

Pervasive Introgression During Rapid Diversification of the European Mountain Genus *Soldanella* (L.) (Primulaceae)

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Abstract.—Hybridization is a key mechanism involved in lineage diversification and speciation, especially in ecosystems that experienced repeated environmental oscillations. Recently radiated plant groups, which have evolved in mountain ecosystems impacted by historical climate change provide an excellent model system for studying the impact of gene flow on speciation. We combined organellar (whole-plastome) and nuclear genomic data (RAD-seq) with a cytogenetic approach (rDNA FISH) to investigate the effects of hybridization and introgression on evolution and speciation in the genus *Soldanella* (snowbells, Primulaceae). Pervasive introgression has already occurred among ancestral lineages of snowbells and has persisted throughout the entire evolutionary history of the genus, regardless of the ecology, cytotype, or distribution range size of the affected species. The highest extent of introgression has been detected in the Carpathian species, which is also reflected in their extensive karyotype variation. Introgression occurred even between species with dysploid and euploid cytotypes, which were considered to be reproductively isolated. The magnitude of introgression detected in snowbells is unprecedented in other mountain genera of the European Alpine System investigated hitherto. Our study stresses the prominent evolutionary role of hybridization in facilitating speciation and diversification on the one hand, but also enriching previously isolated genetic pools. [chloroplast capture; diversification; dysploidy; European Alpine system; introgression; nuclear-cytoplasmic discordance; ribosomal DNA.]

Hybridization is a key evolutionary process shaping diversification and speciation processes (Soltis and Soltis 2009; Taylor and Larson 2019; Nieto Feliner et al. 2020). Recent methodological developments allowed uncovering numerous, often unexpected, cases of hybridization and introgression (Mallet 2007; Whitney et al. 2010; Abbott et al. 2013, 2016; Mallet et al. 2016; Taylor and Larson 2019). Identification of historical hybridization in various groups of organisms has opened avenues to investigate the long-term consequences of gene flow in evolution, including hybrid speciation (Greig et al. 2002; Harris and Nielsen 2016; vonHoldt et al. 2016; Taylor and Larson 2019), adaptive introgression (Choler et al. 2004; Suarez-Gonzalez et al. 2018), but also adaptive radiation (e.g., Meier et al. 2017). A direct consequence of speciation induced by gene exchange is genomic mosaicism (Jiggins et al. 2008). The substantially increased allelic diversity

of introgressed populations can facilitate their adaptation to novel and challenging environments. Hence, this mechanism can directly trigger the emergence of new species and fuel adaptive radiations (Pease et al. 2016; Meier et al. 2017). On the other hand, excessive introgression can reintegrate previously separated gene pools into a single biological entity, and thus hampering diversification (Bog et al. 2017; Kearns et al. 2018).

Key extrinsic triggers facilitating hybridization are dynamic changes in the environment, which enable previously allopatric populations to shift their ranges and come into secondary contact (Stebbins 1985). Mountain landscapes are highly diverse, forming steep altitudinal gradients featuring mosaics of niches with specific microclimates and bedrock. Such fine-scale, ecologically-heterogeneous systems promote secondary contacts of originally allopatric or parapatric species and hence provide ample opportunities for gene exchange (e.g.,

Raven 1973; Körner and Spehn 2002; Emadzade et al. 2015; Antonelli et al. 2018; Pouchon et al. 2018; Kandziora et al. 2022). The Quaternary climatic oscillations led to pronounced range contractions and expansions, especially in the mountain regions of the Northern hemisphere (Stebbins 1985; Hewitt 1996, 2000, 2004; Flantua et al. 2019). Consequently, the extensive range shifts along vertical and horizontal gradients created opportunities for secondary contacts and gene flow among species and populations lacking substantial mating barriers. Hybridization events associated with Quaternary glaciations in the European Alpine System (EAS, hereafter; Ozenda 1985) have primarily been evidenced among closely related species (Paun et al. 2008; Boucher et al. 2016; Klein and Kadereit 2016; Olšavská et al. 2016; Melichárková et al. 2020). Although the impact of gene flow on plant speciation at generic level remains understudied in European mountains, a crucial role for hybridization has been recently demonstrated, for instance, in South American mountain plant groups (Cortés et al. 2018; Morales-Briones et al. 2018; Pouchon et al. 2018; Kandziora et al. 2022).

The present study investigates the role of hybridization in the diversification of the mountain-dwelling genus *Soldanella* L. (snowbells; Primulaceae). Based on morphological and molecular data, it was hypothesized that the evolutionary history of this genus has been affected by hybridization (Vierhapper 1904a, 1904b; Pawłowska 1972; Meyer 1985; Zhang et al. 2001; Zhang and Kadereit 2002; Steffen and Kadereit 2014). Classical phylogenetic reconstructions uncovered shallow and poorly resolved topologies that hampered explicit detection of hybridization and introgression (cf. Zhang et al. 2001; Zhang and Kadereit 2002; Steffen and Kadereit 2014; Bellino et al. 2015). The weak morphological and genetic divergence between snowbell species from the Carpathians and Balkans might indicate incomplete speciation or widespread hybridization-induced erosion of species boundaries (Zhang et al. 2001; Zhang and Kadereit 2002). Interestingly, a traditional hypothesis that the widespread *S. alpina* is of hybrid origin has not received support in a recent study (Steffen and Kadereit 2014). Nevertheless, the role of hybridization in snowbells evolution remains speculative and needs to be addressed by high-resolution genomic data.

Using an exhaustive species sampling and a combination of NGS-based restriction site-associated DNA sequencing (RAD-seq), whole-plastome sequencing, and cytogenetic data (rDNA FISH) we aim here to investigate the prevalence of hybridization across the history of an EAS-dwelling plant genus, given that such a system must have been exposed to significant Quaternary climatic fluctuations. Specifically, we aim to test the following two hypotheses: 1) predominantly Carpathian and Balkan *Soldanella* species, usually featuring large ecological amplitude, have been affected by hybridization more than alpine zone-dwelling snowbell species, and 2) hybridization contributed to diversification of *S. alpina*, but also triggered the breakdown of species boundaries in *Carpathian* species.

MATERIALS AND METHODS

Study System

The genus *Soldanella* consists of 18 species, of which four include two subspecies and one includes three subspecies (Zhang et al. 2001; Zhang and Kadereit 2002; Niederle 2003, 2016; Bellino et al. 2015) and is distributed across EAS, including the Alps, Apennines, Balkan mountains, Carpathians, Cordillera Cantábrica, Bohemian Massif, and the Pyrenees. Most species are endemic to a single mountain range, while others, such as *S. alpina* are more widespread (Zhang et al. 2001; Zhang and Kadereit 2002). Snowbells are caespitose to clonally growing, evergreen perennials with campanulate or funnel-shaped flowers with lacinate lobes, and capsule forming fruits. The genus includes snow bed specialists (*S. minima* and *S. pusilla*) occupying arctic-alpine environments in the alpine or subnival zone (Supplementary Fig. S1—<https://doi.org/10.5061/dryad.mcvdnc41>; Zhang et al. 2001; Zhang and Kadereit 2002), but the majority of snowbells have large ecological amplitude and occur from sheltered, humid forests to open habitats in the alpine zone (Zhang and Kadereit 2002; Štubňová et al. 2017; Valachovič et al. 2019). From a karyological perspective, most of species have the same ploidy and chromosome number with $2n = 40$. A dysploid cytotype with $2n = 38$ was, however, reported in several species (Štubňová et al. 2017).

Sampling Design

We sampled all members of the genus *Soldanella*, covering its entire distribution range (Fig. 1) including localities from which taxa were originally described (Supplementary Table S1). Fresh, intact leaves were preserved in silica gel. The RAD-seq based phylogenomic inference was performed with a minimum of four accessions per species, originating from different populations. Exceptions represented taxa with a narrow distribution (Supplementary Table S1). For RAD-seq analyses, we included 123 individuals from 118 populations. The plastome analyses were based on a selection of 42 *Soldanella* accessions, plus 47 outgroup accessions (Supplementary Table S1). For cytogenetic analyses, all taxa except *S. minima* subsp. *samnitica* and *S. callabrella* were used, representing 50 accessions from 42 localities (Supplementary Table S1). Vouchers labeled with the population and individual code number have been deposited in the SAV herbarium.

DNA Isolation and RAD-seq Library Preparation and Sequencing

Genomic DNA was extracted using a DNeasy plant mini kit (Qiagen, Hilden, Germany) or a cetyltrimethylammonium bromide (CTAB) procedure (Doyle and Doyle 1987), with modifications proposed by Schönswetter et al. (2002), and purified using a NucleoSpin gDNA clean-up kit (Macherey-Nagel).



FIGURE 1. Sampling map of *Soldanella* accessions used in the present study. Taxa specialized in alpine zone are represented by squares, while species that grow in forests or have a large ecological amplitude are represented by circles. The bold line on the symbol depicts species with a dysploid cytotype, whereas the dashed line denotes species revealed to be non-monophyletic through inferred tree analyses (Supplementary Figs. S2 and S3). Photos with their credits from left to right: *S. alpina* subsp. *alpina* (Jaromír Kučera—JK), *S. minima* subsp. *minima* (JK), *S. angusta* (Eliška Gbúrová Štubňová), *S. carpatica* (Marek Slovák), *S. pindicola* (JK) and *S. villosa* (JK).

Preparation of single-digest RAD libraries followed a protocol adapted from Brandrud et al. (2017). The starting amount of genomic DNA was 120 ng per individual. Considering genome size (1C = 1.5–2.0 pg, Štubňová et al. 2017), 48 barcoded samples were pooled together in one library. Final libraries were sequenced on an Illumina HiSeq 2500 at the sequencing facility of the Vienna BioCenter Core Facilities (VBCF; [https://](https://www.viennabiocenter.org/)

www.viennabiocenter.org/) as 100 bp single-end reads. Individuals with less than 1.5 million high-quality reads were re-sequenced.

Filtering SNPs from RAD-seq Data

Raw Illumina reads were demultiplexed in two steps, first using BamIndexDecoder v.1.03 of the Picard Illumina2bam package (<http://ggq1.github.io/>

illumina2bam/index.html) and subsequently with *process_radtags* implemented in STACKS v.1.45 (Catchen et al. 2013), including quality filtering to retain only full-length sequences of 94 bp (without inline barcodes).

The filtered reads were further analyzed with *denovo_map.pl* from STACKS. To find the STACKS parameters that maximized the recovery of reliable polymorphic loci, whereas avoiding as much as possible over-splitting and collapsing of any paralogous loci, various settings were tested (Supplementary Table S2), following Heckenhauer et al. (2018). The optimal settings were $m = 5$, $M = 1$, $n = 1$, allowing for indels. These parameters resulted in the highest number of polymorphic loci with one to nine SNPs that were covered in at least 90% of individuals (Supplementary Table S2). To maximize the recovery of loci across the coverage and phylogenetic breadth in the data, we prepared a pseudo-reference based on consensus haplotypes of loci, following pipelines successfully applied in previous studies on species without reference genomes (e.g., Bateman et al. 2018; Heckenhauer et al. 2018; Brandrud et al. 2019).

The reads were further aligned against the pseudo-reference using BWA mem (v.0.7.17-r1188; Li and Durbin 2010), flagging shorter split hits as secondary. The aligned files were sorted by reference coordinates and read groups were added with Picard v.2.18.17 (<https://broadinstitute.github.io/picard/>). Indels were realigned in GATK v.3.7-1 (McKenna et al. 2010). Variants were finally called with the *ref_map.pl* script from STACKS with default parameters. Vcftools v.0.1.13 (Danecek et al. 2011) was used for SNPs filtration with various settings (Supplementary Table S3). The maximum allowed level of missing data was 5%.

RAD-seq based Phylogenetic Inference

The phylogenetic inference was performed using maximum likelihood (ML) and Bayesian inference (matrix RAD_A; Supplementary Table S3). The ML analyses were computed in RAxML v.8.1.3 (Stamatakis 2014), with the GTR + CAT approximation of rate heterogeneity, the standard Lewis acquisition bias correction (Lewis 2001) as recommended for concatenated SNPs, and 1000 rapid bootstrap replicates with a thorough ML search.

Bayesian phylogenomic trees were constructed in BEAST v.2.6.3 (Drummond and Bouckaert 2015), using the mutation model as selected by the in-built program, the relaxed lognormal molecular clock, and the Yule speciation model. Analyses were run for 300 million generations, sampling and logging every 15,000 generations. ESS values of parameters were checked for convergence in the software TRACER v.1.7 (Rambaut et al. 2018). Finally, TREEANNOTATOR v.2.6.3 (Bouckaert et al. 2014) was used to generate a maximum clade credibility tree discarding 25% of sampled trees as burn-in. The obtained trees were visualized with FIGTREE v.1.4.2 (available from <http://tree.bio.ed.ac.uk/software/figtree/>).

Detecting Genetic Admixture and Conflicting Signals

Neighbor-Net analysis (Bryant and Moulton 2004) was conducted in SPLITSTREE v.4.10 (Huson and Bryant 2006) using Matrix RAD_A. A co-ancestry heatmap based on genotype likelihoods was constructed with the genotype-free method of calling variants implemented in ANGSD v.0.921-26-g58a85440.910 (Korneliussen et al. 2014) on the indel-realigned mapping files. Only reads with base and mapping minimum qualities of 20 were used. Alleles were inferred from genotype likelihoods with a GATK-derived algorithm (i.e., GL 2). Only variable sites ($P < 0.000001$) that were detected in at least 50% of individuals and showed a minor allele frequency of 0.015 were kept. A covariance matrix was further calculated with PCangsd v.0.99 (Meisner and Albrechtsen 2018) and was plotted using the heatmap.2 function in gplots v.3.0.1.1 (Warnes et al. 2020). Population structure and recent admixture events were also inferred using NGSadmix v.32 (Skotte et al. 2013) based on data matrices containing a single SNP per locus. Namely, matrix RAD_C included all analyzed snowbell individuals, while Matrix RAD_D (79 individuals) was created to search for a finer genetic structure within taxa from the Carpathians, NE Alps, Balkan, and southern Italy. Clustering patterns were estimated for both datasets with K values from 1 to 15. Each analysis was run ten times for each K and started from a different seed.

Historical Hybridization and Introgression

To reconstruct the relationships among snowbell taxa in the face of historical gene flow, TREEMIX v.1.13 (Pickrell and Pritchard 2012) was used for Matrix RAD_B containing a single SNP per locus. To generate a TREEMIX plot, the input file from vcf and the population script from STACKS were utilized. TREEMIX was run with *S. villosa* as an outgroup and visualized in R. Migration edges were sequentially added to a ML tree of population ancestry, with 20 possible migrations tested. The contribution of each migration vector was identified using an f index representing the fraction of the variance in the sample covariance matrix ($W \partial$) accounted for by the model covariance matrix (W).

To identify candidate species involved in introgressive hybridization, we computed D-statistic (i.e., ABBA-BABA-derived test), f_4 -admixture ratio, and F_{branch} (f_b) for all trios using Dsuite v.0.4r43. These statistics have been specifically developed to infer introgression in the presence of ILS (Patterson et al. 2012; Malinsky et al. 2021). The f_b statistic is a heuristic strategy for summing f_4 -admixture ratios over the entire tree topology to detect introgression events, in particular affecting internal branches (Malinsky et al. 2021). Matrix RAD_A was utilized for D-statistics computations. *Soldanella villosa* was selected as an out-group and species relationships were outlined on a pruned RAxML tree (Fig. 2), which was obtained with the 'drop.tip' command implemented in the R library ape (Paradise et al. 2004).

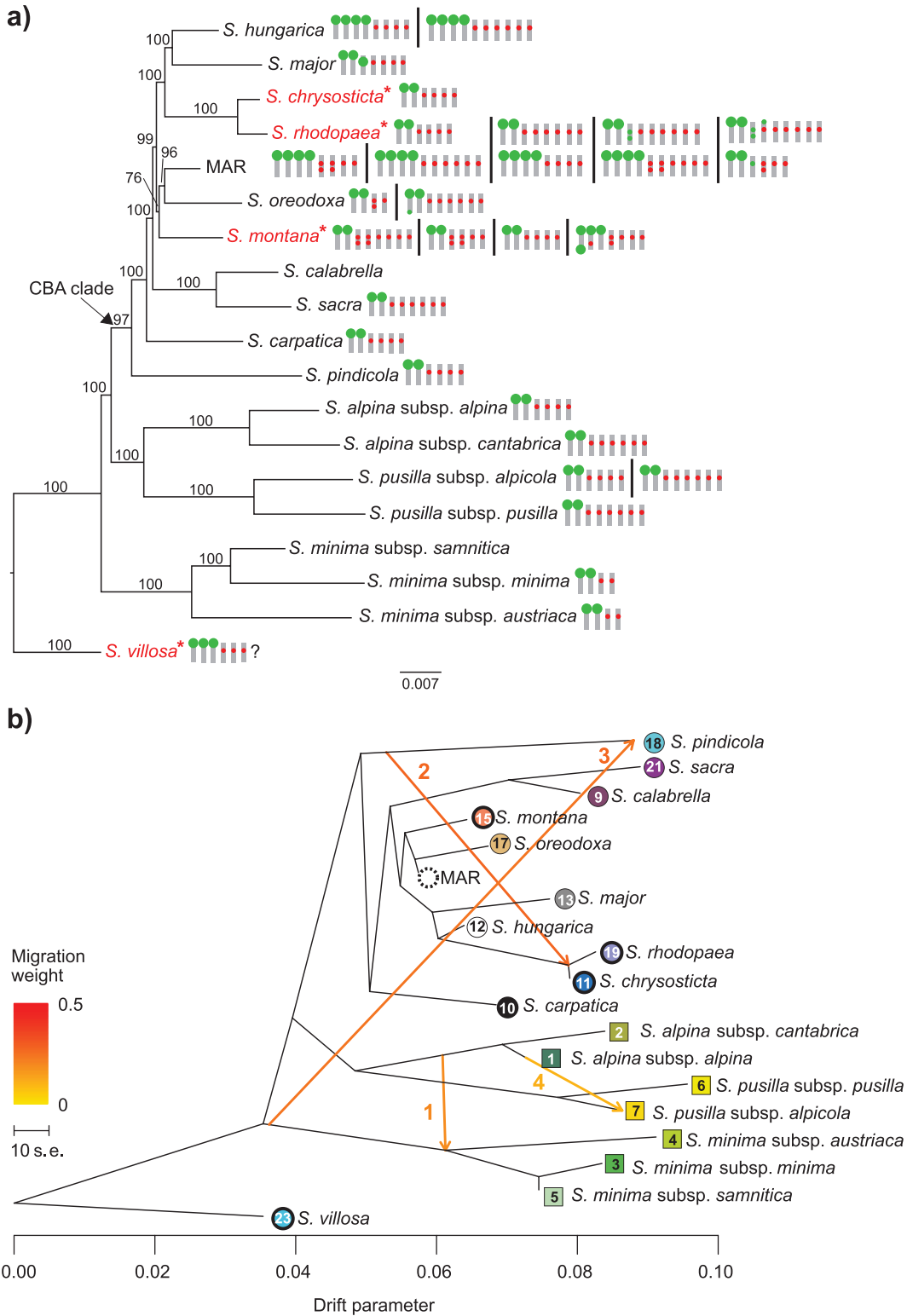


FIGURE 2. (a) Collapsed best-scoring ML tree of *Soldanella* based on 39,416 SNPs and 119 individuals (Matrix RAD_A). Bootstrap support is indicated above branches. Chromosomes carrying 5S (internal marks) and 35S (terminal bullets) ribosomal DNA loci detected by FISH analyses are shown on tree terminals. All detected karyotypes are shown for each taxon. The question mark in *S. villosa* indicates an approximate number of 5S loci. Species marked by asterisk have a dysploid cytotype with $2n = 38$, while the rest have $2n = 40$. The polyphyletic *S. marmarossiensis*, *S. angusta*, and *S. rugosa* are grouped into an arbitrary taxon “MAR”. (b) The phylogenetic network of relationships between *Soldanella* taxa as inferred by TREEMIX, based on 11,804 SNPs and 119 individuals. The first four migration edges between species are marked by arrows indicating the direction toward the recipient group and colored according to the ancestry percentage received from the donor. Symbols at the tips follow taxon identity from Fig. 1.

Plastome Data—Divergence Time Estimation and Phylogenetic Analyses of Plastome Data

For building a plastid phylogeny, we used genome skimming data generated by the sequencing project PhyloAlps, and recently published by [Smyčka et al. \(2022\)](#). For further details, see [Supplementary data 1](#).

Annotated plastomes of *Soldanella* taxa together with outgroup taxa were processed in Geneious v.7.1 (Biomatters, Auckland, New Zealand). Divergence times were estimated in a Bayesian framework using BEAST v.2.5.2. ([Drummond et al. 2012](#)) and a combination of primary (fossil-based) and secondary calibration points from the studies of [Magallón et al. \(2015\)](#) and [de Vos et al. \(2014\)](#) (for more details see [Supplementary data 1](#)).

Matrix cp_B that included 42 *Soldanella* accessions and *Hottonia* as an outgroup (70 genes, 43 individuals, 103,787 bp) was used for computation of the ML analysis conducted in RAxML v.8.1.3 ([Stamatakis 2014](#)), with the GTR + CAT model and 1000 rapid bootstrap replicates, each with a subsequent thorough ML search.

Chromosome Preparation and Fluorescence In Situ Hybridization

Chromosome spreads were prepared for 50 individuals ([Supplementary Table S1](#)) as described by [Mandáková and Lysak \(2016a\)](#) and in [Supplementary data 1](#).

The BAC clone T15P10 (AF167571) of *Arabidopsis thaliana* bearing 35S rRNA gene repeats was used for in situ localization of nucleolar organizer regions (NORs), and the *Arabidopsis* clone pCT4.2 (M65137), corresponding to a 500-bp 5S rDNA repeat, was used for localization of 5S rDNA loci. The rDNA probes were labeled with biotin-dUTP and digoxigenin-dUTP by nick translation as previously described ([Mandáková and Lysak, 2016b](#); see also [Supplementary data 1](#)).

RESULTS

RAD-seq Data

An average of 3,162,605 ($\pm 1,253,129$ SD) high-quality RAD-seq reads were obtained per individual after demultiplexing and filtering. Altogether 23,498 polymorphic loci (94–112 nt long) were included in the synthetic reference after optimizing parameters and filtering. On average, 25.8% ($\pm 2.2\%$ SD) of the reads could be mapped to this reference, for a final mean coverage of 34.7 \times . The raw dataset contained 322,278 SNPs. Four samples (including *S. chrysosticta* subsp. *serbica*) were discarded due to high levels of missing data. Hence, the final dataset consisted of a total of 119 *Soldanella* individuals. The RAD-seq reads have been deposited in the NCBI Short Reads Archive (BioProject ID PRJNA820454, SRA: SRR18507145-SRR18507263).

Phylogenetic Tree Reconstruction

ML and BI analyses served to uncover overall phylogenetic relationships among snowbell taxa. *Soldanella villosa* appeared in a sister position to the rest of the snowbells after midpoint rooting, which is in line with outcomes of previous studies (e.g., [Zhang et al. 2001](#); [de Vos et al., 2014](#)). Species occupying the alpine zone (*S. alpina*, *S. minima* and *S. pusilla*) branched off before the radiation of a clade containing species mostly from the Balkans and Carpathians ([Fig. 2a](#), [Supplementary Figs. S2 and S3](#)). By contrast, *S. villosa* and *S. minima* were clustered in sister positions to the rest of the taxa in BI analyses, although with poor support (PP = 0.51; [Supplementary Fig. S3](#)). The rest of the snowbell taxa from the Carpathians, Balkans, and southern Apennines formed a clade, referred to as the CBA clade hereafter. Individuals belonging to the same taxon were grouped into strongly supported clades (BS 100%, PP 1.0), except for some Carpathian species that were reciprocally non-monophyletic (*S. marmarossiensis*, *S. angusta*, and *S. rugosa*). Interestingly, the dysploid cytotype ($2n = 38$) has very likely evolved independently in four species ([Fig. 2a](#)).

Genetic Admixture and Conflicting Signal Detection

Neighbor-Net results ([Supplementary Fig. S4](#)) displayed multiple reticulations across the network, with parallel splits connecting particularly *S. alpina*, *S. minima*, *S. pusilla*, and *S. villosa* reciprocally, but also with the rest of snowbell taxa.

The co-ancestry heatmap indicated the existence of two distinct species groups: alpine species + *S. villosa*, and the CBA clade ([Supplementary Fig. S5](#)). *Soldanella pindicola* appeared to share relatively equal co-ancestry with both groups. The most pronounced traces of genetic admixture were visible between *S. alpina* and *S. minima*, as well as between *S. alpina* and *S. pusilla*. Intensive mutual gene flow was also indicated for the Carpathian taxa, with a lack of taxon boundaries among *S. angusta*, *S. marmarossiensis* and *S. rugosa*.

The first run of admixture analysis including all taxa found higher ΔK peaks ([Evanno et al. 2005](#)) when individuals were clustered into 2, 4, 10, and 13 groups ([Supplementary Fig. S7a–e](#)). When clustered into two groups, *S. pindicola* appears admixed between two major species groups formed by *S. minima*, *S. alpina*, *S. pusilla* and *S. villosa* on one hand, and the rest of snowbells on the other hand ([Supplementary Fig. S7a](#)). In the case of four groups, *S. rhodopaea*, *S. chrysosticta*, *S. calabrella*, and *S. sacra* were genetically intermediate between *S. pindicola*-*S. carpatica* and the other Carpathian species ([Supplementary Fig. S7b](#)). The second run including only CBA taxa uncovered interpretable results at $K = 2, 3, 5, 6,$ and 8 ([Supplementary Fig. S8 a–e](#)). The new insight with respect to the first run was provided by results with three and five clusters, where *S. rhodopaea* and *S. chrysosticta* were remarkably admixed with *S. pindicola*, and *S. calabrella* shares genetic material with

S. carpatica, respectively (Supplementary Fig. S8b,c). Finally, *S. angusta*, *S. hungarica*, *S. marmarossiensis*, and *S. rugosa* share alleles from various gene pools especially for the analyses with high number of clusters (Supplementary Fig. S8c–e).

The TREEMIX graph rooted with *S. villosa* revealed nearly the same topology as the ML and BI trees (Fig. 2b). Residuals from the covariance matrix did not significantly improve after including four migration edges, as also indicated by the curve of variance (Supplementary Fig. S6a,b). The first migration edge showed a historical gene flow between lineages ancestral to *S. alpina* and *S. minima* (weight 19.3%), the second edge suggested a migration from *S. pindicola* to the ancestor of *S. chrysosticta* + *S. rhodopaea* (weight 27.6%), and the third edge indicated a shared ancestry of *S. pindicola* with the ancestor of *S. minima* (weight 24.4%). Finally, the last migration edge suggested a historical gene flow between populations of *S. alpina* subsp. *alpina* and *S. pusilla* subsp. *alpicola* (weight 10.2%).

D and derived statistics uncovered a pervasive, but generally mild gene exchange, whereby several hybridization/introgression events have most likely occurred between ancestral lineages and others occurred between currently existing taxa (Fig. 3, Supplementary Table S4). *Soldanella alpina*, *S. minima* and *S. pusilla* appear to have interbred frequently in different phases of their evolutionary histories. Strong signals of likely historical gene flow and admixture were identified between *S. alpina* and *S. minima*, and also between *S. alpina* and *S. pusilla* subsp. *alpicola* (Fig. 3). Some level of gene flow is indicated also between species with the euploid and dysploid cytotypes (i.e., between the euploid *S. pindicola* and the dysploid *S. chrysosticta-rhodopaea* clade, as well as between the dysploid *S. montana* and the euploid MAR clade and *S. hungarica* Fig. 3b,c).

As determined by *D*-statistics, the most extensive hybridization occurred between the following pairs: *S. alpina* and *S. minima*, *S. alpina* and *S. pusilla* subsp. *alpicola*, *S. pindicola* and the *S. chrysosticta-rhodopaea* clade (Fig. 3b). The *f*₄ statistics revealed that *S. major* and *S. hungarica*, or more likely their ancestor, hybridized with the MAR clade, which has affected a substantial portion of their genomes (i.e., around 30%). Genetic admixture, hybridization, and introgression in snowbells as resolved by various analytical approaches are summarized in Supplementary Table S5.

Plastid Phylogeny and Molecular Dating

The topology of the inferred plastome tree contrasts strongly with topologies obtained from RAD-seq data (Fig. 4, Supplementary Fig. S9). Plastome haplotypes of the vast majority of species reflected neither the current taxonomic concept nor the morphology or ecology of the taxa studied. However, individual haplotype subclades were clustered based on narrower or wider geographical units. Plastome-based molecular divergence time estimation indicates that the genus *Soldanella* is of a Middle Tertiary origin. Its MRCA split from *Hottonia*

in the early Miocene (21.2 Ma, Supplementary Fig. S9). After a prolonged diversification stasis in the late Miocene (6.3 Ma), the MRCA lineage was divided into two major sister clades. Ancestral lineages of recent taxa branched off at the end of the Pliocene (3.1 Ma) and the beginning of the Pleistocene (2.3 Ma). All recent species are of a Pleistocene origin, mostly being younger than 1 Ma.

Variation in Chromosome Number and rDNA Loci

The euploid chromosome number ($2n = 40$) dominated in the genus, whereas the dysploid chromosome number ($2n = 38$) was proved in *S. chrysosticta*, *S. montana*, *S. rhodopaea*, and *S. villosa*. A high variation in the number and localization of 35S and 5S rDNA loci was observed (Fig. 2a; Supplementary Fig. S10). The rDNA FISH revealed 16 unique and clearly interpretable patterns. Probes corresponding to 35S rDNA provided 2–5 hybridization signals in chromosome complements. Interstitial 35S rDNA loci were identified in *S. major* and the MAR clade (Supplementary Figs. S10b,g,h,k). NORs on both termini of a single chromosome were observed in *S. oreodoxa* and *S. montana* (Supplementary Fig. S10i and n).

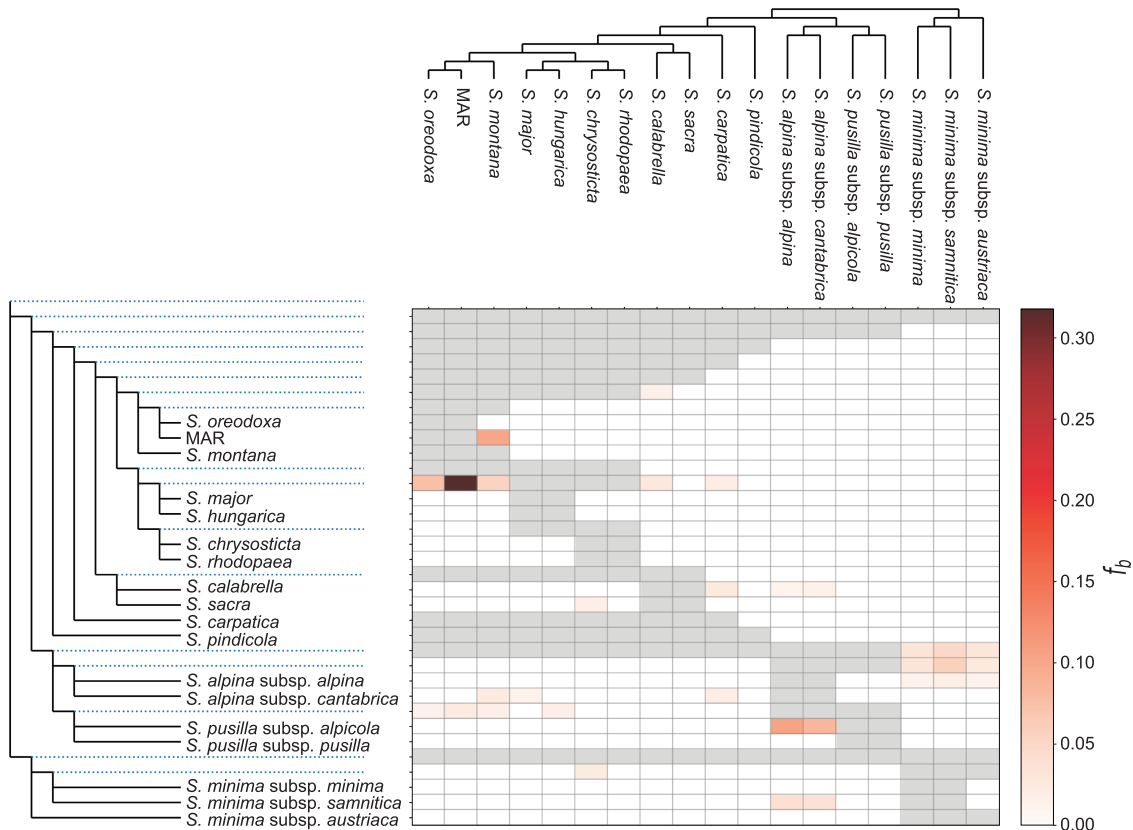
All 5S rDNA loci were observed in the interstitial position on chromosomes. However, the number of 5S rDNA hybridization sites varied extensively from two in *S. minima* to eight in the MAR clade (Supplementary Fig. S10p and d). In *S. montana*, *S. oreodoxa* and the MAR clade, duplicated 5S rDNA loci were observed within one or two chromosomes (Supplementary Fig. S10d,f,j,k,m–o). Two evolutionary trends are obvious: 1) a single NOR-bearing chromosome pair and two 5S-bearing chromosome pairs represent the most frequent rDNA pattern (Fig. 2a and Supplementary Fig. S10c) and 2) the highest intraspecific rDNA variation occurs only in the young Carpathian species (Fig. 2a). No correlation in the rDNA chromosome positioning was identified between the euploid and the dysploid cytotype, except for the sister species pair of *S. chrysosticta* and *S. rhodopaea* (Fig. 2a; Supplementary Fig. S10c).

DISCUSSION

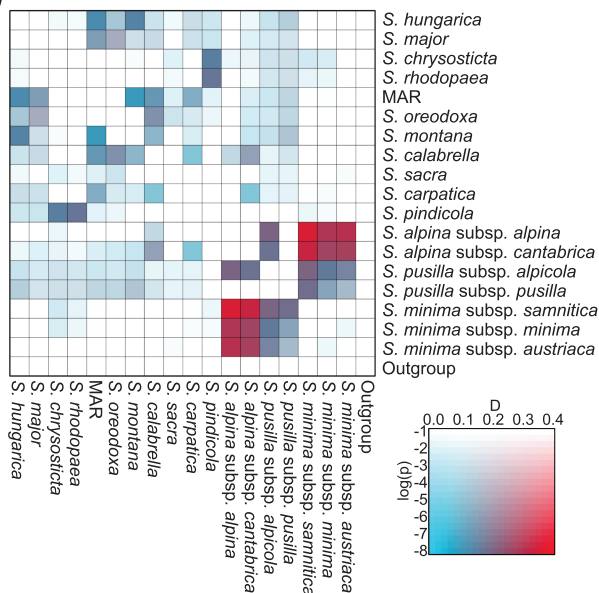
Pervasive Introgression in Snowbells

Recurring waves of hybridization and introgression have played a vital role in the evolutionary history and speciation processes in the genus *Soldanella*. Hybridization did not affect only forest species, primarily from the CBA clade as we hypothesized (cf. Zhang et al. 2001), but it was even more extensive in species inhabiting alpine habitats. Even though we employed a variety of methods to infer gene flow, the detected patterns and intensity of gene flow were not fully consistent across methods. This might be explained by extremely complicated patterns caused by recurrent hybridization across various time horizons. In addition,

a)



b)



c)

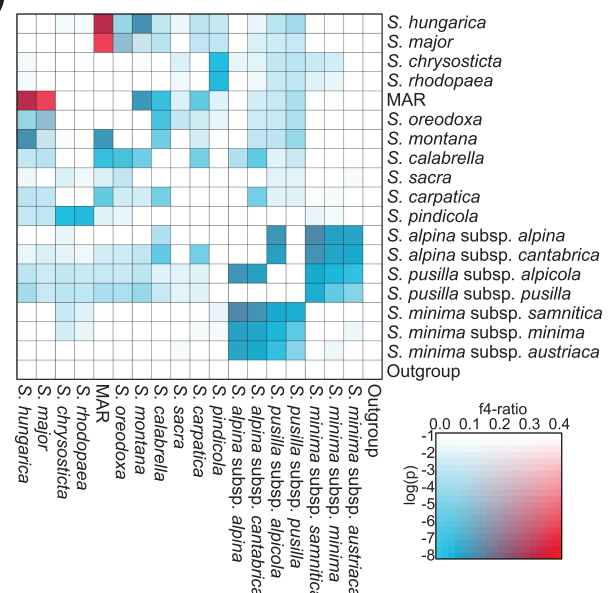


FIGURE 3. Results of D -suite statistics. (a) f_4 -branch statistics showing deeper introgressions among taxa and lineages. Gray shadings indicate untestable pairs, given our sampling and the phylogenetic tree. (b) D -statistics showing that most taxa have been significantly affected by introgression. (c) f_4 statistics showing the proportion of the genome affected by admixture. All analyses were based on Matrix_RAD_A (39,416 SNPs and 119 individuals). The polyphyletic *S. marmarossi*, *S. angusta*, and *S. rugosa* are grouped into an arbitrary taxon named “MAR” (Supplementary Figs. S2 and S3).

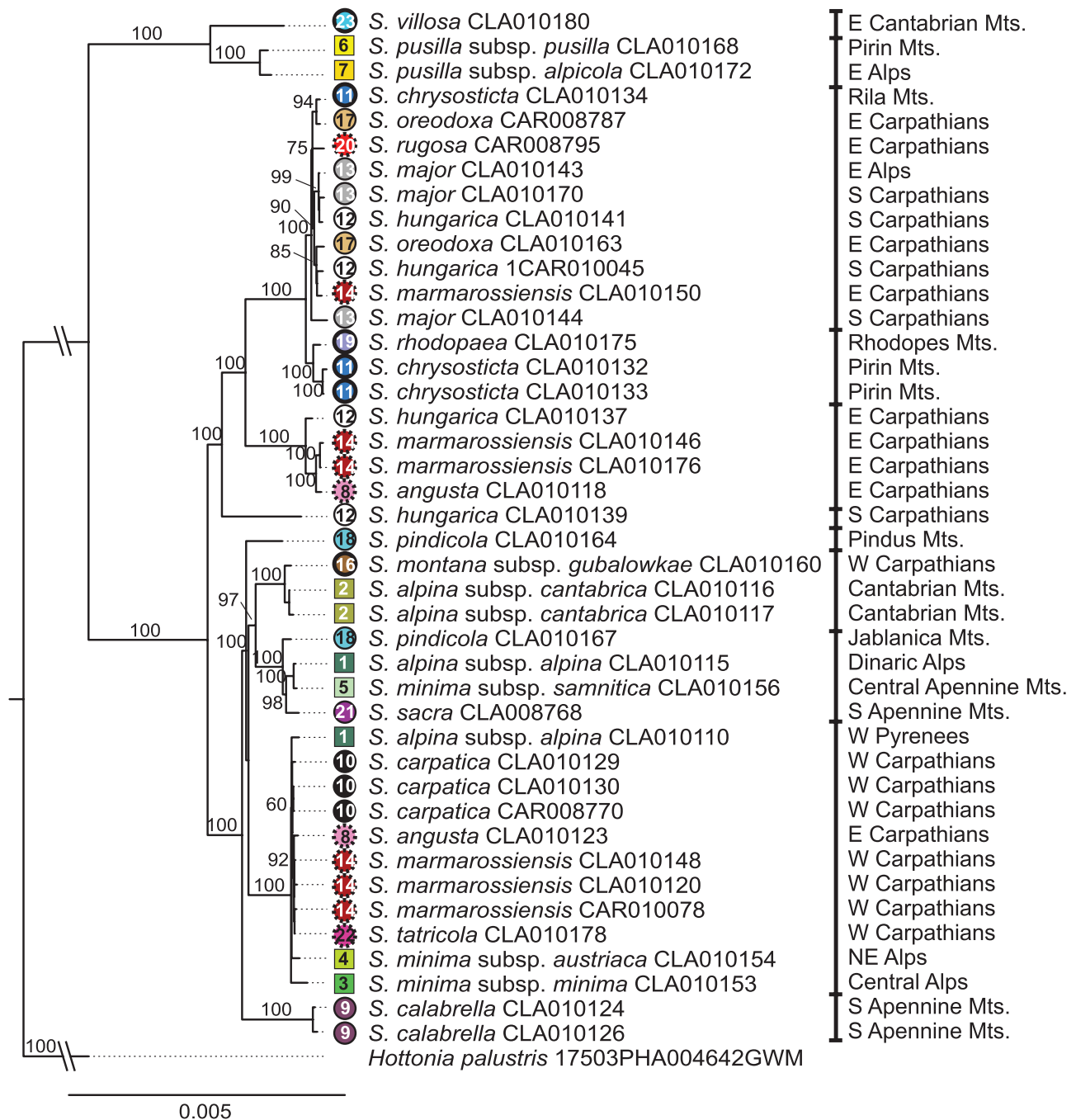


FIGURE 4. Best-scoring ML tree of *Soldanella* based on plastome data including 43 accessions and 103,787 bp (Matrix cp_B). Bootstrap support is indicated above branches. The branch lengths to the outgroup and ingroup are interrupted and not to scale. Symbols at the tips follow Fig. 1.

the employed methods are sensitive to gene flow at different time horizons. This could result in various degrees of masking of ancestral introgression across analytical approaches (e.g., Dsuite or TREEMIX versus ADMIXTURE, cf. Lawson et al. 2018). Extensive introgression was, apart from the genetic admixture, suggested also by the exceptional diversity in the number of 35S and 5S loci and their positions on chromosomes (Fig. 2a; Supplementary Fig. S10). Speciation-related

chromosomal re-patterning further increases/decreases the number of rDNA sites or their chromosomal repositioning. The dynamics of rDNA loci may be thus regarded as a strong indicator for significant hybridization processes (Raskina et al. 2008; Waminal et al. 2021). The genus underwent several descending dysploidy leading to the recurrent formation of dysploid cytotypes (from $2n = 40$ to 38) and speciation (cf. Blösch et al. 2009; Moraes et al. 2016; Singhal et al. 2017; Melichárková

et al. 2020). Although descending dysploidy can act as an instant reproduction barrier between the newly formed dysploid and its euploid parental cytotype (Mandáková and Lysak 2018; Winterfeld et al. 2018), hybridization events between dysploid and euploid *Soldanella* species were revealed by our analyses (Fig. 2b,3, and Supplementary Figs. S5, S7b, and S8b).

To our best knowledge, such a high level of within-genus hybridization has never been observed in mountain plants from the EAS. Snowbells lack specific dispersal mechanisms (e.g., anemophily, anemochory, or extensive clonality), which could otherwise promote secondary contacts among distinct populations and thus explain the extensive introgression detected. Indeed, it has been suggested that the reduced floral morphology of alpine-dwelling snowbells indicates a shift from insect pollination towards self-pollination (Steffen and Kadereit 2014), which would rather hamper such a pervasive gene exchange.

During the rapid diversification of snowbells, however, there may not have been enough time to build strong reproductive barriers between species, which might have been further weakened by repeated episodes of introgression. Importantly, the majority of snowbell taxa, especially from the CBA clade, share large ecological amplitudes that range from foothill sheltered and humid forest understory to exposed alpine meadows and snow bed communities (Štubňová et al. 2017; Valachovič et al. 2019). Recurrent elevation shifts triggered by the glacial-interglacial range contraction most likely facilitated secondary contacts of the mountain and alpine populations both within and between species (cf. Smyčka et al. 2022). In combination with the absence of inter-specific reproductive barriers, these frequent secondary interactions significantly boosted the probability of effective hybridization between snowbell populations. Nevertheless, historical range shifts did not need to play a substantial role in promoting gene flow in alpine species; at least at present they frequently occur in parapatry or sympatry (Zhang et al. 2001; Zhang and Kadereit 2002), which enhances the frequency of secondary contacts among them.

Strong Cytonuclear Discordance Reflects Introgression and Chloroplast Capture

The most blatant case of reticulation visible at first sight in the snowbell phylogeny is the cytonuclear discordance documented by the incongruent plastome (Fig. 4 and Supplementary Fig. S9) and nuclear SNP-based phylogenies (Fig. 2a, Supplementary Figs. S2, S3). Cytonuclear discordance is mostly attributed to ILS and/or hybridization (e.g., Folk et al. 2017; García et al. 2017; Pouchon et al. 2018; Heckenhauer et al. 2019; Rose et al. 2020). ILS is often prominent in rapidly radiating lineages, and affects phylogenetic inferences by a lack of hierarchical phylogenetic structuring, accumulation of speciation events in short periods, and sharing haplotypes across clades without a geographic pattern (Wendel and Doyle 1998; Acosta and Premoli 2010; Xu

et al. 2012; Pouchon et al. 2018). Nevertheless, given the present results, we suggest that the major source of the cytonuclear discordance during the evolutionary history of snowbells could rather be attributed to extensive hybridization and introgression than to ILS (see results of Dsuite statistics, Fig 3; see Malinsky et al. 2021). However, a certain level of ILS is expected, especially in young taxa. Extensive introgression among *S. alpina*, *S. pusilla*, and *S. minima* (Figs. 2b and 3, Supplementary Figs. S4 and S5) finally resulted in distinct cytonuclear discordances and their altering reciprocal positions in the nuclear and plastome phylogenies (Figs. 2a and 4, Supplementary Figs. S2, S3, and S9). Significant genetic admixture, as would be expected in nuclear signals of recent hybrid entities, was, however, not revealed in these taxa using admixture analysis (Supplementary Figs. S7a–e; cf. Acosta and Premoli 2010; Yi et al. 2015; Folk et al. 2017; Pouchon et al. 2018; Rose et al. 2020). In reality, ADMIXTURE parsimoniously describes variation between individuals, but it does not implement a historical model and assumes that all populations are descended from a single ancestral population. Under such a relatively simple scenario, identified admixture can be misleadingly interpreted or even omitted. Moreover, if all members of a certain taxon are equally admixed or affected by genetic drift, there is no variation for the model to explain, and such admixture might be missed (see Lawson et al. 2018). Furthermore, introgression traces might have vanished from the biparentally inherited nuclear genome via long-term unidirectional backcrossing, leaving signals only in the plastid genomes as a result of chloroplast capture (Soltis and Soltis 1995; Fehrer et al. 2007). More specifically, the ancient hybridization between parental species gave rise to F1 hybrids that consequently might have undergone asymmetrical, repetitive backcrossing towards only one parental species, causing the nuclear material of the other parental species to be essentially eradicated. Backcrossed populations thus maintained the nuclear material from one parent while the chloroplast was obtained from another parent via chloroplast capture (Acosta and Premoli 2010; Yi et al. 2015; Folk et al. 2017; Pouchon et al. 2018; Rose et al. 2020).

Extensive and Recurrent Introgression Facilitates but also Prevents Speciation

Hybridization can accelerate diversification via the formation of hybrid species (Seehausen 2004; Litsios and Salamin 2014; Stankowski and Streisfeld 2015; Sefc et al. 2017; White et al. 2020), but it can also slow down diversification via the breakdown of species reproductive barriers followed by extensive introgression. This could further lead to swamping of previously separated lineages and extinction of parental taxa via genetic swamping (Huxel 1999; Gómez et al. 2015; Todesco et al. 2016). We did not identify key hybridization events that would directly trigger the evolution of some snowbell clades as shown in other organisms (Meier et al. 2017; Irisarri et al. 2018; Svardal et al. 2020). Nevertheless,

we uncovered several introgression events which most likely contributed to the evolution of numerous snowbell species, especially within the CBA clade. The cascade of diversification and speciation events facilitated by ancestral introgressions might thus have significantly contributed to the emergence of most Carpathian and Balkan taxa.

The widespread *S. alpina* was traditionally considered a hybrid species, but this status has been left unsupported by previous genetic data (Zhang et al. 2001; Steffen and Kadereit 2014). The present analyses showed that this species underwent recurrent introgression with the alpine-dwelling snowbell specialists *S. pusilla* and *S. minima* (Fig. 3, Supplementary Figs. S3 and S5). Furthermore, polyphyly of *S. alpina* plastomes across the geographically delimited sub-clades implies that *S. alpina* might have hybridized and captured plastids recurrently not only from the parapatric *S. minima* and *S. pusilla*, but also from a more distant and recently allopatric species (Fig. 4, Supplementary Fig. S9). This could imply local extinctions of one of the parental taxa after hybridization events followed by long-term unidirectional introgression of the hybrid offspring with *S. alpina*, a phenomenon coined “ghost introgression” (Ottenburghs 2020). Given the foregoing, piecing together a puzzle of hybridization episodes involving *S. alpina* is still exceedingly difficult, which precludes solid conclusions as to whether or not it is a hybridogeneous entity.

Unexpected and exciting is the genetic mosaicism found in *S. pindicola* and the *S. chrysosticta*–*S. rhodopaea* clade, occupying mountains in the SE and E Balkans, respectively. *Soldanella pindicola* was likely the recipient of the genetic material from an ancestor close to *S. minima* (Supplementary Fig. S2b) and, at the same time, served as a donor of a significant proportion of the genetic material to the ancestor of the *S. chrysosticta*–*S. rhodopaea* clade (Figs. 2b and 3, Supplementary Figs. S5, S7b and S8b). Indeed, the extensive admixture found in *S. pindicola* and the *S. chrysosticta*–*S. rhodopaea* clade may be indicated also by their increased absolute genome sizes (Štubňová et al. 2017). Given the significant genetic admixture present in the genomes of all three Balkan species (see Figs 2b and 3b,c, as well as Supplementary Figs S7a,b and S8b), we can conclude that they can be considered to be of hybridogeneous origin.

Substantial introgression has been observed among the young Carpathian species, namely, among *S. major* and *S. hungarica* from the southern parts of the Carpathians, and the *S. marmarossiensis*–*angusta*–*rugosa* group occurring across almost the entire Carpathian arc (Fig. 3; Supplementary Fig. S5). Apart from the genetic evidence, extensive gene flow among these taxa and their lineages is also reflected by the astonishing diversity of their 35S and 5S karyotype patterns (Fig. 2a; Supplementary Fig. S10). The extensive gene flow resulted in pronounced weakening of their species boundaries, which finally hampered and/or slowed down their speciation processes. From an evolutionary standpoint, it appears that *S. angusta*, *S. marmarossiensis*,

and *S. rugosa*, which are currently recognized as separate species (Zhang et al. 2001; Zhang and Kadereit 2002), might represent a single polymorphic biological entity. Additional taxonomically focused research based on comprehensive population sampling is required to determine whether certain Carpathian taxa are hybridogeneous or merely highly affected by more recent introgression.

CONCLUSIONS

Despite the extensive research on the evolutionary history of plants in the EAS, there is still a gap in our understanding of how introgression has impacted the diversity and speciation of mountain plants. An unprecedented degree of introgression in comparison with other plants from the EAS was detected in almost all snowbell species. While the detected introgression potentially bolstered the formation of new snowbell species, in other cases it likely promoted reintegration of previously isolated gene pools and slowed down speciation in the young Carpathian taxa. The wide ecological amplitude of multiple snowbell taxa, growing in habitats from foothill forests to snow-beds in the alpine zone, was most likely one of the key factors facilitating allopatric lineage interconnection and gene flow.

SUPPLEMENTARY MATERIAL

Data available from the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.mcvdnck41>

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