51.7684	82.1381	7
17	*V. taigischensis*	Russia	53.0584	93.3399	3
TOTAL	17 morphotypes (10 taxonomically described pure forms; 7 taxonomically described putative hybrids forms; 3 individuals were not identified, they are listed in suppl. Table S1)				

photographed using a Zeiss Axioimager epifluorescence microscope and a CoolCube camera (MetaSystems). The individual images were merged and processed using Photoshop CS software (Adobe Systems).

DNA extraction and library preparation for high-throughput sequencing. — To extract total genomic DNA, we used an innuPREP Plant DNA extraction kit (Analytik Jena, Jena, Germany) following the manufacturer's instructions. We used NanoDrop spectrophotometer (ThermoFisher Scientific, Waltham, Massachusetts, U.S.A.) and Qubit dsDNA BR Assay Kit (ThermoFisher Scientific) with a Qubit 3.0 Fluorometer (Life Technologies, Carlsbad, California, U.S.A.) to check the quality and quantity of the extracted DNA, respectively, for all the samples.

We prepared genomic DNA libraries for high-throughput sequencing following (Siadjeu & al., 2018). Briefly, 200 ng of total genomic DNA from each sample were included in restriction digestion with one Unit *Msl*I (New England Biolabs, Ipswich, Massachusetts, U.S.A.), which is not methylation-sensitive. This reaction was carried out using 1× NEB4 buffer in 30 µl (reaction volume) for 1 h at 37°C and heat inactivated at 80°C for 20 min. Restriction digestion was followed by adaptor ligation. We transferred 15 µl of digested DNA to 96-well PCR plate, mixed with 3 µl of 192 L2 ligation adaptors (Ovation Rapid DR Multiplex System, Tecan, Leek, The Netherlands), and 12 µl master mix (4.6 µl D1 water, 6 µl L1 ligation buffer mix, and 1.5 µl L3 ligation enzyme). The ligation reaction was incubated at 25°C for 15 min and

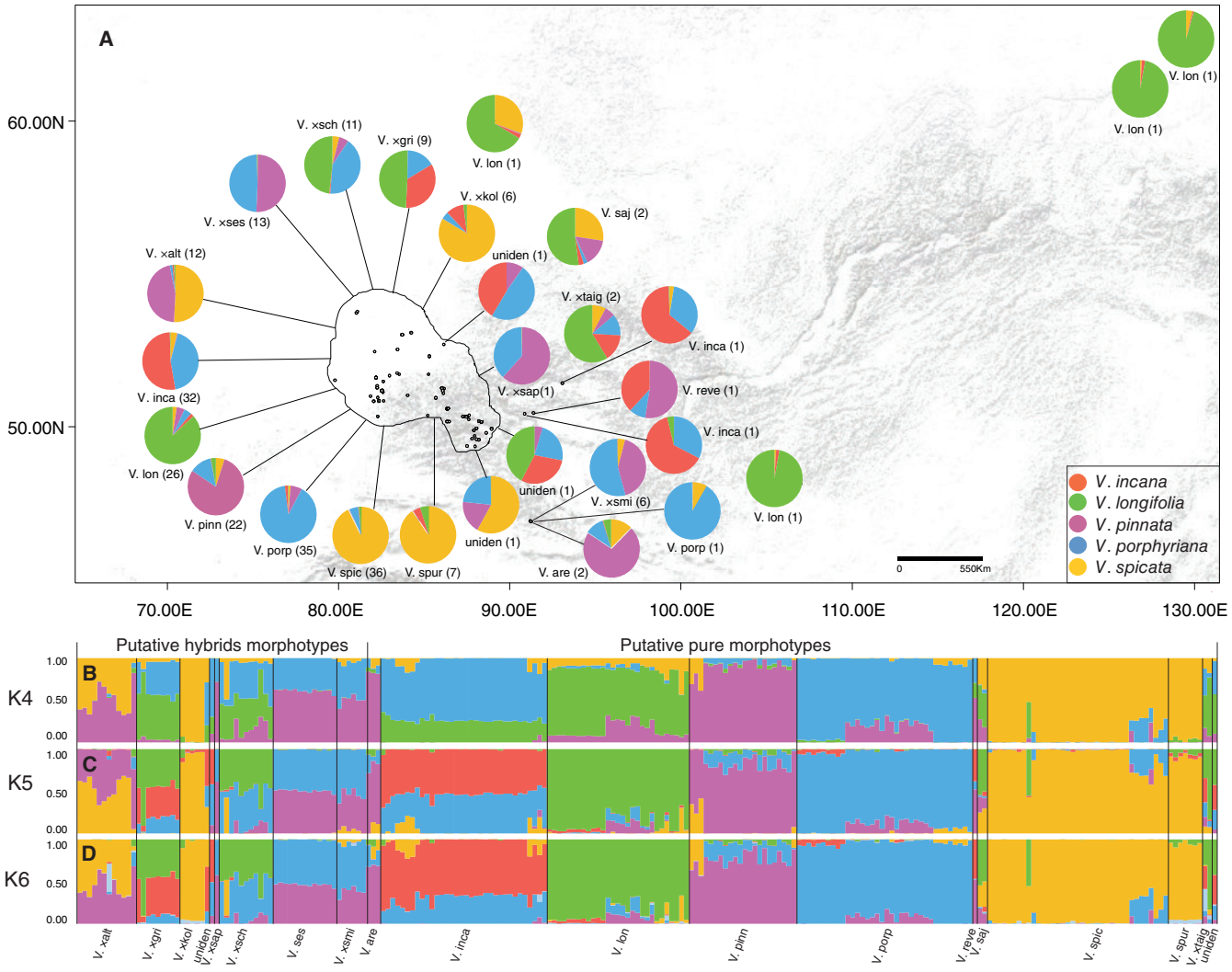

Fig. 1. A, Genetic composition based on $K = 5$ of first-level STRUCTURE results and number of individuals included from that particular locality. Genetic composition of the localities with more than one individual has been averaged. Key to the colors is given in a separate inset. **B–D**, Probability of ancestry of each individual (horizontal axis; total 233 individuals) to each of $K = 4, 5, 6$ populations (vertical axis). The five populations correspond mostly to the morphotypes hypothesized for species and putative hybrids. V. ×alt, *V. ×altaica*; V. ×gri, *V. ×grisea*; V. ×kol, *V. ×kolyvanensis*; V. ×sap, *V. ×sapozhnikovii*; V. ×sch, *V. ×schmakovii*; V. ×ses, *V. ×sessiliflora*; V. ×smi, *V. ×smirnovii*; V. are, *V. arenosa*; V. inca, *V. incana*; V. lon, *V. longifolia*; V. pinn, *V. pinnata*; V. porp, *V. porphyriana*; V. reve, *V. reverdattoi*; V. saj, *V. sajanensis*; V. spic, *V. spicata*; V. spur, *V. spuria*; V. ×taig, *V. ×taigischensis*; uniden, unidentified.

heat inactivated at 65°C for 10 min. To the ligation product of the first reaction, we added 20 µl of the "final repair" master mix and heated it at 72°C for 3 min. Ligation reaction was followed by purification with magnetic beads (beads protocol). The purified ligated products for each individual were amplified by PCR (10 cycles) using 4 µl of 5× MyTaq buffer (Bioline), 0.2 µl polymerase and 1 µl (10 pmol/µl) of standard Illumina TrueSeq amplification primers. Total volume of the PCR was 20 µl with 10 µl of ligation product. To remove small fragments from each individual library, all amplicons were purified again with magnetic beads (beads protocol). Finally, the purified individual libraries were normalized and pooled. We included an additional purification step to remove the PCR polymerase through Qiagen MinElute Columns (beads protocol) from the pooled library, and reactions were sent to LGC Genomics (Berlin, Germany) for sequencing. The sequencing was carried out on an Illumina NextSeq500 using Illumina V2 Chemistry (Illumina, Berlin, Germany). The size of the library was kept between 300 and 400 bp. We used paired-end (PE) sequencing to maximize the chance of distinguishing homologs from paralogs. Both the forward and reverse reads were followed for 150 bp, resulting in 300 bp.

Sequencing quality, stacking reads into loci and SNPs calling. — We used GBS-SNP-CROP to generate polyploid-aware bi-allelic SNPs (Melo & al., 2016). GBS-SNP-CROP is explicitly designed for sample sets including individuals of varying ploidy levels and has the potential to genotype bi-allelic SNPs and exclude the multi-allelic variants by imposing a population-level allele frequency filter via a user-defined "Alternative Allele Strength" parameter. For each potential SNP position, this parameter considers the total read depth, across the whole population, of all four bases, from primary (the allele with the highest depth at that position) to quaternary (the allele with the lowest depth). A potential SNP is retained for further downstream analysis if and only if it is strongly bi-allelic. The GBS-SNP-CROP workflow first processes the raw GBS data to exclude the sequences with noise/bad quality; secondly builds a mock reference (if reference genome is not available); maps the high-quality reads to generate standardized alignment files; and lastly, calls the SNPs. The pipeline has seven Perl scripts utilizing VSEARCH and PEAR for clustering and merging of paired-end reads respectively (Zhang & al., 2014; Rognes & al., 2016). To make a mock reference we used only the diploid individuals (confirmed by flow cytometry) with a high number of reads after quality filtering (suppl. Table S1). At the end we got a total of 233,987 SNPs with 99.9999% confidence, which means 0.000001 error rate and Alternative Allele Strength parameter 0.90. For phylogeny estimation we used all these 233,987 SNPs with 75% missing data allowance before the post-processing step in VCFtools v.3.0. For STRUCTURE analyses, we only used the unlinked bi-allelic SNPs allowing every SNP to be present again in at least 75% of the individuals with minor allele frequency equal to 0.05 utilizing VCFtools (Danecek & al., 2011). For assigning individuals to respective hybrid classes, we applied the avg-PIC (polymorphism information content averaged over the reference and alternate SNPs) to get 200 highly informative SNPs (examples see in Georges & al., 2018; Buono & al., 2021; Baiakhmetov & al., 2021). For this purpose, we first converted the VCF file to genlight in vcfR and then in dartR retrieved the 200 SNPs (Knaus & Grünwald, 2018; Gruber & Georges, 2019) using the script from Buono & al. (2021). For the demographic analyses carried out in G-PhoCS v.1.2.3 (Generalized Phylogenetic Coalescent Sampler), we used the Stacks v.2.41 (Catchen & al., 2013) pipeline (detailed bioinformatics processing can be found in suppl. Appendix S1). This dataset included only three individuals per species from the pure parents without any missing data of K5 (316 loci). These individuals were selected based on genetic clustering after assessing the species boundaries in Altai *Veronica* (details are in the following section). We submitted the data to an open data publishing platform, Dryad (DOI: 10.5061/dryad.gb5mkkwmg)

Assessment of species boundaries in Altai *Veronica*. — We performed different analyses to distinguish pure individuals from admixed individuals (of different combinations of species). Initially, we included all 233 individuals (suppl. Table S2) in a Bayesian modelling approach in STRUCTURE v.2.3.4 (Pritchard & al., 2000) by considering them as one metapopulation. STRUCTURE infers the presence of distinct genetic populations (here considered species) by assigning individuals to a number (K) of genetic clusters. We used STRUCTURE without any predefined spatial or genetic population information to avoid any a priori bias (Janes & al., 2017) encountered in F_{ST}-based assessments (Wright, 1951; Weir & Cockerham, 1984; Balloux & Lugon-Moulin, 2002). We used the admixture model assuming correlated allele frequencies after an initial burn-in of 10% generations followed by 500,000 MCMC (Markov chain Monte Carlo) generations. The alpha and F_{ST} values were uniform after the first 100,000 MCMC generations. For each K the analysis ran for 10 iterations in which K was set from 1 to 8. The analysis was based on 233 individuals and 7340 SNPs (details above). The best K was determined based on the Ln P(D)-values reaching a plateau (Pritchard & al., 2000) and using Evanno ΔK statistics (Evanno & al., 2005) as implemented in STRUCTURE HARVESTER (Earl & vonHoldt, 2012). We clumped results of the best K in CLUMPP v.1.1.2 (Jakobsson & Rosenberg, 2007) and displayed in an Excel spreadsheet (order and individuals' membership in suppl. Table S2). In subsequent analyses, we excluded putative pure hybrid individuals and one unidentified individual with clear admixture from the main dataset (233 individuals). This reduced the dataset to 174 individuals. The run was carried out in the same fashion (K = 1, 2, …, 8). Lastly, we performed a hierarchical STRUCTURE analysis with two different groups; the first group included individuals of *V. arenosa*, *V. longifolia* and *V. pinnata*, whereas the second one included individuals of *V. incana*, *V. porphyriana*, *V. reverdattoi*, *V. sajanensis*, *V. spicata*, *V. spuria*, and *V. taigischensis* (suppl. Table S2). We also ran the second-level

STRUCTURE analysis for the second group where the first group included individuals of *V. incana*, *V. porphyriana* and *V. reverdattoi* (totaling 71 individuals), while the second group consisted of individuals from *V. sajanensis*, *V. spicata*, *V. spuria*, and *V. reverdattoi* (49 individuals). The individuals were grouped based on the results of the first-level STRUCTURE results.

To supplement the results of the STRUCTURE analyses, we performed discriminant analysis of principal components (DAPC: Pritchard & al., 2000; Jombart & al., 2010; Grünwald & Goss, 2011) in the R-package adegenet v.1.3-1 (Jombart & Ahmed, 2011). DAPC is a multivariate approach and is less assumptive compared to STRUCTURE. For example, STRUCTURE assumes that all SNPs are unlinked and that populations are panmictic (Pritchard & al., 2000). In addition, DAPC performs better than STRUCTURE in cases of complex population structures and their corresponding genetic clusters/groups (Jombart & al., 2010). In the actual DAPC, we first divided the dataset into between-group and within-group components. This step is useful to increase discrimination between/among groups by transforming data, into the most important principal components (PCs), followed by grouping of individuals into clusters using discriminant analysis (DA). We determined the most important PCs by applying spline interpolation statistics. The best *K* was displayed with the function find.clusters (Jombart & Ahmed, 2011).

To additionally supplement the results of DAPC and STRUCTURE, we reconstructed an unrooted phylogenetic tree using maximum likelihood statistics based on the best substitution model (GTR) as assessed in jModelTest v.2 (Guidon & Gascuel, 2003; Darriba & al., 2012) in IQ-TREE v.1.6.12 (Nguyen & al., 2015) with 1000 ultrafast bootstrap replicates (Hoang & al., 2018). We reconstructed maximum likelihood trees based on both the full dataset (233 individuals, total 233,987 SNPs, with 199,736 parsimony-informative sites, 27,037 singleton sites, and 7214 constant sites) and the one including only the putative pure individuals (174 individuals, total 233,987 SNPs, 173,631 parsimony-informative sites, 40,812 singletons, and 19,544 constant sites). In the actual analyses, we removed the constant sites and considered the ascertainment bias which means to inform the algorithm that each SNP is a discrete character (details see in Lewis, 2001).

Lastly, we performed AMOVA as employed in ARLEQUIN v.3.5 (Excoffier & Lischer, 2010) to compare genetic differentiation among the clusters/groups/species that resulted from STRUCTURE, DAPC and maximum likelihood tree. We excluded again the putative hybrid individuals from this analysis (resulting in 174 individuals, see details in suppl. Table S2). The significance of fixation indices was calculated using 10,000 permutations with those SNPs present in at least 75% of the individuals. For AMOVA, we used the F_{ST}-based distance method of pairwise differences as suggested (Weir & Cockerham, 1984; Weir, 1996; Excoffier & al., 2013). Calculating F_{ST} is a standard measure of the genetic variance among populations (Whitlock & McCauley, 1999).

Assignment of hybrid classes. — We divided all individuals into five groups (hereafter we will call each group a scenario) based on the STRUCTURE results (including all 233 individuals) in combination with the initial hypothesis (Fig. 1, suppl. Fig. S1). These scenarios included *V. pinnata* × *V. spicata* and putative hybrids *V.* ×*altaica* (scenario 1); *V. incana* × *V. longifolia* and putative hybrids *V.* ×*grisea* (scenario 2); *V. longifolia* × *V. porphyriana* and putative hybrids *V.* ×*schmakovii*, *V. sajanensis* and *V. spuria* (scenario 3); *V. pinnata* × *V. porphyriana* and putative hybrids *V.* ×*sessiliflora* (scenario 4); and *V. pinnata* × *V. porphyriana* and putative hybrids *V.* ×*smirnovii* (scenario 5). Note that Kosachev & German (2004) suggested *V.* ×*smirnovii* to be a hybrid of *V. porphyriana* with *V. laeta* but *V. pinnata* is the closest relative of the latter in our sampling. To assign hybrid individuals to classes of F1-hybrids or later-generation hybrids (F2-hybrids, or backcrossed), we implemented two different approaches: (i) We inferred Q values for each individual in a Bayesian framework as employed in STRUCTURE (for only $K = 2$). This analysis ran with half million MCMC generations discarding the first 10% chains. (ii) We used a model-based method to identify hybrid individuals employed in NewHybrids v.1.0 (Anderson & Thompson, 2002; Anderson, 2003). NewHybrids v.1.0 assigns individuals to six main categories of parent1, parent2, F1-hybrids and later-generation hybrids (F2-hybrids, and backcrossed individuals to parent1 and parent2; Anderson, 2003) using data on multiple, unlinked markers without any prior information. NewHybrids v.1.0 is suitable for both markers with fixed allelic differences between the species and markers without fixed differences using the framework of Bayesian statistics (Anderson & Thompson, 2002). To run Newhybrids v.1.0, we parallelized the program by using the R package parallelnewhybrid (Wringe & al., 2017; https://github.com/bwringe/parallelnewhybrid). We selected only a set of 200 SNPs loci, the ones which were fixed and different or most divergent in terms of allele frequency profiles (Georges & al., 2018; Buono & al., 2021). We ran NewHybrids with default parameter settings, the Jeffreys prior for θ, 10,000 sweeps and 10,000 MCMC with 10% burn-in. To countercheck the results, we ran the analyses on 200 SNPs selected randomly. The reference population (parental individuals) were those that had posterior probabilities ≥0.85 selected from the results of STRUCTURE (K5, all individuals), except *V. incana* and *V. pinnata* (suppl. Tables S2, S3).

Demographic inferences. — To infer the demographic history of all five species involved in hybridization, we used the program G-PhoCS v.1.2.3 (Gronau & al., 2011). The main parameters considered were effective population size of each species, divergence times, and migration rates. G-PhoCS infers a species' demographic history associated with the population (here species) phylogeny based on inferred genealogies. Migration rates are estimated based on scenarios of post-divergence gene flow, defined by ordered pairs of branches in the population phylogeny, and allow different rates associated with two directions of gene flow. All parameters were

inferred using MCMC to jointly sample model parameters and genealogies. G-PhoCS models gene flow along defined migration bands based on unphased diploid genotypes integrating over all possible phases. Using MCMC methods, G-PhoCS pools information across loci in obtaining an approximate posterior distribution for the parameters of interest. The major challenge in using G-PhoCS is the utilization of unphased, de novo diploid loci, which may have biases that result from differences in power and accuracy in single nucleotide variant detection. Such biases may stem from differences in sequencing technologies, depth of coverage and data optimization during de novo assemblies. We alleviated this problem by using stringent filtering steps during the bioinformatics pipeline and by assuming that the selected loci were neutral. We used the coalescent-based FDIST method from Arlequin v.3.5 (Excoffier & Lischer, 2010) to investigate selection on neutral loci. For the actual G-PhoCS analysis, we restricted our dataset to three individuals per species due to high computational demands including the dataset of 316 loci (we used the same dataset as there were no selected loci in this dataset) without missing data. The inferences were then conditioned on the trees (tree constructed for the reduced dataset with 40% missing data: suppl. Fig. S2). The actual analysis employed 1 million generations of MCMC. We used the program TRACER v.1.7 (Rambaut & al., 2018; http://tree.bio.ed.ac.uk/software/tracer/) to ensure convergence of all parameters. To convert estimates of divergence time (τ) and population size (θ) from mutations per site to years (T) and effective numbers of individuals (N_e), we used $\theta = 4N_e\mu g$ and $T = \tau \cdot g / \mu$ (in which μ is mutation rate per nucleotide site per year and g is generation time) following (Lindblad-Toh & al., 2005; Gronau & al., 2011; Oswald & al., 2019; Yu & al., 2021). We assumed a mutation rate of 2.44E-9 substitutions per base per year (Richardson & al., 2001; as followed by Rønsted & al., 2002). We considered a generation time of 10 years based on personal observations. Migration rates are based on the migration rate per generation parameter ($M_{sx} = m_{sx} \cdot \theta_x / 4$; details in Gronau & al., 2011), which is the proportion of individuals in population x that arrived by migration from another population per generation (as explained in Oswald & al., 2019). Gene flow was calculated using the total migration rate. In cases in which the total rate is low, total migration rate approximates the probability of gene flow between the two species. However, for higher rates, we adjusted probabilities into rates with the equation $P = 1 - e^{-m}$ (where P = the probability of gene flow, e = exponent and m = total migration rate; following vonHoldt & al., 2016).

■ RESULTS

Species boundaries and cohesion. — The assignment test in STRUCTURE with the dataset including all 233 individuals recovered $K = 5$ as optimal both by Evanno ΔK and likelihood scores (suppl. Fig. S3), which differs from the expected $K = 10$ based on species assignment (Table 1). The result of three different groupings, $K = 4, 5, 6$ recovered more or less highly genetically cohesive groups including *Veronica longifolia*, *V. pinnata, V. porphyriana* and *V. spicata*; however, the individuals from *V. incana*, in K4 showed admixture of *V. longifolia* and *V. porphyriana*, but were recovered as a separate group in K5 and K6 with high genetic portion from *V. porphyriana* (Fig. 1). Removing the putative hybrids and including only putative purebreds (174 individuals, ~75% of total dataset), the STRUCTURE result of $K = 5$ again showed more or less the same results (Fig. 2B, suppl. Fig. S3).

The DAPC recovered 20 principal components as the best to group our data and clearly resolved all the individuals into $K = 5$ (Fig. 2C; suppl. Fig. S4). The DAPC results were supported by the unrooted trees inferred by IQ-TREE (Fig. 2A). The morphometric analysis (suppl. Appendix S1.) also supported the results of the genetic analyses in delimiting five main groups similar to those in Fig. 1. Genetic differentiation among species based on AMOVA was $F_{ST} = 0.16$ (Table 2).

The hierarchical STRUCTURE analysis retrieved five cohesive groups including *Veronica incana*, *V. longifolia*, *V. pinnata*, *V. spicata* and *V. porphyriana*. In addition to these five groups, *V. arenosa* merged with *V. pinnata*, while *V. sajanensis*, and *V. spuria* with *V. spicata*. However, *V. reverdattoi* and *V. taigischensis* did not fit to any clear group (suppl. Table S2).

Chromosome localization of rDNA loci and classes of putative hybrids. — Chromosome numbers and rDNA loci were determined in six species. *Veronica longifolia*, *V. pinnata*, *V. porphyriana*, *V. ×schmakovii*, and *V. spicata* were found to have $2n = 2x = 34$ chromosomes and one 5S rDNA-bearing chromosome pair, whereas *V. incana* had a tetraploid chromosome number ($2n = 4x = 68$) with a corresponding double number of 5S loci, i.e., two 5S-bearing chromosome pairs. In contrast to the conserved number of 5S rDNA, we found a large interspecific variation in the number of 35S rDNA: four NOR-bearing chromosomes in *V. porphyriana*, six in *V. ×schmakovii* and *V. spicata*, seven in *V. pinnata*, eight in *V. longifolia*, and at least nine in the tetraploid *V. incana*. In *V. incana*, additional very weak signals were observed (Fig. 3). Each gDNA of *V. longifolia* and *V. porphyriana* hybridized to one half of the chromosome complement of *V. ×schmakovii*. Therefore, GISH strongly suggests a homoploid hybrid origin of *V. ×schmakovii* from parental genomes closely related to *V. longifolia* and *V. porphyriana*. This is also supported by the number of 35S rDNA-bearing chromosomes in *V. ×schmakovii* (six), an intermediate value between *V. longifolia* (eight) and *V. porphyriana* (four).

The hypothesis of hybridization between and among species of *Veronica* in the Altai Mountains based on their morphotypes was mostly supported by STRUCTURE, and NewHybrids (Fig. 4; suppl. Table S3). The first-level STRUCTURE analysis is a bit more inconclusive in that respect showing admixture of various levels involving both the biparental hybrids and potential triparental hybrid (Fig. 2). The maximum likelihood tree based on all individuals also

revealed that the putative hybrid individuals are mostly sister to one of the suggested parents (suppl. Fig. S5). The genetically admixed individuals were mostly the ones identified as hybrids a priori by morphological characteristics (suppl. Fig. S1); however, we also recovered admixture in individuals considered to be pure species, e.g., *V. sajanensis, V. spuria*, *V. taigischensis*, and *V. reverdattoi*. The STRUCTURE results showed that in most cases the putative hybrid individuals in all five scenarios had posterior probabilities ranging between $0.15 < Q < 0.50$ or $0.50 < Q < 0.85$, which suggests a hybrid

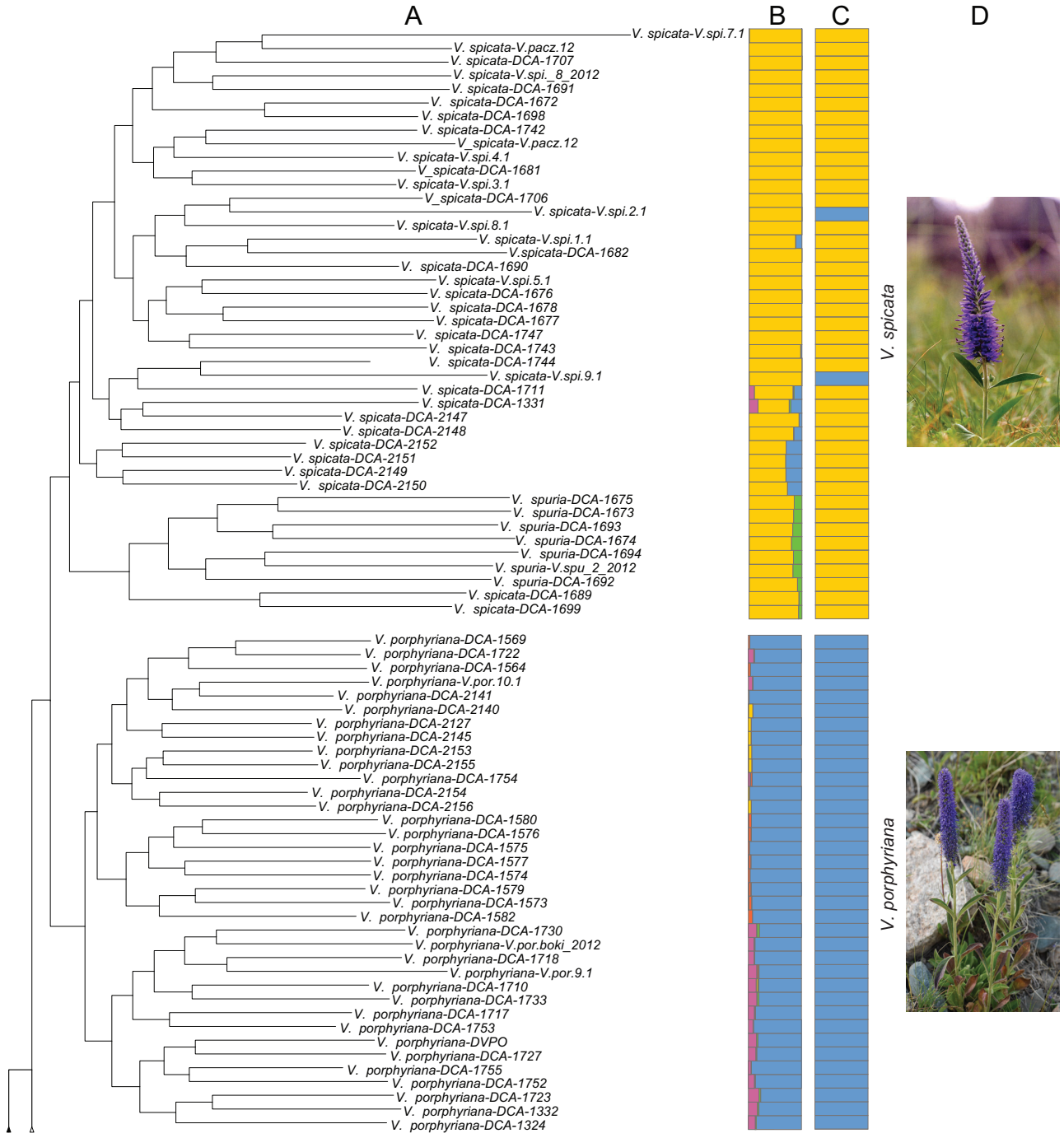

Fig. 2. Assignments of individuals to *K* clusters/populations based on Bayesian, multivariate and likelihood approaches as implemented in STRUCTURE, DAPC (in the adegenet package) and IQ-TREE programs respectively. **A**, Maximum likelihood unrooted tree reconstructed in IQ-TREE, calibrated with 1000 ultrafast bootstrap replicates. The numbers near the nodes represent bootstrap confidence. This analysis was based on 174 putative pure individuals. **B**, Probability of ancestry of each individual (horizontal axis; total 174 individuals) to each of $K = 5$ populations (vertical axis). The five populations correspond mostly to the morphotypes hypothesized for putative pure species. **C**, Discriminant analysis of principal components using the same dataset as in (B). **D**, Corresponding morphotypes of $K = 5$. — Photos: P.A. Kosachev.

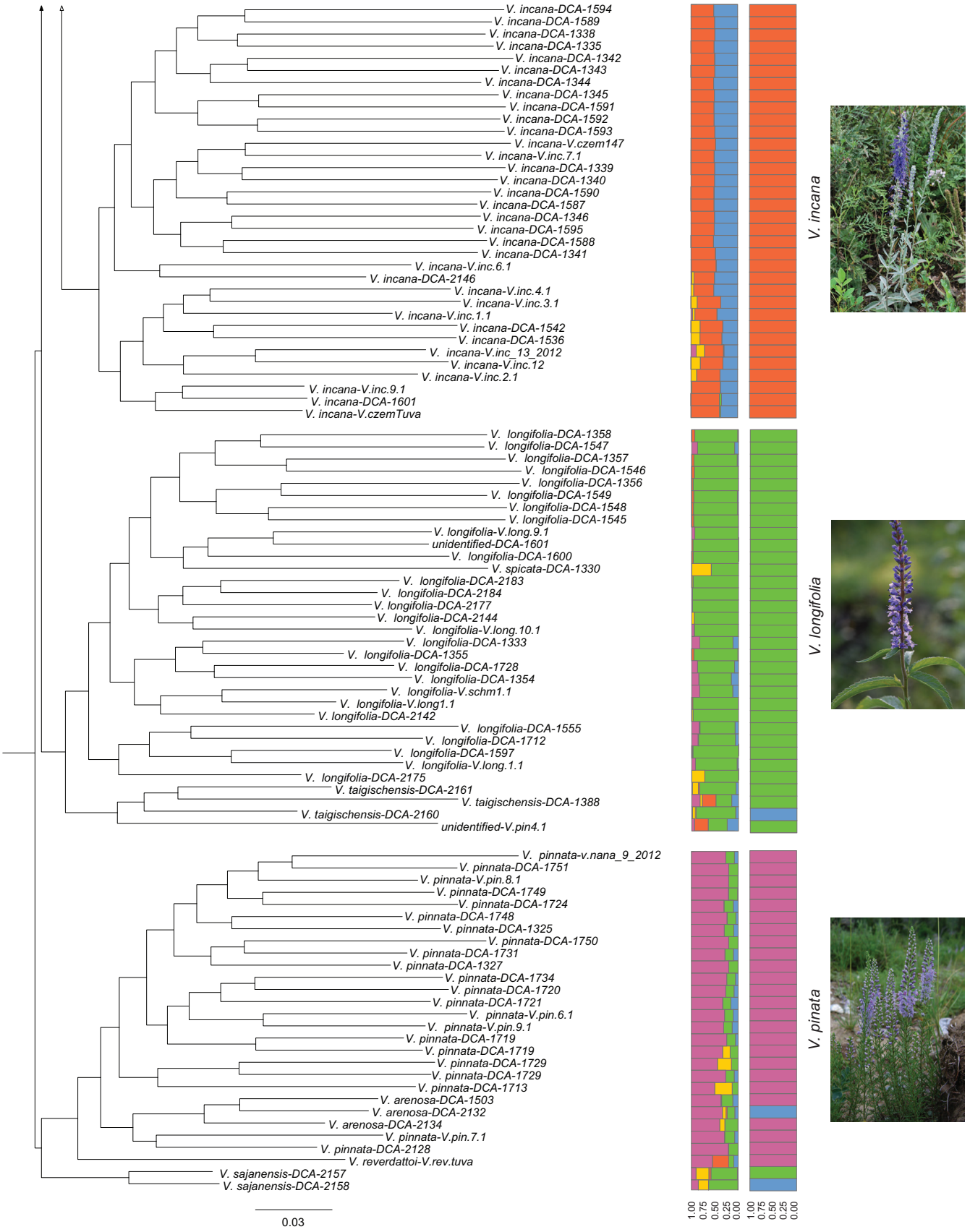

Fig. 2. Continued.

swarm from F1 to later-generation hybrids (Fig. 4; suppl. Table S3). The analysis implemented in NewHybrids to group the hybrid individuals supported the results of STRUCTURE with the exception of scenario 5 and recovered hybrids of different classes but dominated by F1 (suppl. Table S3).

Demographic history of pure species. — Using G-PhoCS to model the associated demographic history in the species of *Veronica* (Fig. 5), we retrieved divergence times between the major clades and branches to be recent (1.14 Mya to 1.57 Mya) at 95% HPD (highest posterior density; see suppl. Table S4 for complete information). Even with the upper HPD at 95%, the divergence time was in the range of 1.35 Mya to 1.84 Mya. Among the five species, the earliest recently diverged species are *V. porphyriana* and *V. incana* (1.14 Mya), while the most divergent species is *V. pinnata* (1.84 Mya) at upper 95% HPD. Similarly, the lowest effective population size is that of *V. pinnata* (4280 individuals) followed by *V. porphyriana* (6150 individuals), while the largest one belongs to *V. spicata* (21,000 individuals) at average 95% HPD. The effective population sizes of their ancestors were also different in comparison with the extant effective population sizes of all the five species, e.g., the smallest one of *V. porphyriana* and *V. spicata* ancestors (1160 individuals), whereas the largest one for the ancestor population of all species (16,100 individuals) (Fig. 5; suppl. Table S4). Our modeling of gene flow in both forward and reverse directions showed high probability of frequent gene flow from *V. longifolia* and *V. pinnata* to *V. incana*, *V. porphyriana* and *V. spicata*. Nevertheless, from *V. incana* and *V. porphyriana* to the other three species, the probability of gene flow was also comparatively high. Similarly, the gene flow among *V. incana*, *V. porphyriana* and *V. spicata* was high in both forward and reverse directions (Fig. 5; suppl. Table S4).

■ DISCUSSION

Based on extensive sampling and GBS analysis, we analyzed the relationships and evolutionary history of *Veronica* subg. *Pseudolysimachium* in the Altai Mountains, a center of diversity in the group. We have included individuals from 10 species (following the taxonomy of Kosachev & al., 2013 and 2015; not considering hybrids inferred based on morphology). The results of Bayesian modelling (STRUCTURE) were not conclusive at first but with the second-level analysis and exclusion of admixed individuals, the results led to five separate clusters, i.e., *V. incana*, *V. longifolia*, *V. pinnata*, *V. porphyriana* and *V. spicata* corresponding to the ribotype groups identified by Kosachev & al. (2016). This was further highly supported and confirmed by DAPC and maximum likelihood phylogenetic analysis. The F_{ST}-based analysis (distance method of pairwise differences) showed higher genetic distances for K5 as well. These results are consistent with the morphometric analysis (suppl. Appendix S1) in which the same five clusters were retrieved, with each cluster distant from the other species and putative hybrids intermediate between these five species. The problem of assigning *V. incana*, *V. porphyriana* and *V. spicata* to separate clusters is mirrored in previous taxonomic concepts in which *V. incana* and *V. porphyriana* were considered subspecies of *V. spicata* (Elenevsky, 1971; Walters & Webb, 1972). The problem can either be explained by high rates of gene flow among these species (Waples & Gaggiotti, 2006), recent divergence (vonHoldt & al., 2010; Rodríguez-Ramilo & Wang, 2012; Waples & Anderson, 2017), or small numbers of individuals or loci (Waples & Gaggiotti, 2006). Here, we consider a combination of the first and second explanation to be the most likely given that the coalescent sampler approach (G-PhoCS) suggested that gene flow among the three species is frequent in both directions assuming that all 316 loci were neutral and unlinked. Gene flow between *V. spicata* and *V. porphyriana* is likely limited due to ecological separation between lowland habitats of the former (below 500 m a.s.l.) and subalpine habitats (reaching as far down as 300 m a.s.l. only in valleys of the Altai). However, this may have been different in the Pleistocene considering that populations of *V. porphyriana* likely occurred at lower elevations in these times. With regards to *V. incana*, sympatric populations with *V. pinnata* and *V. longifolia* have been encountered during our fieldwork.

Table 2. Details of AMOVA in each group with different combination without including the putative hybrid.

	Source of variation	Variance components	Percentage of variation	Fixation index
Global AMOVA based on $K = 5$ results of STRUCTURE including all the putative pure 174 individuals	Among Species	14	16	
	Within Species	74	84	
	TOTAL	88	100	F_{ST}: 0.16*
Hierarchical AMOVA based on $K = 5$ results of STRUCTURE including all the putative pure 174 individuals	Among Groups	7.2	8.13	F_{CT}: 0.08*
	Among Species	7.1	8.01	F_{SC}: 0.09*
	Within Species	74.3	83.86	F_{ST}: 0.16*
	TOTAL	88.6	100	

F_{ST}, correlation within populations relative to total; F_{CT}, correlation within groups relative to total; F_{SC}, correlation within populations relative to groups. * $P < 0.001$, 10,000 permutations.

However, it should be noted that no hybrids between *V. spicata* and *V. porphyriana* and *V. incana* were noted in the field or in the molecular investigations. The hybrid between *V. incana* and *V. porphyriana* (*V. ×czemalensis* Kosachev & Albach) has been described based on morphology but those specimens assumed to belong to this hybrid have all been found to be genetically *V. incana* or hybrids with other species (see also Kosachev & al., 2016). Additionally, demographic inferences suggest that divergence between all five species is relatively recent. Our inference for the divergence of all five species coalesced at 1.57 Mya (at average 95% HPD); 1.31 Mya (at lower 95% HPD); and 1.84 Mya (at upper 95% HPD) (suppl. Table S4). These divergence times are almost in line with previous estimates (1.7 Myr) based on molecular dating on a single gene region (Meudt & al., 2015). However, the divergence between *V. spicata* and *V. incana* was estimated to be much younger (130,000 yrs; Meudt & al., 2015) compared to our estimate of 1.21 Mya (suppl. Table S4). The issue is difficult to resolve now because of the different taxon sampling, as well as DNA markers and dating methods used.

Frequent hybridization in *Veronica* subg. *Pseudolysimachium* has been reported by many researchers based on morphological characteristics or anonymous molecular markers (Härle, 1932; Trávníček & al., 2004; Bardy & al., 2011; Kosachev & al., 2019). Here, for the first time we are providing a

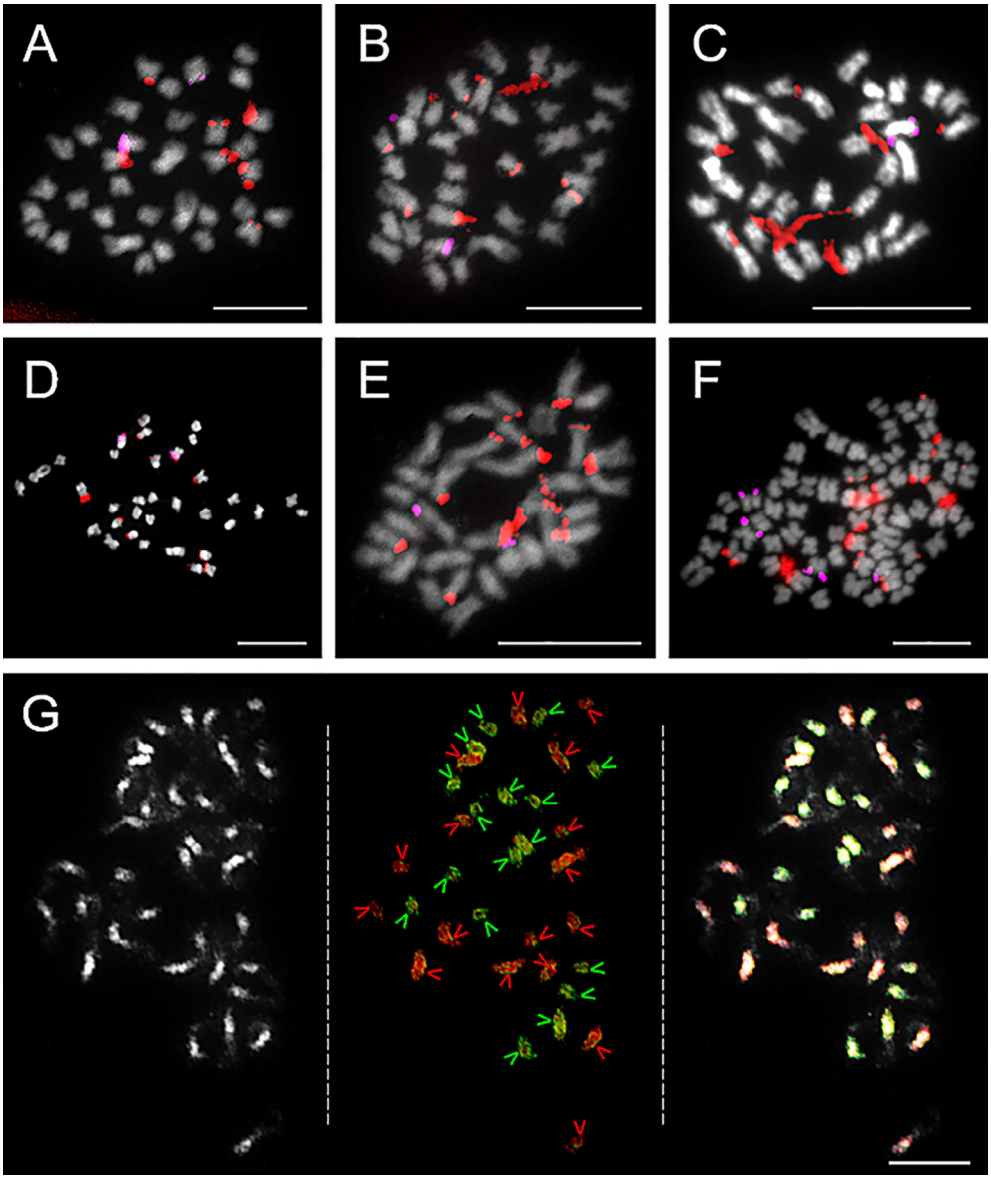

Fig. 3. Chromosome localization of rDNA and genomic *in situ* hybridization (GISH) in *Veronica*. Mitotic chromosome complements of: **A**, *V. porphyriana*; **B**, *V. ×schmakovii*; **C**, *V. spicata*; **D**, *V. pinnata*; **E**, *V. longifolia*; and **F**, *V. incana* hybridized with 35S (red fluorescence) and 5S (purple) rDNA probes. **G**, Mitotic chromosomes of *V. ×schmakovii* hybridized with gDNA of *V. longifolia* (red) and *V. porphyriana* (green). — Chromosomes were counterstained with DAPI. Scale bars, 10 μm.

genome-wide investigation of hybridization, gene flow and limits of reproductive isolation in species of *V.* subg. *Pseudolysimachium* distributed in the Altai Mountains. Our results inferred F1 hybrids as the most dominant group of hybrids. Based on the first-level analysis in STRUCTURE, we retrieved different combinations of admixed individuals involving different species (*V. spicata* × *V. pinnata*; *V. longifolia* × *V. incana*; *V. longifolia* × *V. porphyriana*; *V. pinnata* × *V. porphyriana*), which supported previous hypotheses based on morphology (Kosachev, 2003; Kosachev & al., 2013). The results recovered some individuals with inconclusive support and suggesting a range of F1 to later-generation hybrids. However, based on the results of GISH, NewHybrids, morphological analyses and field experience, we are hesitant to explain the results by extensive backcrossing. Analyses based on reference genomes should be conducted to investigate whether there is a bias towards loci of one species or another in our STRUCTURE analysis. In addition, experimental crossings will be necessary to evaluate whether these hybrid forms encountered in the Altai Mountains are fertile and form consistent and recognizable morphologies, rather than swarms with continuous morphological variation. Based on this, we also suspect that the support for scenario 5 in the STRUCTURE analysis (*V.* ×*smirnovii* = *V. pinnata* × *V. porphyriana*) is an artifact of the lacking to include correct parents. NewHybrids supported the involvement of *V. porphyriana*, as suggested by morphology (Kosachev & German, 2004) but not of *V. pinnata*, which is notable since Kosachev & German (2004) suggested *V. laeta* (not sampled by us) to be the second parent. Phylogenetic analyses (suppl. Fig. S5) support a closer relationship of *V.* ×*smirnovii* with *V. arenosa* than with *V. pinnata*. More in-depth comparisons of STRUCTURE and NewHybrids following Vähä & Primmer (2006) are necessary but it seems that STRUCTURE has more problems assigning individuals with less divergent parents and NewHybrids with more divergent parents.

The individuals showing admixture are mostly those identified as hybrids by morphological characteristics. Nevertheless, we also found that our specimens of *Veronica sajanensis*, *V. spuria*, *V. reverdattoi* and *V. taigischensis* were inferred to be admixed individuals (Fig. 2A), although these species have not been suggested to be hybrids before. We abstain from hypothesizing that these four species are, indeed, hybrids since the low sample size in these taxa may have obscured results (Wang, 2017). The case of *V. spuria* being so close to *V. spicata* is especially interesting since European specimens are usually considered closer to *V. longifolia* (Kosachev & al., 2016; Daubert & al., in prep.) but Kosachev & al. (2016) already recognized that their second specimen, a specimen from the Ural Mountains, is phylogenetically closer to *V. spicata*. Thus, despite potential false positives caused by low sample size of unambiguous species included, there are many, different hybrids in the subgenus and the question remains why this taxon has such a high rate of hybridization and weak reproductive barriers. The major points may be that plants from the subgenus are perennial (with possibly quite long generation time of more than 10 years) and outcrossing, two parameters that have been shown to correlate strongly with a high hybridization rate

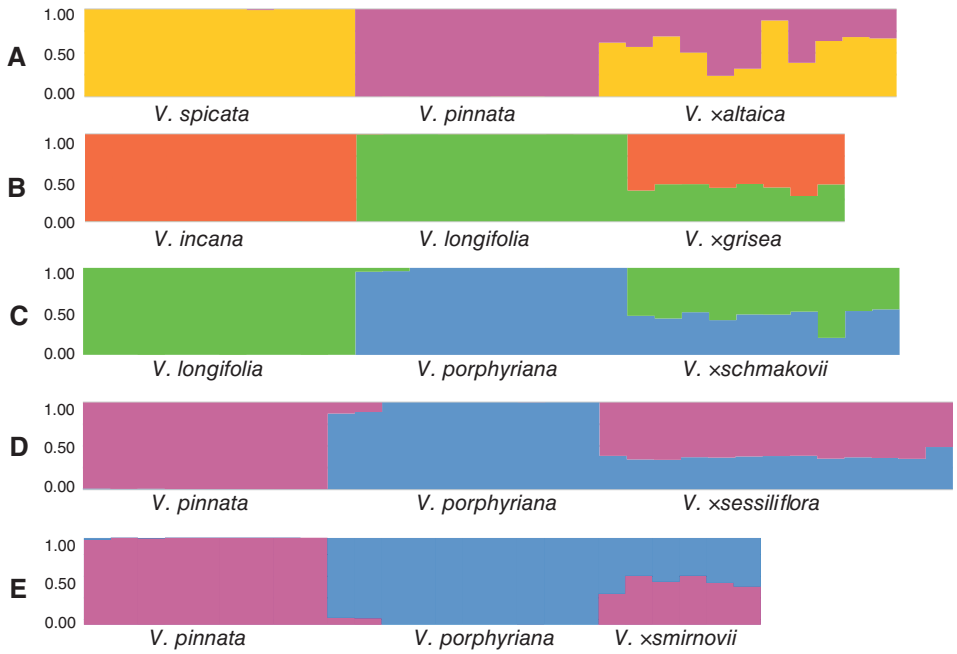

Fig. 4. STRUCTURE results showing the probability of ancestry of each individual (horizontal axis) to each of $K = 2$ populations (vertical axis) in all the five scenarios. **A**, *Veronica spicata* × *V. pinnata*; **B**, *V. incana* and *V. longifolia*; **C**, *V. longifolia* and *V. porphyriana*; **D** & **E**, *V. pinnata* and *V. porphyriana* involving putative hybrids of *V.* ×*schmakovii* and *V.* ×*sessiliflora*. Details of the exact posterior probabilities of each putative hybrid individual and their corresponding parents are given in suppl. Table S3.

(Mitchell & al., 2019). According to Carlquist (1974), overlapping flowering times, long flowering season of the parental species, and the presence of generalist pollinators (e.g., butterflies and syrphids in the case of *V.* subg. *Pseudolysimachium*; personal observation) facilitate the interspecific cross-pollination necessary for hybridization.

Our demographic model in G-PhoCS suggested a high probability of gene flow from *Veronica incana* and *V. porphyriana* to the other three species. This is consistent with general expectations in cases of hybridization in so far as alleles generally introgress from the native species to the immigrants (Barton & Hewitt, 1985; Allendorf & al., 2001; Buggs, 2007; Currat & al., 2008). Although we do not have evidence for *V. incana* being a long-time resident of the Altai Mountains – it is widespread from eastern Europe to eastern Asia, *V. porphyriana* is a subalpine species, restricted to the Altai Mountains and certainly originated from *V. spicata* as a form adapted to subalpine habitat (Elenevsky, 1971). In fact, *V. porphyriana* differs from *V. spicata* mainly in the strongly glandular indumentum (Kosachev & al., 2013), which is a general phenomenon in alpine plants (Wu & al., 2021). Thus, after the ice ages it is easily imaginable that *V. porphyriana* extended its range upwards followed by the widespread, lowland *V. longifolia*, *V. pinnata* and *V. spicata* migrating to the Pleistocene refugia of *V. porphyriana* and then crossing with it. This may be an ongoing process since both, *V. pinnata* and *V. longifolia*, still co-occur at the lower elevational range of *V. porphyriana*. According to Allendorf & al. (2001), Barton & Hewitt (1985), Buggs (2007), and Currat & al. (2008), in such cases, gene flow of alleles generally occurs from the native species to the immigrants. Adaptive introgression led by hybridization may have played its role in this regard providing adaptations to these newly immigrated species to cope with the mountainous climate of the Altai. However, to substantiate this hypothesis, we would need a more in-depth genomic scan to analyze differences in general homozygosity between species and identify potential adaptive alleles. Furthermore, different studies suggested that in such cases

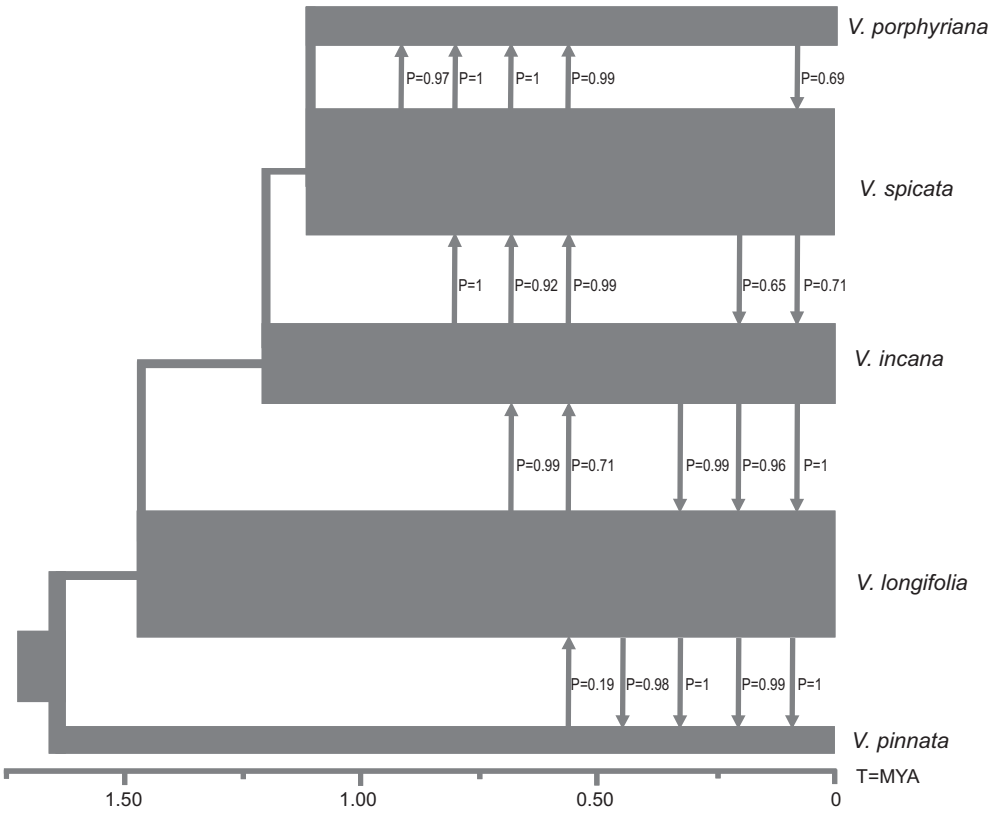

Fig. 5. Results of the G-PhoCS analysis for effective population sizes, and gene flow using only pure individuals (no admixture). The inferred current effective population size (N_e) of each species are given for all the five species. The direction of the arrows represents the probability of migration among the species both in forward and reverse directions. The width of the bars represents the effective population size of each species. For population size estimation, we used the equations N_e (effective population size) = θ / 4μg; and T (divergence time) = $\tau \cdot g / \mu$; where substitution rate/site/year (μ) = 2.44E-9, generation time for population (g) = 10 years. Migration rates are based on per generation parameter ($M_{sx} = m_{sx} \cdot \theta_x / 4$), which is the proportion of individuals in population x arrived by migration from another population per generation. Gene flow has been calculated using the total migration rate, cases where the total rate is low, it approximates the probability of gene flow between the two species. However, for higher rates, we adjusted probabilities into rates with the equation $P = 1 - e^{-m}$ (where P = the probability of gene flow, e = exponent, and m = total migration rate; following vonHoldt & al., 2016). The phylogenetic tree on which the G-PhoCS analysis has been based is given in suppl. Fig. S2. For complete details, see in Materials and Methods as well as suppl. Tables S4 and S5 for migration rates (m_{sx}), τ and θ values, and divergence times.

hybridization involving adaptive introgression is also a potential threat, in which the more common species can assimilate the more restricted species (for example, see Todesco & al., 2016) and lead to its population decline and extinction. Owens & Samuk (2020) studied this process in more detail and found that adaptive introgression can readily weaken the ecologically mediated reproductive barriers and facilitate homogenization of reproductive isolating alleles (see examples about the weakening of ecologically mediated reproductive barriers in Vonlanthen & al., 2012; Chunco, 2014). We currently have no evidence for this to be the case in the species in the Altai Mountains and species boundaries in the subgenus seem to be maintained by ecological adaptation of the species to extreme habitats (Kosachev & al., 2019).

■ CONCLUSIONS

Here, we have assessed species boundaries and cohesion, frequent hybridization and demographic histories of *Veronica* subg. *Pseudolysimachium* within its center of diversity in the Altai Mountains. Our results support the hypothesis of frequent hybridization, and gene flow between these species, as all putative hybrid individuals were inferred to be most likely F1 hybrids, although there were hybrid individuals which were difficult to place in any hybrid class. These results are in line with our field observations and the results of GISH investigations. We conclude that *V.* subg. *Pseudolysimachium* in the Altai Mountains represents a complex of species characterized by low species cohesion with weak genetic barriers. It is desirable to investigate the genomes of these species more thoroughly to understand the genomic patterns of natural selection and adaptive ecological impact of hybridization. More generally, our study highlights the potential of hybridization in the Altai Mountains. We hypothesize that the large Siberian plains and topographically diverse foreland of the Altai Mountains provide an ideal setting for hybridization between lowland and highland congeners, with the potential for introgression of alleles that confer tolerance to cooler climates of lowland species migrating to the Altai Mountains.

■ AUTHOR CONTRIBUTIONS

Conceived the idea: DCA, PAK, and GK; Performed experiments: EMQ, TM and MAL; Contributed reagents/materials/analysis tools: EMQ, DCA, and PAK; Analyzed data and authored the drafts of the paper: GK, DCA, TM and MAL. All authors contributed, made multiple revisions, and approved the final draft.

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■ LITERATURE CITED

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