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RESEARCH

Exploring genetic admixture in putative hybrid zones of *Pinus mugo* Turra and *P. sylvestris* L. in Slovakia

Miroslav Klobučník[1] · **Cock van Oosterhout**[2] · **Martin Galgóci**[1,3] · **Andrej Kormuťák**[1]

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Abstract

The *Pinus mugo* complex (*P. mugo* Turra s. l. or *P. mugo* aggregate) is an important fragment of European dendroflora, which is characterized by high variability, reticulate evolution, and several hybrid zone populations in different parts of Europe. Here we tried to explore the admixture structure of four putative hybrid zones between *P. mugo* Turra (s. str.) and *P. sylvestris* L. in Slovakia, using Bayesian ancestry inference with microsatellites. Unexpectedly, compared to nine reference populations, the results showed no elevated allelic diversity in our four locations. However, there was a high marker information content for ancestry based on the reference samples (δ_C or $D_K = 0.451$). The individual admixture proportions of *P. sylvestris* averaged at 0.529, 0.419, and 0.292 in the three locations, with the distribution ranging from unimodality to bimodality. These data support the presence of hybrid zones, but only in association with peatland habitats. In a typical calcicolous pine relict examined, no hybrids were detected, which contrasts earlier reports. The unexpected absence of elevated diversity, including the differences in hybrid zones' modality within the same habitat, might be explained by population bottlenecks due to anthropogenic peatland degradation during the 1960s to 1980s, when intensive drainage in the region occurred. The study highlights the evolutionary, ecological and conservation value of the studied bog populations.

Keywords *Pinus mugo* · *Pinus sylvestris* · Hybridization · Admixture · Population structure · Microsatellites

Introduction

Peatlands such as bogs, fens, and mires are very rare, threatened, and often relic ecosystems (Stanová 2000; Joosten and Clarke 2002). Owing to short-term or single-sector human priorities, they have been massively degraded worldwide. Currently, they cover around 3% of the land and freshwater surface of the planet, yet still hold 10% of global freshwater resources and the amount of carbon exceeding that of the world's forests (Joosten and Clarke 2002). For these reasons, anthropogenic peatland degradation is recognized as a global threat (Parish et al. 2008). Apart from their important water regulation and climate change mitigation functions, peatlands are of great production value as well as of non-material value for education and research (Joosten and Clarke 2002). In *Pinus mugo* and *P. sylvestris*, genetic analyses of relic bog populations may uncover their recent evolutionary processess, history, taxonomic and conservation genetic issues. These species are also very interesting because of their integrate mode of reticulate evolution.

Reticulate evolution in *Pinus mugo* Turra sensu lato (Pinaceae) made the taxonomic history of these pines particularly complex in European dendrology (Hamerník and Musil 2007). At present, this group is treated as a species aggregate of *P. mugo* Turra sensu stricto (typically shrub forming subalpine belt) and *P. uncinata* Ram. ex DC. (typically arborescent). The latter is further divided into *P. uncinata* subsp. *uncinata*, present mostly on carbonate or silicate substrates of higher altitudes, and *P. uncinata* subsp. *uliginosa* (Neumann) Businský, present specifically at peatlands in low altitudes. Apart from internal hybrids, interspecific hybridization in central and southeastern Europe has

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✉ Miroslav Klobučník
miroslav.klobucnik@savba.sk

[1] Plant Science and Biodiversity Centre SAS, Institute of Plant Genetics and Biotechnology, Akademická 2, Nitra SK-950 07, Slovak Republic

[2] School of Environmental Sciences, University of East Anglia, Norwich Research Park, Norwich NR4 7TJ, UK

[3] Faculty of Natural Sciences and Informatics, Constantine the Philosopher University in Nitra, Nábrežie mládeže 91, Nitra SK-949 74, Slovak Republic

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also been recognized outside the aggregate, e.g., between *P. mugo* Turra (s. str.) and closely related, ecologically euryvalent species *P. sylvestris* L. (=*P.* × *celakovskiorum* Asch et Graebn 1897) (Businský 2008). However, despite their taxonomic and conservation importance, the genetic admixture within most of these small populations remains to be clarified.

In Slovakia, the presence of putative hybrids *P.* × *celakovskiorum* is associated primarily with highly acidic, nutrient-poor ombrogenous peatlands (i.e., raised bogs) in Zuberec, Suchá Hora (Musil 1975, 1977a, b; Viewegh 1981), and Tisovnica (Staszkiewicz 1993), which represent extreme environments for both parental species. A few hybrid individuals have also been reported in the Obšívanka gorge, a mountainous habitat with steep limestone walls and rock formations that characterize the area (Businský 1998). The only genetic data supporting their hybrid nature comes from isozymes and genetic structure analysis in terms of Nei's population differentiation index. Interestingly, the authors suggested Suchá Hora to be a mixed stand of pure species (Maňka et al. 2015). In contrast, Klobučník et al. (2022) found a considerable admixture in this population using a dominant markers (inter-Primer Binding Sites, iPBS).

Secondary contact between divergent populations and the formation of hybrid zones has been of interest to evolutionary biologists for many decades. Hewitt was the first to refer to these zones as 'natural laboratories', as they offer unique experimental material for studying the characters and processes involved in speciation (Hewitt 1988). The distribution of genetic admixture in hybrid zones may further provide insight into the taxonomy of the parental forms. For example, with bimodal distribution, i.e., when parental-like genotypes predominate (both within and between loci, resulting in heterozygote deficits and linkage disequilibria), the parents can be considered fully separated species regardless of the species concept used. This bimodality is also characteristic of species that show strong prezygotic isolation (reviewed in Jiggins and Mallet 2000). Therefore, unimodal hybrid zones with prevalent intermediates are more likely to form at the subspecies level, or between closely related species. In case of more diverged species, a longer period of their existence would increase the opportunity for the species to coexist in sympatry and reinforcing prezygotic barriers to evolve, cf. reinforcement (Butlin and Tregenza 1997; Grant and Grant 2002; Noor 1999). Hence, estimates of individual admixture proportions (IAPs) can be very informative to elucidate the evolutionary history of complex taxa, including their hybrid zones.

In order to revise their taxonomic status, the present study explores the admixture structure of four putative hybrid zones between *P. sylvestris* and *P. mugo* (s. str.) in more detail, using nuclear Simple Sequence Repeat (SSR) markers (microsatellite DNA). The second goal is to assess the impact of reticulate evolution on the phylogeny of these populations. Thirdly, we study the within-population genetic variation to examine the extent of genetic erosion on species in this highly threatened ecosystem. Based on above, including the well-known gametophytic incompatibility of these species (e.g., Christensen and Dar 1997; Kormuťák et al. 2005; Kormuťák et al. 2008), we may expect hybrid swarm status (unimodality) for all sympatric bog populations, with higher allelic diversity compared to the parental species. Genetic data for these populations are also expected to show stronger phylogenetic signal for reticulation. By contrast, introgression favoring *P. mugo* should be more likely in the Obšívanka gorge.

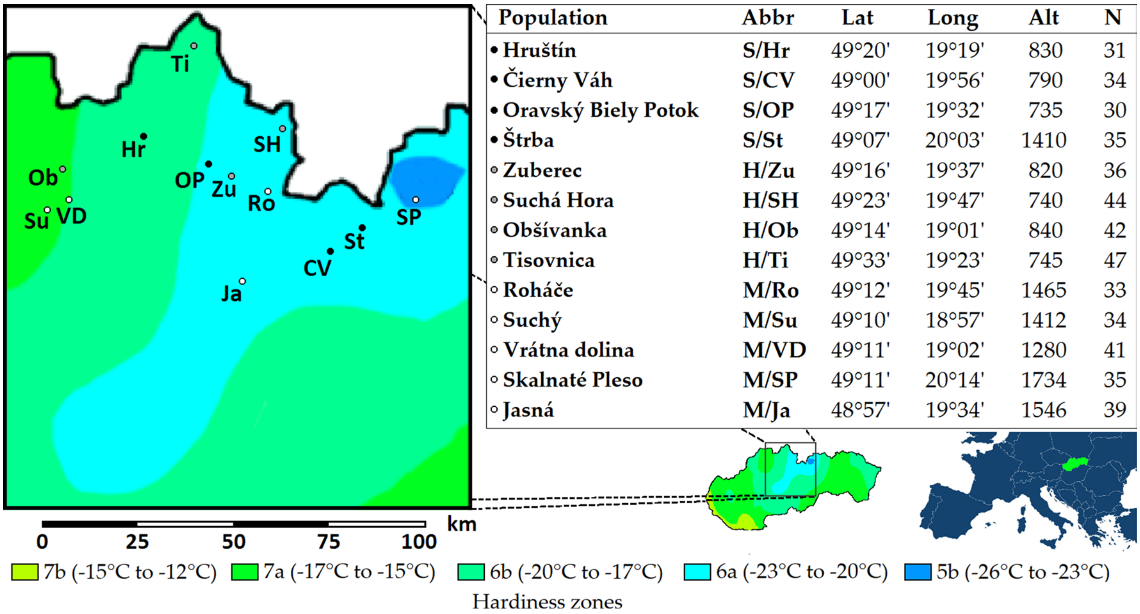
Materials and methods

Sampling and DNA isolation

In total, four *P. sylvestris* and five *P. mugo* reference allopatric populations in Slovakia along with the four populations identified earlier as hybrid zones (Klobučník et al. 2022) were subjected to SSR analysis. The reference populations have originated from the typical habitats of *P. sylvestris* in Hruštín, Čierny Váh, Oravský Biely Potok, and Štrba, while those of *P. mugo* from the locations Roháče, Suchý, Vrátna dolina, Skalnaté Pleso, and Jasná. Each of these populations was presumed natural based on personal communications with nature conservationists. The putative hybrid zones in Nature Reserves (NR) Medzi bormi (in Zuberec), Rudné (in Suchá Hora), and Tisovnica (in Oravská Polhora) represent 'degraded raised bogs still capable of natural regeneration' (NATURA 2000 habitat code: 7120). The last one, Obšívanka (NR Tiesňavy, Terchová), is a gorge-like area representing 'Carpathian relict calcicolous *Pinus sylvestris* forests' (NATURA 2000 habitat code: 91Q0) (State Nature Conservancy of Slovak Republic 2023) (Fig. 1).

The samples were collected from young needles from May to August, 2017–2018. The sampling was done randomly with respect to individual's phenotype. The pre-defined reference populations were sampled with a minimum distance of 15 m between individuals to reduce the risk of clonality. In the putative hybrid zones, a special emphasis was given to sampling all phenotypic variation due to limited population sizes. Here, the samples were collected all over the area, up to about 10 ha in Tisovnica.

Preliminary grouping of needle samples was done during needle collection based on visual assessment of individual tree habitus. Monocormic (single-stemmed) arborescent forms (4–25 m tall) were presumed to be pure *P. sylvestris*,



Population	Abbr	Lat	Long	Alt	N
• Hruštín	S/Hr	49°20'	19°19'	830	31
• Čierny Váh	S/CV	49°00'	19°56'	790	34
• Oravský Biely Potok	S/OP	49°17'	19°32'	735	30
• Štrba	S/St	49°07'	20°03'	1410	35
◦ Zuberec	H/Zu	49°16'	19°37'	820	36
◦ Suchá Hora	H/SH	49°23'	19°47'	740	44
◦ Obšívanka	H/Ob	49°14'	19°01'	840	42
◦ Tisovnica	H/Ti	49°33'	19°23'	745	47
◦ Roháče	M/Ro	49°12'	19°45'	1465	33
◦ Suchý	M/Su	49°10'	18°57'	1412	34
◦ Vrátna dolina	M/VD	49°11'	19°02'	1280	41
◦ Skalnaté Pleso	M/SP	49°11'	20°14'	1734	35
◦ Jasná	M/Ja	48°57'	19°34'	1546	39

Fig. 1 Locations of pre-defined populations on a map of northern Slovakia. Abbr – Population abbreviations (S – *P. sylvestris*, H – putative hybrid zones, M – *P. mugo*); Lat – latitude (N.); Long – longitude (E.); Alt – altitude (m); N – sample size (map adapted from Matej Games, CC BY-SA 4.0<https://creativecommons.org/licenses/by-sa/4.0>, via Wikimedia Commons)

and polycormic (multi-stemmed) shrub forms (2–3 m tall) were assessed as pure *P. mugo*. Transitive forms were defined primarily by a combination of these two traits. When arborescence and polycormy were combined into a single phenotype (4–15 m tall), the individual was labelled as an intermediate, with a similar genetic contribution from each parental species. Introgressive subforms were determined by a higher resemblance to one of the species, using other characteristics such as morphology and colour of needles, cones, and bark (e.g., monocormic *P. sylvestris*-like morphotypes lacking typical orange bark on the top of a tree were presumed introgressive, similar as with polycormic but taller *P. mugo*-like shrub forms).

After harvesting, the needles were stored at −81 °C and subsequently used in DNA isolation following the CTAB protocol (Murray and Thompson 1980). For assessing DNA integrity and concentration we used 1% agarose gel (1×TBE) with 0.5 µg.ml-1 ethidium bromide and NanoDrop spectrophotometer (BioSpec-nano, Shimadzu). To compare the results from different methodologies, the DNA samples were the same as those used in our previous study based on iPBS approach (Klobučník et al. 2022).

Genotyping

Genotyping was carried out for seven SSR marker loci, namely PtTX2146 (Auckland et al. 2002), SPAG7.14 (Soranzo et al. 1998), LOP1 (Liewlaksaneeyanawin et al. 2004), PtTX3025 (Elsik et al. 2000), PSYL36, PSYL42, and PSYL57 (Sebastiani et al. 2012) (Some of these markers were also tested by our colleagues in a previous study, Koubová et al. 2014). The former three were amplified separately in singleplex polymerase chain reactions (PCR) by the Schuelke approach (Schuelke 2000), using M13(-21) primer labelled with HEX, NED, and PET, respectively. Multiplexing was done for the remaining four marker loci. Details of the primers with PCR concentrations and annealing temperatures are in Table S1 (Supplementary material). All PCR reactions were carried out in TProfessional Thermocycler (Biometra) with HOT FIREPol® DNA polymerase (Solis BioDyne), dNTP Mix (Thermo Fisher Scientific), and with the same source of PCR Grade Water (Solis BioDyne). Conditions of both singleplex and multiplex PCRs are given in Table S2 (Supplementary material).

In fragment analysis, PCR products of PtTX2146, SPAG7.14, and LOP1 were firstly mixed in a single tube (1 µl each) with 11.5 µl of Hi-Di™ formamide (Applied Biosystems) and 0.5 µl of GeneScan™ 500 LIZ® Size Standard (Applied Biosystems). Similarly as with multiplexes, the mixtures of singleplex PCR products were denatured at 95 °C for 3 min. The samples were subsequently genotyped using a SeqStudio™ Genetic Analyzer and GeneMapper® version 4.0 (Applied Biosystems). The genotyping was done at Breeding Services of Slovak Republic, s.e., Lužianky, Slovakia.

Data analysis

PopGene 1.32 (Yeh et al. 1999) was used to estimate within-population genetic parameters including allelic richness (observed number of alleles, n_a), allelic diversity (effective number of alleles, n_e), observed and expected (unbiased) heterozygosities (H_O, uH_E), heterozygote deficiency or excess (F_{IS}), Hardy-Weinberg equilibrium (HWE) departure, and two-locus linkage disequilibria (*LD*). All input files were generated with GenAlEx 6.51b2 (Peakall and Smouse 2012). Private alleles (n_p) were calculated manually. To test for significant differences in genetic diversity at both population and taxonomic levels, these parameters were tested by nested ANOVA in R 4.1.1 (R Core Team 2021) (hybrid zones were expected to show an elevated allelic diversity, especially n_p, with higher F_{IS} and *LD* in case of bimodality). Among loci, the deviations in F_{IS} were tested by one-way ANOVA to investigate a possible outcome of assortative mating and/or selection. Two loci that showed evidence of significantly higher F_{IS} (SPAG7.14, LOP1) were examined further. We note that the lack of technical replicates has some limitations, which we tackled using two approaches to differentiate whether the observed deviations are due to the HWE departure, null alleles, or other genotyping errors. First, the presence of null alleles was evaluated using Micro-Checker 2.2.3 (van Oosterhout et al. 2004). This software also examines other sources of error in microsatellite data, including large allele drop-out and errors due to stuttering peaks. The expected number of non-amplified samples (i.e., putative null allele homozygotes) was then calculated and compared to the observed number of non-amplified samples using a binomial test. Second, we used the program INEST 2.3 (Chybicki and Burczyk 2009) to calculate unbiased $F_{IS\ (\mathrm{INEST})}$ values in the presence of null alleles and genotyping failures (model *nfb*), and performed the Bayesian procedure of model comparison (*nfb* vs. *nb*) according the authors' recommendations (MCMC iterations = 500 000, thinning = 1000, burn-in = 50 000). INEST was also used to test for population bottlenecks in the putative hybrid zones (Wilcoxon signed-rank test for the deficiency in M-ratio, SMM model, 10 000 simulations).

Genetic structure at the taxonomic and population level was resolved using the phylogenetic software Phylip 3.698 (Felsenstein 2009), SplitsTree 4.19.2 (Huson and Bryant 2006), and Dendroscope 3.8.10 (Huson and Scornavacca 2012). First, we calculated pairwise Cavalli-Sforza chord distances between individual locations, D_{CS} (Cavalli-Sforza and Edwards 1967), in Phylip. These data were chosen as an input file for cluster analyses. The reason is that, unlike for example Nei's (1972) D_S (assuming mutation-drift equilibrium and constant effective population size) or Goldstein's (1995) $\delta_\mu{}^2$ (assuming stepwise mutation model with a large role of mutational process), the chord distance is a geometric distance, it makes no biological assumptions and has the Euclidian property necessary for many hierarchical cluster analyses (thereby providing more reliable relative measures for tree construction) (Takezaki and Nei 1996; Paetkau et al. 1997; Kalinowski 2002; reviewed in Reif et al. 2005). The clustering was done by four phylogenetic network-building algorithms that provide either implicit or explicit representation of evolutionary history. The implicit network was computed by the Neighbor-Net algorithm in SplitsTree. This method allows one to evaluate conflicting signal in the data due to different reticulate evolutionary processes such as hybridization, horizontal gene transfer, ancient gene duplication, gene loss or incomplete lineage sorting, but rarely allows an accurate identification of hybrids and their parents (Huson and Bryant 2006; Willems et al. 2014). For this reason, we also incorporated alternative methods, including the Cluster Network, Galled Network and Level-k Network algorithms using Dendroscope. These networks were constructed based on Neighbor-Joining midpoint-rooted gene trees calculated in Phylip. The threshold for consensus construction was 25% in all the three methods.

To quantify individual-based structure in terms of IAPs, we used the Bayesian clustering approach in STRUCTURE 2.3.4 (Pritchard et al. 2000). Before the clustering, intra- and interspecific population differentiation (ancestry informativeness of markers) within individual loci was measured as fixation index, F_{ST} (PopGene), and Gregorius-Roberds's differentiation index, D_K (Gregorius and Roberds 1986), respectively. Following Halder et al. (2009), markers with an absolute allele frequency difference, δ_C (Shriver et al. 1997; or D_K, which is the same as Shriver's δ_C), of ≥ 0.3 were considered as ancestry informative (AIMs).

The STRUCTURE settings included the Admixture model (*LOCPRIOR* = 1) (Hubisz et al. 2009) and Correlated allele frequencies model (Falush et al. 2003), with the number of assumed clusters (*K*) of 1 to 6 and 11 replicates for each *K*. Burn-in length was set to 100 000 followed by 500 000 Markov chain Monte Carlo iterations. For all other options, the default settings were used. The optimal *K* was determined by the method of Evanno et al. (2005) as implemented in Structure Harvester 0.6.94 (Earl and von Holdt 2012). To generate consensus results, the individual matrices of cluster membership coefficients (*Q*) were permuted by the FullSearch method in Clumpp 1.1.2 (Jakobsson and Rosenberg 2007). The resulting IAP estimates were tested for normal distribution by the Shapiro-Wilk test (Statistics Kingdom 2017), with $p < 0.05$ rejecting normality. Finally, to simplify the interpretation of the results, the values were binned into five equally sized intervals representing distinct genotype classes: pure *P. mugo* (0.0–0.2), *P. mugo*-like introgressants (0.2–0.4), intermediates (0.4–0.6), *P. sylvestris*-like introgressants (0.6–0.8), and pure *P. sylvestris* (0.8–1.0). Consistent with Jiggins and Mallet (2000), unimodality was considered the null hypothesis in evaluating hybrid zone modality.

Results

Within-Population genetic variation

The values of the population genetic summary statistics were remarkably similar across all 13 samples ('pre-defined populations'), and no significant differences were detected by nested ANOVA ($p = 1.0$ for n_a, n_e, uH_E, and 0.999 for H_O). In addition, no significant differences were detected between taxonomic groups ($p = 0.993$ for n_a, 0.839 for n_e, 0.501 for uH_E, and 0.428 for H_O). The observed number of alleles was the same in most of the putative hybrids when compared with the median values of both *P. sylvestris* and *P. mugo* reference data. This observation is noteworthy because hybrids are expected to show an elevated allelic diversity. However, only a slight increase in allelic diversity and heterozygosities was detected. In the H/Ob sample (for sample codes, see Fig. 1), the observed heterozygosity actually dropped below the reference values, despite its higher allelic diversity (H_O/uH_E ratio = 0.56/0.65 in *P. mugo*, and 0.58/0.64 in *P. sylvestris* vs. 0.38/0.73 (H/Ob), 0.63/0.77 (H/SH), 0.66/0.73 (H/Zu), and 0.67/0.69 (H/Ti) in the study samples) (Fig. 2). (All these statistics are provided numerically in Tables S3 and S4, Supplementary material).

Mating system

As with diversity, we found no significant differences in F_{IS} either ($p = 0.713$ for populations, and 0.929 for taxa). Compared to the reference samples, there was only a slightly increased median value in H/Ob but also in H/SH. A similar pattern was observed when calculating F_{IS} using INEST. The H/Ob sample was characterized by the highest number of loci with significantly positive F_{IS} (five vs. two loci in the reference samples). Moreover, this sample showed the highest number of non-random associations between alleles ($LD = 25$), although the corresponding values in both *P. mugo* and *P. sylvestris* were statistically similar (ANOVA $p = 0.145$ and 0.168, respectively). In contrast, the H/Zu and H/Ti samples exhibited low heterozygote deficiency and linkage disequilibria relative to the reference values. In LOP1, we also found a significant departure of the H/Zu sample from HWE due to negative F_{IS} (Table 1). All putative hybrid zones displayed significant or nearly significant deficiency in M-ratio, indicating population bottlenecks (Wilcoxon signed-rank test: H/Ob *Z-score* $= -2.197$, $p = 0.008$; H/Zu *Z-score* $= -1.352$, $p = 0.082$; H/Ti *Z-score* $= -1.992$, $p = 0.016$; H/SH *Z-score* $= -1.521$, $p = 0.056$).

Surprisingly, the F_{IS} values at LOP1 and SPAG7.14 were significantly positive in almost all populations as opposed to the remaining markers. As evidenced by one-way ANOVA, both loci also deviated in absolute values ($F_{(6;82)} = 8.217$ and $p < 0.001$ when the loci were included, but $F_{(4;58)} = 1.050$ and $p = 0.389$ when excluded), indicating either (i) null alleles causing the higher F_{IS}, (ii) real HWE departure due to linked non-neutral loci, (iii) or a combination of both. It is unlikely that this departure is caused by Wahlund effect because that would affect all loci.

There were a total of 14 and 6 non-amplified samples found in SPAG7.14 and LOP1, respectively. This compares to, on average, 8 non-amplifications detected also in the other loci. Non-amplification may indicate null allele homozygotes, or alternatively, samples that failed for other reasons.

Micro-Checker estimated the null allele frequency for the SPAG7.14 locus to be $r = 0.156$ (weighted mean across populations). The mean (5–95%) expected number of null allele homozygotes was equal to 12 (6–17). Assuming approximately 8 samples would have failed to amplify at this locus (the same PCR failure rate as observed in every other loci), the number of null allele homozygotes was estimated to be $14 - 8 = 6$. In other words, the observed number (6) falls just within the expected range at SPAG7.14 (6–17), providing weak evidence for null alleles causing the higher F_{IS}.

In contrast, we found only 6 non-amplified samples at LOP1, which is less than the average across the other loci. Hence, we do not have any evidence to support the null allele hypothesis in this case. The deviation of LOP1 from other markers may rather suggest a linkage with assortative mating locus (or loci). However, it was the typical habitat of the species (i.e., reference samples), including the mountainous study area H/Ob, where heterozygotes at LOP1 declined relative to the expected frequencies. Consistent with interspecific heterozygote advantage, there was no decline in heterozygotes in the bog populations, especially in H/Zu. This population was also the only hybrid zone having a private allele (171 bp at PSYL42 locus, frequency of 0.014) (Table 1).

Furthermore, real HWE departure was also supported using the program INEST by comparing the full model (*nfb*) with the pure model that accounts only for null alleles (*n*) and genotyping failures (*b*). This comparison showed that the latter better fit the data in 5 out of 13 samples (Table 1). However, the differences in Deviance Information Criterion (DIC) between these models were negligible: 1236.1 vs. 1235.8 in S/CV, 1091.7 vs. 1090.9 in S/OP, 1530.2 vs. 1528.7 in H/Ti, 1075.4 vs. 1073.9 in M/Ro, and 1172.1 vs. 1169.7 in M/SP. Additionally, no sample showed stronger support for the *nb* model when calculating $F_{IS\ (\mathrm{INEST})}$ for SPAG7.14 or LOP1 exclusively, further reinforcing the evidence against the null allele hypothesis.

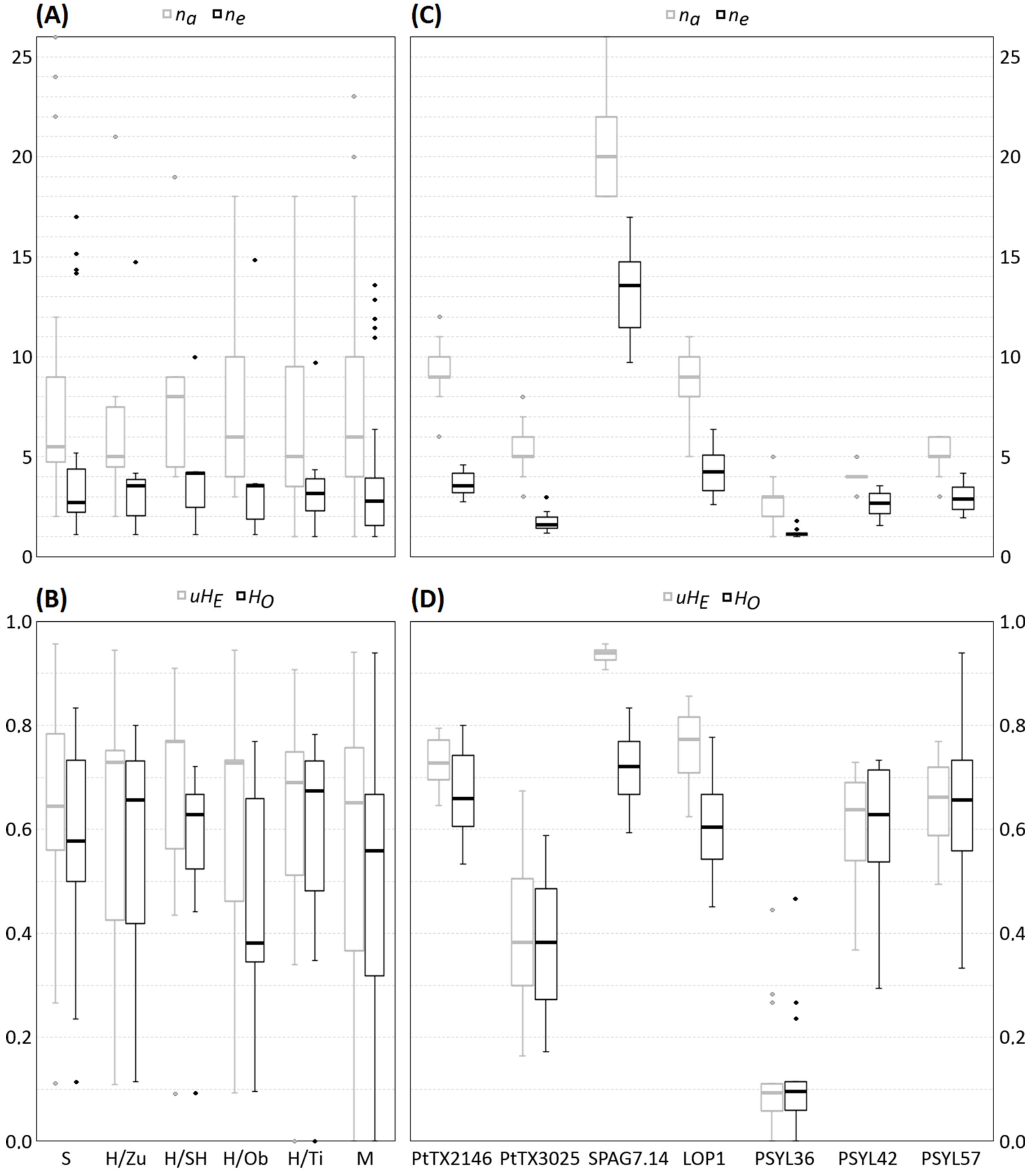


Fig. 2 Within-population genetic variation estimated for individual population samples (**A**-**B**) and markers (**C**-**D**). n_a, n_e – observed and effective number of alleles; uH_E, H_O – expected and observed heterozygosity. Shown are median (thick bar), interquartile range (box), minimum and maximum (whiskers), and outliers (dots). The symbols S and M represent *P. sylvestris* and *P. mugo* reference data for comparison with the putative hybrid zones (for sample codes, see Fig. 1)

Phylogeny and admixture structure

Three levels of genetic structure were tested in the whole dataset, i.e., species, population, and individual. The former two are described using the Neighbor-Net network in Fig. 3, which clearly distinguished between *P. sylvestris* and *P. mugo* (the original distance matrix is given in Table S5, Supplementary material). In the standard Neighbor-Joining with 1000 bootstrap replicates, the extended majority-rule consensus tree showed 99.8% of support for the *P. sylvestris* clade and 99.6% for that of *P. mugo*. However, all other nodes were only poorly supported (<75%) indicating no genetic structure at the population/location level (the data not shown). The network also shows that the putative hybrid zones clustered together in the central part between *P. sylvestris* and *P. mugo*, with H/Zu and H/SH being closer to the

Table 1 Private alleles, linkage disequilibria, and heterozygote deficiency/excess

				F_{IS}								$F_{IS\,(INEST)}$
Pop.	*N*	n_p	LD	PtTX2146	PtTX3025	SPAG7.14	LOP1	PSYL36	PSYL42	PSYL57	Median	
S/Hr	31	4	12	0.035*	0.128	0.171*	0.440*	0.040	0.040	−0.028	0.040	0.086**
S/CV	34	3	20	0.168	−0.049	0.194*	0.322*	0.104*	−0.029*	−0.027	0.104	0.071*
S/OP	30	0	7	0.162	0.093	0.115*	0.186	−0.066	−0.034	−0.136	0.093	0.053*
S/St	35	2	16	0.051	0.184	0.139*	0.140	−0.049	−0.059	−0.035	0.051	0.084**
S											0.072	
H/Zu	36	1	9	−0.051	−0.075	0.315*	−0.080*	−0.061	0.046	−0.008	−0.051	0.034**
H/SH	44	0	7	0.133	−0.028	0.199*	0.208	−0.033	0.080	0.113*	0.113	0.051**
H/Ob	42	0	25	0.118*	0.056	0.175*	0.061*	−0.040	0.286*	0.539*	0.118	0.126**
H/Ti	47	0	4	0.032	−0.036	0.249*	0.089	N/A	−0.051	−0.099	−0.002	0.025*
M											0.058	
M/Ro	33	3	18	0.149*	0.076	0.166*	0.305*	N/A	−0.089*	−0.295*	0.113	0.035*
M/Su	34	0	4	−0.036	−0.064	0.270*	0.193*	−0.030	0.349*	0.012	0.012	0.079**
M/VD	41	1	13	0.083*	−0.065	0.221*	0.268*	−0.029	0.104	0.150	0.104	0.098**
M/SP	35	1	9	0.062	−0.200	0.359*	0.209*	−0.015	0.054*	−0.178*	0.054	0.038*
M/Ja	39	2	19	−0.035*	−0.061	0.310*	0.150	−0.049	−0.102	0.005	−0.035	0.081**

Note: *N* – sample size; n_p – number of private alleles; *LD* – Burrow's composite measure of linkage disequilibria between pairs of loci ($p < 0.05$; chi-square $df = 1$); F_{IS} – heterozygote deficiency/excess (* $p < 0.05$ for the chi-square test of HWE); $F_{IS\,(INEST)}$ – unbiased F_{IS} corrected for null alleles and genotyping failures (* significant based on 95% highest posterior density interval, with ** indicating stronger support for the inbreeding model (*nfb*) over the *nb* model, which accounts only for null alleles and genotypig failures)

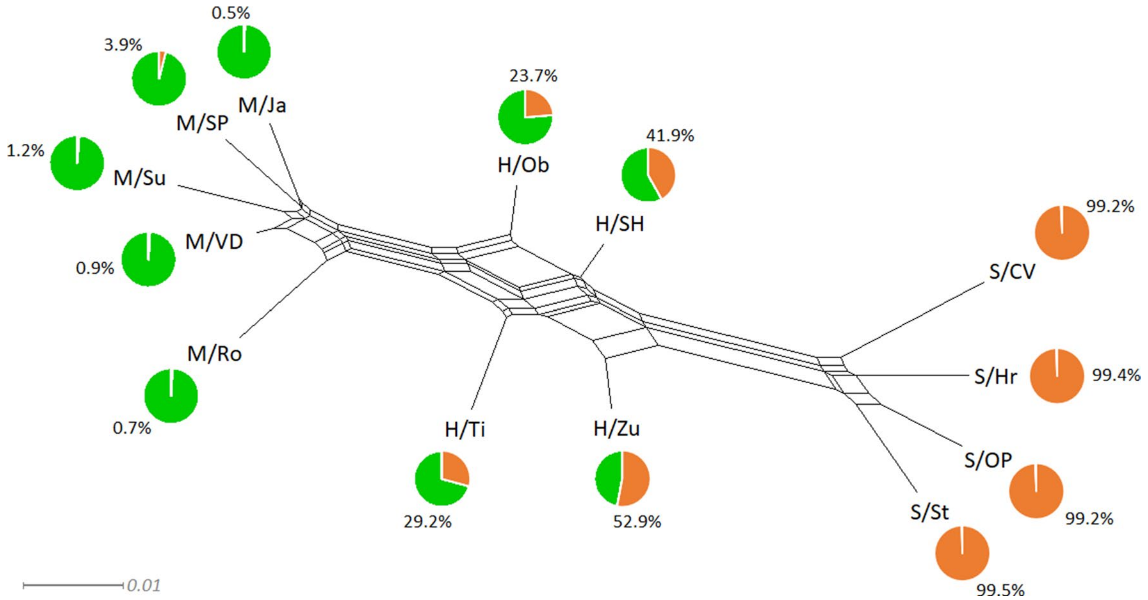


Fig. 3 Neighbor-Net split network generated based on Cavalli-Sforza distances among sampled locations. For sample codes, see Fig. 1. The corresponding pie graphs show the average admixture of *P. sylvestris* for each location, as revealed by STRUCTURE analysis

former. The presence of multiple splits and parallel branching indicates that the data possess some amount of phylogenetic signal that cannot be explained by a single tree-like topology (Fig. 3).

A more explicit approach to investigate phylogenetic incongruence and potential hybridization is presented below. Here, we used the cluster network algorithm that shows the clusters in a *hardwired representation*, the galled network with topologically restricted *softwired representation*, and the level-k network that further minimizes the number of reticulations that may explain the conflict. Interspecific reticulation was confirmed only for the H/Ti lineage, as shown by all the three networks. H/Zu and H/SH were grouped at the bases of the trees, with no reticulation in their ancestry. However, the average admixture was the most intermediate in these locations (see the next section). The most recent divergence was found for the H/Ob sample that clustered closer to *P. mugo* (Fig. 4).

Overall, the phylogenetic reconstruction confirmed our hypothesis that we have two distinct species in the data, but there was no evidence for the genetic structure at the population level. This situation including the network topologies was very similar when the two loci, SPAG7.14 and LOP1, with significant heterozygote deficiency relative to other markers, were excluded from the dataset (the data not shown).

Next, we performed an admixture analysis using the Bayesian individual-based clustering in STRUCTURE. All markers (except PSYL36) were found to be informative for this analysis. For example, the genetic structure of *P. sylvestris* ($F_{ST\ (S)}=0.027$) and *P. mugo* ($F_{ST\ (M)}=0.029$) were both low, but for all these samples, it was moderate ($F_{ST\ (SM)}=0.107$). The interspecific allele frequency differences averaged at $D_{K\ (S\text{–}M)}=0.451$ across loci, which is higher than the cutoff value for AIMs ($D_K = 0.3$) determined by previous empirical studies (see Halder et al. 2009). The proportion of markers with $D_{K\ (S\text{–}M)} \geq 0.3$ was 6/7 (85.7%) (Table 2). (The most, and particularly informative, were PSYL57 and PtTX2146. This is also evident from the major allele frequencies given in Table S6, Supplementary material). Therefore, we did not remove any loci to speed up the simulations.

Two genetic clusters representing ancestral populations were found in our data, as evidenced by the highest ΔK value at $K=2$ ($\Delta K=88.67$) and its rapid decrease at $K=3$ ($\Delta K=1.12$). In the dataset with the SPAG7.14 and LOP1 loci excluded, the optimum was $K=3$. However, there was only a very little evidence to support this solution ($\Delta K=14.22$) (Table S7, Supplementary material) (According to Cullingham et al. 2020; the values of $\Delta K>53$ indicate good support for $K=2$ but for the $K=3$ scenario, the ΔK value indicating significant support increases to ~400). Hence, this alternative was not considered reliable.

With the prior information on sampling locations (*LOCPRIOR*), reference individuals were structured almost completely into the two distinct species. We found only one individual of intermediate admixture among these samples (*P. sylvestris* contribution = 0.513, location M/SP). On the other hand, a considerable admixture was found within the study locations H/Zu, H/SH and H/Ti (0.529, 0.419 and 0.292 of *P. sylvestris* average contribution, respectively) (Fig. 5).

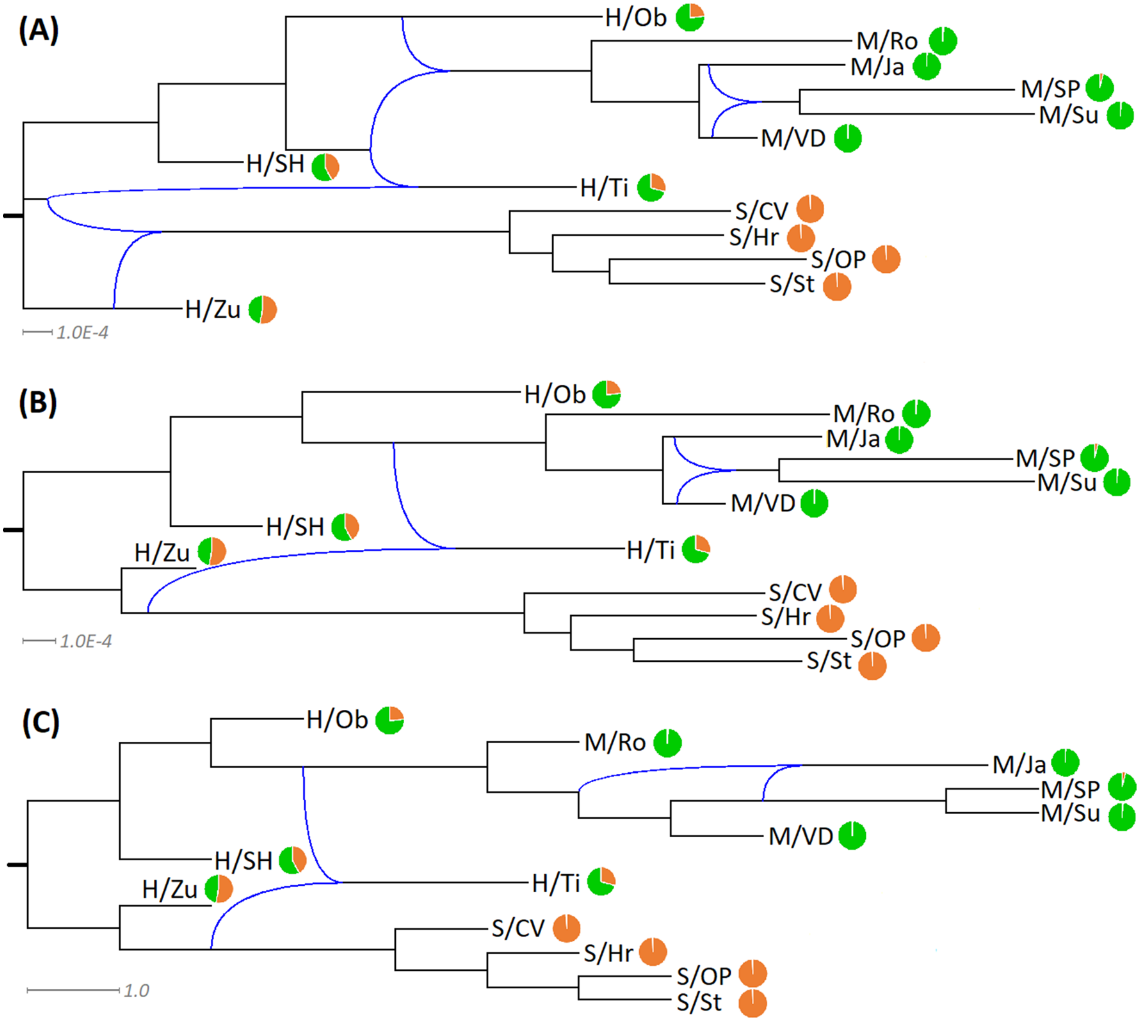


Fig. 4 Phylogenetic networks constructed using three different algorithms, based on Cavalli-Sforza distances among sampled locations. (**A**) Cluster network, (**B**) Galled network, (**C**) Level-k network. The threshold for consensus construction is 25% (for sample codes, see Fig. 1; with an average admixture added from Fig. 3)

Table 2 Intraspecific differentiation and marker informativeness for ancestry

SSR Marker	$F_{ST\,(SM)}$	$F_{ST\,(S)}$	$F_{ST\,(M)}$	$D_{K\,(S-M)}$
PtTX2146	0.113	0.026	0.016	0.689
PtTX3025	0.093	0.026	0.040	0.332
SPAG7.14	0.031	0.022	0.029	0.300
LOP1	0.086	0.030	0.033	0.548
PSYL36	0.087	0.041	0.016	0.143
PSYL42	0.154	0.027	0.038	0.442
PSYL57	0.189	0.023	0.030	0.699
Mean	0.107	0.027	0.029	0.451

Note: F_{ST} – Nei's fixation index calculated for the whole set of reference samples (SM) and for *P. sylvestris* (S) and *P. mugo* (M) groups separately; D_K – Gregorius and Roberds's differentiation index between these groups (S-M)

In evaluation of the hybrid zones' modality, we found a significant departure from normal distribution in all samples, but especially in H/SH ($p<0.001$) and H/Ti ($p<0.003$). The *p*-value for normality was relatively higher in H/Zu ($p=0.039$). This location was characterized by a much higher frequency of intermediates than the frequencies of other genotype classes, failing to reject the unimodal hypothesis. In contrast, the admixture distributions revealed a 'flat' hybrid zone status for the location H/SH and a bimodal, or rather introgressive structure for H/Ti. Finally, in the H/Ob location, the data provided no evidence to support the status of a hybrid zone (Fig. 6).

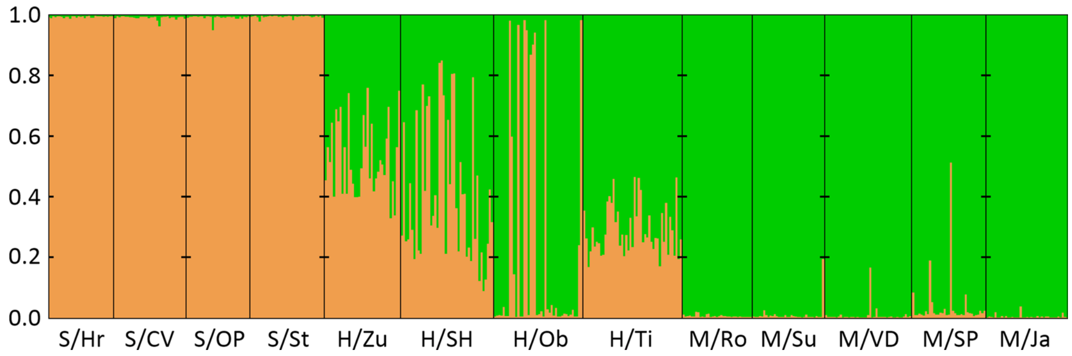


Fig. 5 STRUCTURE plot showing individual admixture proportions of 481 pines when $K=2$. The orange colour represents *P. sylvestris* ancestral population, and the green colour represents that of *P. mugo*. Individuals classified by phenotype as pure *P. sylvestris* and pure *P. mugo* are arranged from left to right, while those sampled in putative hybrid zones are in the middle (for sample codes, see Fig. 1)

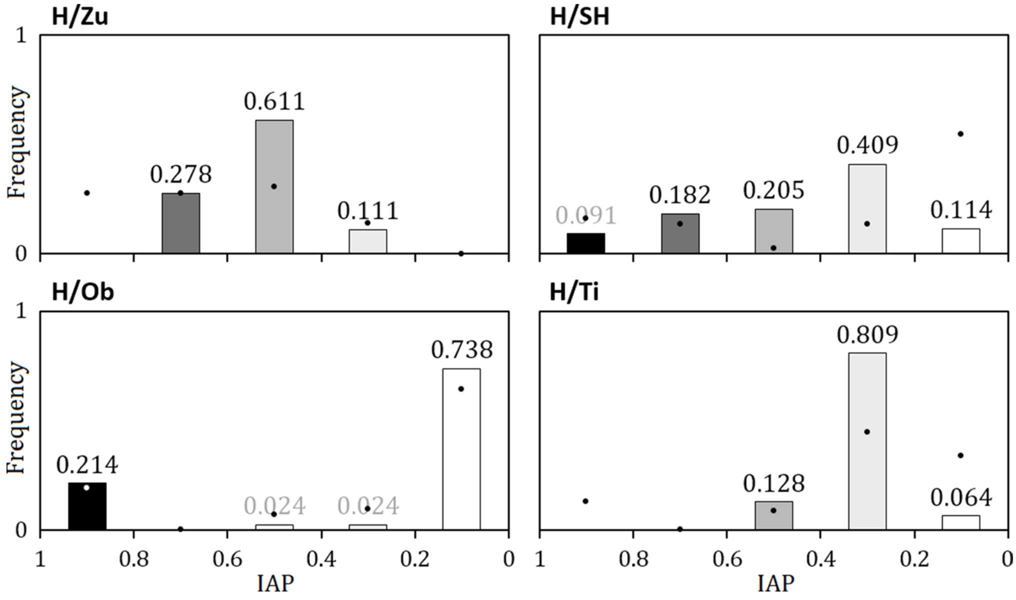


Fig. 6 Distribution of individual admixture proportions (IAP) within the putative hybrid zones, as estimated using the program STRUCTURE. The X-axis shows IAP values grouped into five genotype classes: pure *P. sylvestris* (black bars, IAP 1.0–0.8), pure *P. mugo* (white bars, IAP 0.2–0.0), intermediates (grey bars, IAP 0.6–0.4), and introgressants (dark grey, IAP 0.8–0.6; light grey, IAP 0.4–0.2). The Y-axis shows the frequencies of genotype classes for each population: Note the significantly unimodal hybrid zone structure of the H/Zu (Zuberec) population, the flat structure of H/SH (Suchá Hora), the introgressive structure of H/Ti (Tisovnica), and the mixture of pure species in H/Ob (Obšívanka). The dots correspond to the frequencies based on our preliminary phenotypic assessment of individuals, illustrating genotype-phenotype comparisons

Discussion

Taxonomy of the four pine populations in Slovakia

The *Pinus mugo* aggregate is one of the most variable and complicated group of woody plants in the history of European dendrology. According to Hamerník and Musil (2007), it comprises four growth habit categories and ten morphological subcategories representing different morphological clines. These clines range from arborescent through transitive to shrub forms, including the hybrids with *P. sylvestris* of variable habitus. The current taxonomic concept recognizes three main operational taxonomic units, *P. mugo*, *P. uliginosa*, and *P. uncinata*, the latter two being considered subspecies of the same species (Businský 2008, 2009). However, the group has not been solved satisfactorily for a century, and there are many synonyms and taxa of uncertain validity described in the past (Hamerník and Musil 2007). The reason for most complications in classic morphological approaches is a low interspecific divergence leading to potential hybridization and reticulate evolution (Lewandowski et al. 2000; Heuertz et al. 2010; Wachowiak et al. 2011, 2015a; Celiński et al. 2017; Łabiszak and Wachowiak 2021). An important confounding factor is external hybridization with *P. sylvestris*, which is reported as the main reason behind the difficulties in evolutionary and taxonomic studies of *P. mugo* aggregate (Christensen 1987a, b; Boratyńska and Boratyński 2007; Wachowiak and Prus-Głowacki 2008).

From a practical perspective, phenotypic traits alone poses other considerable problems for interpreting genetic variation of the aggregate. These traits can only indicate the presence of hybrids, and they are of no use for detailed hybrid zone analyses (Minder et al. 2007). For example, interspecific hybrids may combine parental traits, and either exhibit intermediate or transgressive trait values (Rieseberg et al. 1999), which is phenomenon observed even in animals (Nichols et al. 2015). Alternatively, hybrid genotypes may show trait values similar to those found in the parental species (cryptic hybrids) (Wachowiak et al. 2015b), probably due to dominance or epistasis.

In case of hybridization between *P. sylvestris* and taxa from *P. mugo* aggregate, one of the first significant progress in the field was achieved after development of paternally inherited cpDNA markers for tracking interspecific gene flow by pollen dispersal (Wachowiak et al. 2000, 2016; Kormuťák et al. 2004). Using these species-diagnostic cpDNA markers, introgression between *P. sylvestris* and *P. mugo* aggregate was found to be asymmetric, favoring *P. mugo* as a pollen donor (Wachowiak et al. 2006, 2016). Unfortunately, the cpDNA markers approach was also questioned by a recent discovery of exceptional, non-paternal inheritance of cpDNA (Kormuťák et al. 2017, 2018). Providing evidence for maternal inheritance of cpDNA in a controlled cross *P. mugo* x *P. sylvestris*, these studies showed that seeds containing haplotype identical to the *P. mugo* mother tree could still result from hybridization with *P. sylvestris*. This reciprocal hybridization may thus be more frequent in nature than previously assumed.

In this paper, we estimated the admixture structure of four putative hybrid zones between *P. mugo* s. str. and *P. sylvestris* (=*P.* x *celakovskiorum*) in northern Slovakia using SSR markers, aiming to define their taxonomic status. We analysed seven nuclear SSRs with a total of 105 alleles, including 17 private alleles. Although the number of markers was small, they exhibited high ancestry information content, as indicated by an absolute allele frequency difference (δ_C or D_K) of 0.451 between reference populations. This demonstrates that, despite their low numbers, the markers provided sufficient resolution to detect hybridization in the study area. Moreover, because SSR-based F_{ST} (and G_{ST}) statistics are limited by very high within-population heterozygosity (Hedrick 1999), fewer alleles at some loci may in fact enhance the statistical power to detect population differentiation necessary for admixture analyses. Additionally, this also helps reduce size homoplasy. Thus, our study fills an important knowledge gap, as genetic admixture in this region has not been formally investigated using markers other than iPBS (Klobučník et al. 2022).

By calculating IAPs (Maximum Likelihood hybrid index), the iPBS study indicated the status of *P.* x *celakovskiorum* with introgressive nature in all populations. In Zuberec, introgression was suggested from *P. mugo* to *P. sylvestris*, while the opposite was true for Suchá Hora, Tisovnica, and Obšívanka (Klobučník et al. 2022). The present SSR-based data on IAPs (STRUCTURE's membership coefficient) are only partially consistent with the previous findings. Both methods suggest an advanced introgression (i.e., only a few intermediates present) towards *P. mugo* in Tisovnica and more (SSR) or less (iPBS) 'flat' hybrid zone in Suchá Hora. The highest discrepancy is related to Zuberec and Obšívanka. Here, the results indicate that the former population is either a unimodal hybrid zone (SSR) or highly introgressed towards *P. sylvestris* (iPBS), and the latter is a mixture of pure species (SSR) or introgressed towards *P. mugo* (iPBS).

Such inconsistencies could be caused by the difference in markers' resolution (Jiggins and Mallet 2000), with more informative markers showing stronger bimodality. However, because the SSR markers were informative for ancestry, and because the level of inconsistencies varies across different populations, we suggest other explanations. Multilocus dominant markers such as iPBS provide accurate IAP estimates only in late-generation or introgressive hybrids (Buerkle 2005). Hence, our SSR-based IAPs indicating unimodality in Zuberec are likely to be more reliable. On the other hand, the frequency of intermediates in this population (61.1%) as revealed by SSRs could have been somewhat overestimated due to interspecific heterozygote advantage. Because of a low number of loci, the possible overdominance near LOP1 suggests that our IAP estimates can be more skewed by this type of selection than those from iPBS data, and that the inconsistency results also from the differences in selection among different genomic regions.

Regarding the unimodality from Zuberec reserve, it should be noted that this population is phenotypically the most obvious case of ongoing hybridization in northern Slovakia. This is evidenced by the biometry data on needle anatomy (Viewegh 1981), cone morphometry (Staszkiewicz 1996), seed quality and germination (Kormuťák et al. 2009), and abortive embryogenesis (Kormuťák et al. 2008). The unimodal hybrid zone hypothesis is also supported by the highest occurrence of polycormic but arborescent morphotypes which represent the habitus traits of both parental species combined into single individuals. Such intermediate morphotype was represented by 11 individuals sampled in this location, and only one was not confirmed to be intermediate genetically (to compare with the other sampling locations, see the genotype-phenotype comparison in Fig. 6).

Apart from this, ten individuals preliminary assessed as pure *P. sylvestris* were found to be of admixed ancestry and thus cryptic hybrids, three of them even representing introgressant of *P. mugo*.

The problem of distinguishing hybrids from pure species *P. sylvestris* and *P. mugo* was also reported by Wachowiak et al. (2015b) in the Bór na Czerwonem reserve, Poland. Using nuclear single-nucleotide polymorphism, the authors identified ten admixed individuals, but only four exhibited hybrid morphology according to the authors' phenotypic assessment, with the other five belonging to *P. mugo* and one to *P. sylvestris*. In our study, we found as many as 20 to 26 individuals in Tisovnica and Suchá Hora, respectively, that have recent admixture in their ancestry but still resemble pure *P. sylvestris* or *P. mugo*. A similar observation was also extensively reported elsewhere (e.g., Jiggins et al. 2008; Kane et al. 2009; Jasińska et al. 2010; Hamilton et al. 2013; Mitchell and Holsinger 2017; Sobierajska et al. 2020), indicating that neutral introgression may be a common phenomenon accompanying spontaneous hybridization. For example, Wachowiak et al. (2011, 2015a) found that *P. mugo* and *P. uliginosa* show zero to very low interspecific differentiation, suggesting that the polycormic/monocormic growth habit by which these taxa differ is likely determined by only a very small number of genetic loci. Alternatively, the quantitative trait loci affecting the growth habit may be co-localized on the same chromosome by multiple genetic variants, as has been found in a study analysing the genomic basis of the growth habit in peanuts (*Arachis hypogaea*) (Li et al. 2019). From this perspective, genotype-phenotype inconsistency is technically inevitable in admixture analyses for different biological reasons, as described above, and should not affect our interpretation of genetic data.

Finally, trees in the Obšívanka location resemble pure *P. mugo* or *P. sylvestris*, and only a few hybrids were suggested there (Businský 1998). This pattern is very similar to the admixture structure as revealed by SSR markers, with no contradictions between genotype and phenotype. Only three individuals of intermediate habitus but genetically same as pure species (one belonging to *P. mugo* and the other two to *P. sylvestris*) were found on this location, which can be explained by phenotypic plasticity. However, the presence of morphologically cryptic hybrids, as suggested earlier by iPBS markers (Klobučník et al. 2022), is still possible given the relative consistency between the iPBS and SSR data in Tisovnica and Suchá Hora. Considering this possibility (as well as our finding of potential linkage between the LOP1 locus and selection for intermediates in bog populations), we acknowledge that our SSR data from Obšívanka do not unambiguously prove the status of a mixed stand of pure species.

Genetic drift in hybrid zones

Peatlands are threatened and overexploited ecosystems (Stanová 2000; Joosten and Clarke 2002) that have been massively degraded worldwide. Our study shows the conservation genetic impacts, evidenced for instance, by the lack of private alleles. Such private alleles are a typical phenomenon in hybrid zones (Barton and Hewitt 1989; Golding and Strobeck 1983). The fact that they are absent in the studied bog populations could be explained by the recent emergence of these habitats, which is dated to the end of the Würm glaciation (12000–8300 years BC) (Jankovská 1997). However, private alleles might also have been lost due to genetic drift or bottlenecks resulting from anthropogenic peatland degradation in the recent past. All these ecosystems were strongly subjected to economic or forest intervention during the 1960–1980 s (Stanová 2000), except for Obšívanka which is a highland with the highest protection degree (4–5th level). In Zuberec and Suchá Hora, large parts of the stands including putative hybrids were removed for the purpose of peat extraction. This activity was later stopped due to its illegal permission. Likewise, Tisovnica is closely surrounded by commercial spruce forest, where some individuals were removed to lighten and support the spruce (Valachovič 2001; Valachovič et al. 2021). As a result, out of 100 or more hectares of peatlands, only 6 ha (Medzi bormi in Zuberec) (Stanová et al. 2015), 1.95 ha (Rudné in Suchá Hora), and 11.62 ha (Tisovnica in Oravská Polhora) of currently protected fragments were preserved (Trnka 2000).

The genetic patterns observed thus far are consistent with a population bottleneck hypothesis. Allelic diversity and observed heterozygosity within these sympatric bog populations is not higher than in allopatric stands of *P. sylvestris* and *P. mugo*. Considering the evidence for admixture from the STRUCTURE analysis, this is surprising since hybrid zones are assumed to have elevated allelic diversity (Golding and Strobeck 1983). Another surprising finding is significant differences in the modality of hybrid zones within the same habitat. According to Jiggins and Mallet (2000), bimodal zones are strongly correlated with assortative mating or fertilization within hybrid populations, resulting in prezygotic isolation. In contrast, the authors suggest that unimodal zones show little assortative mating in a diverse range of taxa, and that this is one of the main reasons for unimodality to occur. Basically, a type of hybrid zone depends on the population biology of parental species, so the modality should not significantly differ among hybrid populations of the same species when the microenvironment is comparable.

Therefore, our data do not support a specific type of hybrid zone for *P. sylvestris* and *P. mugo*. Rather, we suggest that the distribution of admixture in Zuberec, Suchá Hora, and Tisovnica was strongly affected by human-mediated bottlenecks, altering both the local diversity and structure. If true, the genetic diversity was originally here much higher compared to allopatric *P. sylvestris* and *P. mugo* populations of similar sizes. As far as structure is concerned, these natural populations could all have been unimodal before genetic drift. This is because gametophytic incompatibility seems to operate only partially between the parental species (Christensen and Dar 1997; Kormuťák et al. 2005, 2008), and it is strong prezygotic isolation that often leads to bimodality (Jiggins and Mallet 2000). Indeed, breaking down into a fluent hybrid swarm is the most likely scenario for the mating system resulting from a secondary contact of *P. sylvestris* and *P. mugo*.

Regardless of modality, however, these small population fragments in Zuberec, Suchá Hora, and Tisovnica are genetically fragile populations. The fact we see the first evidence of genetic erosion is particularly worrying because the impact on habitat fragmentation and loss tends to be felt many generations after the start of the initial population size decline (Pinto et al. 2023). Given that the generation time of tree species tends to exceed that of animal species, it is likely that other species with shorter generation times may have suffered even more severe conservation genetic consequences resulting from the destruction of the peatland habitat.

The investigated pine populations also represent suitable research objects for hybrid zone studies such as admixture mapping and searching for polymorphism that may play a role in adaptation and speciation. More specifically, they provide invaluable material for understanding differentiation in spite of high gene flow, leading to the evolution of reproductive isolation. To ensure their permanent survival, we suggest the following. First, it is necessary to stop anthropogenic activities related to peat extraction and afforestation with spruce, which is fundamental for the bog populations to sustain their genetic diversity. Second, we propose suppressing the succession of woody plants and brightening light-demanding pines on the boundaries of studied locations; considering the peat bogs' ability of natural regeneration (State Nature Conservancy of Slovak Republic 2023), this could help hybrid genotypes to expand into free ecological space, thus preventing the fixation of potentially adaptive alleles and increasing the microevolutionary potential of the populations (Rogers and Jorde 1995; von Haeseler et al. 1996; reviewed in Flegr 2018).

In summary, our genetic data unequivocally confirm the presence of hybrid zones of *P. mugo* s. str. and *P. sylvestris* (i.e., *P.* × *celakovskiorum*) at peatland locations in northern Slovakia. Furthermore, the data indicate that the hybrid zone structures differ substantially, and there is no higher allelic diversity than in allopatric pure-species stands. These observations can be attributed to genetic drift due to population bottlenecks, which constrained their genetic diversity. Therefore, although peat extraction and degradation of peatlands started in 18th century, it seems that it was the artificial peatland drainage culminating in the area during the 1960s to 1980s that significantly shaped the structures of these hybrid zones and, ultimately, their fate. The study thus represents not only a considerable contribution to the discussion on the evolution and taxonomy of *P. mugo* aggregate, including the question of hybridization, but may also serve as a basis for conservation assessment and strategies for the studied pine populations.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10592-025-01696-0.

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Author contributions A.K. conceived the idea. M.G. and A.K. harvested the plant material and isolated the DNA samples. M.K. designed the analyses, conducted the genotyping, data processing and interpretation, and wrote the manuscript. C.v.O. helped with the statistics, data interpretation, and writing of the manuscript. All authors read and approved the final version of the paper.

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Data availability Microsatellite data generated in this study are provided within the supplementary information files (SSR_data.xlsx).

Declarations

Competing interests The authors declare no competing interests.

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