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Conservation genetic evaluation of *Juniperus communis* sensu lato in Slovakia

Miroslav Klobučník[1]✉, Andrej Kormuťák[1], Ján Jurčík[1] & Martin Galgóci[1,2]

This study explores the population structure, hybridization, and adaptation of *Juniperus communis* sensu lato in the context of its current habitat fragmentation, using inter-primer binding site markers and needle morphometry. Three native juniper taxa in Slovakia were analyzed: *J. communis* ssp. *communis*, *J. communis* ssp. *nana*, and their putative hybrid, *J. communis* nothovar. *intermedia*. The results reveal a moderate overall structure (Φ_{PT} = 0.115, p = 0.001), indicating high gene flow within ssp. *communis* and low gene flow between ssp. *communis* and ssp. *nana*. However, after correcting for non-neutral loci, the inter-subspecies differentiation dropped significantly, suggesting adaptive divergence despite substantial gene flow. This conclusion was further supported using admixture regression of needle morphometry and Φ_{PT}–Q_{ST} comparison. We show that adaptive genetic differentiation may play a more significant role than phenotypic plasticity in these traits, a key consideration in preventing outbreeding depression in conservation management. Importantly, we also provide evidence for intermediate admixture and distinct selection pressures within two populations of nothovar. *intermedia* (Stolica, Kralova Studna), which may qualify their recognition as evolutionarily significant units (ESUs).

Keywords *Juniperus communis*, *Juniperus nana*, *Juniperus intermedia*, Population structure, Adaptation

Habitat loss and fragmentation are important factors threatening biodiversity. This situation is usually associated with the isolation and decline of plant populations, leading to restricted gene flow, pollination failure, the Allee effect, genetic drift, and high inbreeding levels[1]. As a result, small populations may experience an increase in homozygosity and frequency of deleterious alleles, reducing overall population fitness and adaptability[1–4]. Preservation of these small populations is important as they may hold historical genetic diversity necessary for species resilience or speciation[5,6].

Previously, most conservation studies of endangered species have focused on herbaceous and insect-pollinated species (e.g.[5,7,8]). Only a few studies have evaluated long-lived and wind-pollinated woody species with potentially high gene flow through pollen and seed dispersal[4,9]. For such species, the genetic effects of habitat fragmentation may not be apparent for a long time[10]. A good example is common juniper (*Juniperus communis* L. sensu stricto), a wind-pollinated and bird-dispersed evergreen shrub of the Northern Hemisphere. As a pioneer woodland species, it occupies natural rocky outcrops and other habitats with skeletal soils and abundant sunlight[11]. The species has the widest distribution range of all conifers but is strongly declining in North Atlantic and Central European countries, resulting in small and fragmented populations[12]. Probably, the most significant problem is the age-related decline in fertility, i.e., low regeneration by seeds[13,14]. However, the underlying mechanisms remain unresolved. Genetic studies did not reveal any genomic erosion that might explain the phenomenon well[13–16]; therefore, other explanations were suggested.

In reality, the genetic effects of habitat fragmentation are a long-term issue. During population decline, genetic drift and inbreeding cause deleterious mutations to be more frequent and homozygous, but this process can take many generations to become apparent after the cause of the decline[17]. This can be particularly important when considering the typical juniper generation time. Several authors, in this vein, have suggested that assisted gene flow interventions may be necessary for this species in the long term[1,15,16]. Additionally, species resilience could also be improved by utilizing subspecies hybridization. Because introgression can greatly surpass both mutation fitness effects and intra-subspecies gene flow, intentional admixture may be a powerful conservation tool for common juniper, which often exist in small and senescent populations. This strategy is, however, relevant only

[1]Institute of Plant Genetics and Biotechnology, Plant Science and Biodiversity Centre SAS, Akademická 2, 950 07 Nitra, Slovak Republic. [2]Faculty of Natural Sciences and Informatics, Constantine the Philosopher University in Nitra, Nábrežie mládeže 91, 949 74 Nitra, Slovak Republic. ✉email: miroslav.klobucnik@savba.sk

to systems where the risk of outbreeding depression is low[18]. It highly depends on a detailed genetic structure and admixture analysis, followed by fitness monitoring.

Our study is designed for the first stage of the process. It includes three native subtaxa: (i) the shrub-like *J. communis* L. var. *communis* (hereafter treated as ssp. *communis*, syn. ssp. *eu-communis* Syme, var. *arborescens* Gaud., var. *montana* Nielr.-non-Ait., var. *vulgaris* Ait.); (ii) the procumbent *J. c.* var. *saxatilis* Pall (hereafter treated as ssp. *nana* (Hook.) Syme, syn. ssp. *alpina* (Suter) Čelak., var. *montana* Ait., *J. sibirica* Burgsd.); and (iii) their putative hybrid *J. communis* nothovar. *intermedia* (Schur) Nyman (syn. *J. c.* ssp. *communis* var. *intermedia* Sanio). However, the hybrid nature of the latter was postulated based only on habitus and needle consistency[19], and no attempts have been made to investigate the genetic structure of the three groups included. Although some needle morphometric differences were found between the parents, nothovar. *intermedia* did not show any major phenotypic differentiation from var. *communis*[20]. In general, the authors found a stronger association of the phenotype with ecology than phylogeny, suggesting that plasticity is more important than genetic ancestry for these traits.

Therefore, our questions are as follows: (i) does the study species exhibit high population genetic differentiation, low intrapopulation genetic diversity, and high inbreeding levels in Slovakia; (ii) are the populations isolated by distance; (iii) does population size affect genetic diversity and inbreeding; (iv) what is the extent of inter-subspecies genetic differentiation, admixture, and selection; and (v) is genetic ancestry less significant in determining phenotypic divergence in needles relative to the environment (if not, are these genetic differences for the traits adaptive or comparable to genetic drift)?

Material and methods

Sampling

In total, nine sampling sites of *J. communis* L. in Slovakia were subjected to population genetic analysis. These represented four pure reference stands of ssp. *communis* (Zahrada, Cervena Skala, Priechod, Dubniky) and one reference stand of ssp. *nana* (Kralova Hola). The remaining four locations contained several or few individuals of nothovar. *intermedia* (Sumiac, Besnik, Stolica, Kralova Studna). All (sub)populations are assumed to be natural according to the botanical mapping by Futák et al.[19], Tocl[21], and other relevant authorities. The location information of the sampling sites is given in Table 1.

As a source material, we used 1-year-old needles collected from May to August 2022. Special attention was devoted to standardizing the needles' age for morphometric analysis. The sampling was performed randomly with respect to the individual phenotypes. During the sampling, the individual stands were also taxonomically identified based on their habitus and needle consistency (Fig. 1), providing a preliminary assessment of the genetic structure (Table 1). After harvesting, the collected needles were immediately measured and stored at − 81 °C until DNA extraction. A voucher specimen was deposited to the Slovak Academy of Sciences herbarium (SAV0019117, SAV0019118, SAV0019119, SAV0019120).

Needle morphometry

Three phenotypic traits were selected for quantitative genetic analysis: needle length (L), needle width (W), and the L/W ratio. The data were analyzed for six locations (C/Za, C/Du, CI/Su, CI/Be, I/KS, N/KH), with $N = 11$ to 42 (133 in total, with ten replicates per individual). The needles were photographed using a Leica MZ10 F microscope equipped with Leica 10446275 PLAN 1.0× lens and a DFC 420 C camera. Leica Application Suite EZ (LAS EZ) 4.6.1 software (Leica, Switzerland; http://www.leica-microsystems.com) was used for this purpose, utilizing a zoom factor of 0.8x. The traits were subsequently measured by the image analysis software Fiji 2.15.1[22].

DNA extraction

DNA was extracted according to the CTAB protocol described by Murray and Thompson[23].

Code	Location	Lat (N)	Long (E)	Alt (m)	Bedrock: soil type
C/Za	Zahrada	48.6389	18.0772	267	Non-calcareous sediment: loess-like soil
C/CS	Cervena Skala	48.8281	20.1906	961	Pink nodular limestone: n.s
C/Pr	Priechod	48.7803	19.2189	509	Gray layered dolomite: n.s
C/Du	Dubniky	48.5444	17.4336	259	Mixed deluvial sediment: n.s
CI/Su	Sumiac	48.8439	20.1342	962	K-feldspar-plagioclase orthogneiss: n.s
CI/Be	Besnik	48.8667	20.2286	974	Mixed deluvial sediment: n.s
CIN/St	Stolica	48.7739	20.2086	1470	Porphyritic granodiorite: n.s
I/KS	Kralova Studna	48.8788	19.0406	1255	Mixed deluvial sediment: clay-gravelly soil
N/KH	Kralova Hola	48.8838	20.1406	1698	Schist and gneissic phyllonites: n.s

Table 1. Details of the juniper sampling sites used in the study. C, N, I—preliminary taxonomic classification (C—ssp. *communis*, N—ssp. *nana*, I—nothovar. *intermedia*); For ssp. *communis*, the habitat (5130) experiences intense grazing and illegal logging. Bedrock and soil type information were obtained from the geological map of Slovakia (https://apl.geology.sk/mapportal); n.s.—not specified.

Fig. 1. Typical habitus of sampled juniper individuals. (**a**) ssp. *communis*, (**b**) ssp. *nana*, and (**c**) nothovar. *intermedia*. Photo: Andrej Kormuťák.

PCR amplification and genotyping

Genotyping was performed by inter-primer binding site (iPBS) amplification with ten primers designed by Kalendar et al.[24]. These primers were chosen according to the PCR efficiency evaluated in that study. The (single-primer) reaction mixtures consisted of ~ 120 ng of DNA, 1 × B2 buffer, 2 mM $MgCl_2$, 0.2 mM dNTP, 0.55 µM primer, 1 U of HOT FIREPol® DNA Polymerase (Solis BioDyne), and PCR Grade water (Solis BioDyne), for a total volume of 25 µL. The PCR program was initiated by a polymerase activation step at 95 °C for 15 min, followed by 35 cycles of 95 °C for 15 s, 51–63.3 °C (Supplementary Table S1) for 60 s, and 72 °C for 60 s, with

a final extension at 72 °C for 5 min. Amplification was carried out in a TProfessional Gradient Thermocycler (Biometra). Products were analyzed in 1.7% agarose gels with 1 × TBE buffer and ethidium bromide (EtBr). The gels were run at 90 V for 7.5 h in a cold room and were scanned by a BioDoc-It (UVP). Bands (loci) were identified using a 100 bp DNA Ladder (Solis Biodyne) and scored for their presence (1) or absence (0).

Reliability of the iPBS data

Before the data analyses, several precautions were taken to ensure the data reliability. First, samples of poor DNA integrity, i.e., fragmented DNA, were identified and discarded from genotyping based on 1% agarose gels with EtBr (Supplementary Fig. S1). Second, individual PCRs were tested based on intragel replicates for the repeatability of the DNA profiles (Supplementary Fig. S2–S4). Third, samples exhibiting odd profiles, i.e., with many extra or poorly visible/absent bands, were run multiple times as intergel replicates. If these replicates were not repeatable, the samples were also discarded. At the locus level, information from intra- and intergel replicates was used to clean up the binary data matrix from unstable (non-repeatable) marker loci. Fourth, negative controls were utilized in each fourth gel to check for exogenous contamination. Fifth, the whole genotyping process was performed with gel images rendered in GIMP 2.10.8[25] according to Fattal et al.[26] to reduce potential bias associated with intergel differences in overall intensity (Supplementary Fig. S5–S9). Additionally, to remove scoring subjectivity, band presence/absence was scored semiautomatically in GelAnalyzer 19.1[27] using the rolling ball background subtraction. No other manipulations, such as removing monomorphic loci, were performed.

Data analysis

Since common juniper can reproduce clonally, we first tested the dataset for clonality using the method 'Find clones' in GenAlEx 6.51b2[28]. However, as only two putative clones from different locations were suggested, they were not removed from the analyses.

Genetic structure and gene flow were quantified in several ways. First, population differentiation was calculated with Weir and Cockerham's F_{ST} analog (Φ_{PT}) using 999 AMOVA permutations in GenAlEx. This value was converted according to Wright's formula $Nm = (1 - \Phi_{PT})/(4\Phi_{PT})$ to provide an indirect estimate of gene flow. Alternatively, we used the Maximum Likelihood population reallocation test for individuals in FAMD 1.31[29], with a minimum log-likelihood difference (*MLD*) of 1 and 2. Second, we calculated Cavalli-Sforza and Edwards's chord distances between locations (D_{CE}) using Phylip 3.698[30], as they provide more reliable measures for both isolation-by-distance and clustering analyses[31–35]. The isolation-by-distance effect was tested by regressing D_{CE} on geographic distances (ArcMap 9.3[36]) using the Mantel test with 9,999 permutations (GenAlEx). Third, we assessed inter-subspecies differentiation, or marker informativeness, for phylogenetic clustering analysis. To do this, we calculated null allele frequencies using the Bayesian approach with a nonuniform among-population prior (*correction factor* = 0.01) (FAMD). Inter-subspecies differentiation was subsequently quantified as Gregorius and Roberds's absolute allele frequency difference (D_j) between the weighted means of ssp. *communis* and ssp. *nana*. Following Halder et al.[37], markers with a D_j value (i.e., Shriver's δ_C) higher than 0.3 were considered ancestry informative (AIMs). The data distribution was tested for normality by the Shapiro–Wilk test[38].

For clustering analyses, we used the galled network algorithm in Dendroscope 3.8.10[39]. The network was based on Neighbor-Joining midpoint-rooted gene trees (from Phylip) estimated for AIMs only, with a consensus threshold of 24%. At the individual level, the samples were clustered in STRUCTURE 2.3.4[40] according to the admixture model (LOCPRIOR) with correlated allele frequencies (all loci were included). The number of assumed clusters was $K = 1$ to 10, with ten replicates for each K. The burn-in length and Markov chain Monte Carlo iterations were set to 100,000 and 500,000, respectively. The optimal K was determined by the Evanno[41] and Puechmaille methods[42] in StructureSelector[43], and the consensus was provided by the LargeKGreedy algorithm (CLUMPAK)[44].

Next, we conducted admixture regression analysis using a partially linear semiparametric model (the *npplreg* function from the *np* package, R[45]), as suggested by Connor and Fuerst ('Model 3')[46]. The aim was to quantify the genetic component of phenotypic variation in the needle L/W ratio across different ancestries. It is important to note that this characteristic should not be confused with heritability, which is a different measure. To check for significant phenotypic differences among ancestry groups, we performed a mixed model nested ANOVA, followed by Tukey's post hoc test (the *lmer* and *glht* functions, R[47,48]).

Intrapopulation genetic diversity was analyzed in AFLP-SURV 1.0[49], using the Bayesian method with non-uniform prior distribution of allele frequencies. The parameters included Nei's gene diversity (*Hj*, or expected heterozygosity) and the percentage of polymorphic loci (*PPL*) at the 5% level corrected for sample size. Moreover, we estimated the inbreeding coefficient (F) with the dominant-marker-based program FAFLPcalc[50]. To investigate the genetic hypothesis of habitat fragmentation, all these measures (*Hj*, *PPL*, F) were tested for Spearman's rank correlation[51] with census population size. As for selection, we carried out two neutrality tests: Tajima's D and Fu's F_S statistics, using 16,000 simulations in Arlequin 3.5[52]. The first test compares the average pairwise difference between haplotypes within a sample (e.g., subpopulation) with the same measure but is expected based on the number of segregating loci assuming neutrality (both should be the same unless selection, population nonstationarity, or heterogeneity of mutation rates among loci occur). The second test is based on the probability of observing k or more alleles in a sample, conditioned on the observed average pairwise difference. It is especially sensitive to population expansion or departure from equilibrium and is significant only if $p < 0.02$. To identify putatively non-neutral loci, we used the F_{ST} approach with the Shapiro–Wilk test.

Natural selection was also tested explicitly for the three phenotypic traits of interest (L, W, and the L/W ratio). This was done by Φ_{PT}–Q_{ST} comparison, where genetic differentiation in a phenotypic trait (Q_{ST}) was compared with genetic differentiation based on neutral markers (Φ_{PT}). To estimate Q_{ST}, we used an approximation by P_{ST} (phenotypic differentiation) calculated with Pstat[53]. The accuracy with which P_{ST} approximates Q_{ST} depends on the ratio of narrow-sense heritability among the groups (c) to the average narrow-sense heritability within

the groups (h^2), i.e., c/h^2. These parameters were estimated using the rrBLUP method, a genomic prediction approach with a linear mixed model[54]. In this model, the phenotypic values (P) were regressed on ssp. *communis* ancestry (Q) as fixed effects, while accounting for additive relatedness (A matrix) as random effects. The A matrix was calculated by multiplying the REAP kinship coefficient[55] by two, assuming an inbreeding level of 0.1 for the kinship calculation. Finally, the resulting variance components were used to estimate the additive genetic proportion of phenotypic variation, $\sigma_u^2/(\sigma_u^2+\sigma_e^2)$, both within and between subpopulations (via the mean P, Q, and A).

Results

Genetic structure and gene flow

The final dataset included 116 marker loci and nine samples of *J. communis* (298 individuals). Overall, there was significant and moderate genetic structure ($\Phi_{PT}=0.115$, $p=0.001$). Most differences were found between the reference ssp. *nana* (N/KH) and the ssp. *communis* samples (C/Za, C/CS, C/Pr, C/Du) ($\Phi_{PT}=0.229$–0.234), with pairwise Φ_{PT} values corresponding to low differentiation within the latter ($\Phi_{PT}=0.014$–0.053) (Supplementary Table S2).

The overall level of gene flow was estimated to be $Nm \approx 1.9$. Similarly, the assignment test showed that two individuals (0.7%) were at least 100 times (i.e., $MLD=2$) more likely to originate from a location different from their sampling site, which increased to 24 individuals (8.1%) when using only a tenfold difference (i.e., $MLD=1$). Within ssp. *communis*, the number of migrants varied from $Nm \approx 4.5$ to $17.6_{(\Phi PT\ method)}$ or from $0_{(MLD=2)}$ to $11_{(MLD=1)}$ (11.2%). We found no evidence for isolation by distance in this group using the Mantel test ($r^2=0.36$, $p=0.164$) (*J. communis* sensu lato was not tested due to potential bias from inter-subspecies genetic differentiation).

The distribution of inter-subspecies differentiation across loci (D_j) is shown in Fig. 2. The histogram indicates that the data deviated from normality significantly (Shapiro–Wilk test: $W(116)=0.69$, $p<0.001$). Of the nine outliers identified, eight markers were also ancestry informative ($D_j>0.3$) and thus used for phylogenetic network analysis.

The resulting network demonstrated a clear distinction between the two subspecies, separating N/KH (the most divergent) from the reference samples of ssp. *communis*. These samples clustered together, however, forming a paraphyletic group. The clade also included CI/Be with a close relationship to CI/Su. Only CIN/St and I/KS were found to originate from hybridization between the main clusters (Fig. 4).

The observed pattern was further corroborated by the STRUCTURE results. The ΔK approach, often considered limited to the uppermost structure, strongly supported two clusters present in the data ($\Delta K>53$; see[56]) (Fig. 3a), which clearly differentiated between ssp. *communis* and ssp. *nana*. The average membership varied between $Q=0.967$–0.994 in the reference ssp. *communis* locations, while the corresponding $Q=0.008$ in N/KH (Fig. 3c). A fine-scale population structure was revealed by the Puechmaille estimators (0.5 threshold). These statistics favored $K=3$ (*MedMed K*, *MedMea K*) or $K=4$ as the less robust solution (*MaxMed K*, *MaxMea K*) (Fig. 3b), showing further structuring within ssp. *communis*. All scenarios were highly consistent among clustering runs (Fig. 3c). Notably, $K=3$ was supported as the next possible option also by the ΔK method, due to highest mean log-likelihood. However, because the study focuses primarily at the taxonomic (subspecies) level, not population membership within, $K=2$ was selected for further discussion. In other words, these ancestry estimates should better reflect the proportion of genes inherited from ssp. *communis* and ssp. *nana*.

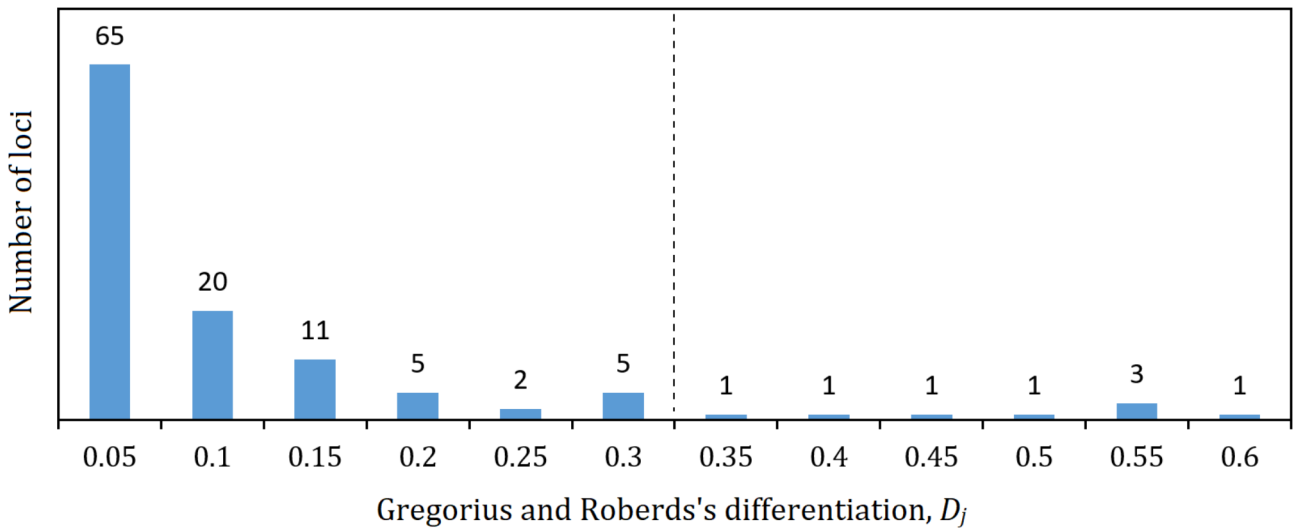

Fig. 2. Distribution of marker information content for ancestry across iPBS loci. The X-axis shows Gregorius and Roberds's allele frequency difference (D_j) between four ssp. *communis* locations and one ssp. *nana* location. The dashed line represents our threshold for considering markers to be ancestry informative.

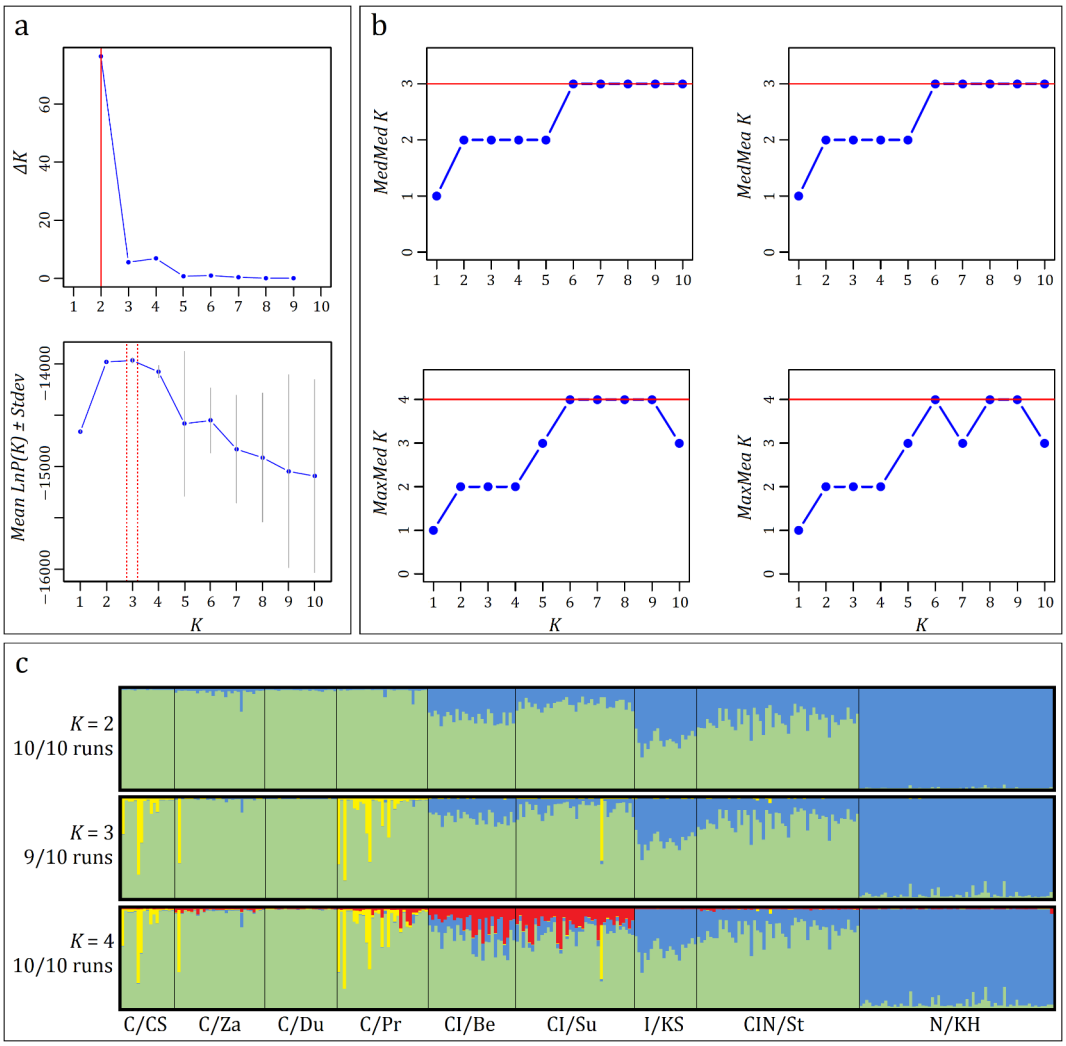

Fig. 3. Determination of the optimal *K* value. (**a**) The Evanno method: *ΔK* and mean log-likelihood with standard deviation. (**b**) The Puechmaille method: Median-of-Median (*MedMed K*), Median-of-Mean (*MedMea K*), Max-of-Median (*MaxMed K*), and Max-of-Mean (*MaxMea K*). The optimal *K* is indicated by red lines. (**c**) Best clustering results from CLUMPAK for all outcomes (*K* = 2, 3, and 4), with consistency among runs.

The highest amount of admixture was observed in the locations of nothovar. *intermedia*. Unlike the galled network, the STRUCTURE analysis detected admixture also in CI/Be and CI/Su. The frequency of intermediates, defined by a range of $Q = 0.4–0.6$, was highest in I/KS (0.800). Conversely, individuals from CI/Be and CIN/St primarily represented the introgressive form of ssp. *communis* ($Q = 0.6–0.8$), with the frequencies of 0.964 and 0.673, respectively. The least admixture among the nothovar. *intermedia* locations was found in CI/Su (Fig. 4).

The admixture regression results are shown in Fig. 5. The purpose of this analysis was to model the total needle area, as measured by the L/W ratio, and to examine the relationship and explanatory power of genetic ancestry for this trait. As indicated by the plot, we found a substantial genetic component to the phenotypic variation ($R^2 = 0.502$). The ancestry-phenotype relationship was mostly linear among the genotypes connecting ssp. *nana* and intermediates. However, from the intermediate form to pure ssp. *communis*, the predicted phenotypic values plateau nonlinearly, with no systematic increase in the trait.

This was also supported by nested ANOVA combined with Tukey's post hoc test using all four ancestry groups (C, CI, I, N). For the two traits that were significantly or almost significantly differentiated ($p_{(L)} = 0.003$, $p_{(L/W)} = 0.098$; but $p_{(W)} = 0.491$), the lowest *p* values were found in the N–C and N–I comparisons, while the I–C was highly non-significant ($p_{(L)} = 0.001$, $p_{(L/W)} = 0.115$; $p_{(L)} = 0.137$, $p_{(L/W)} = 0.262$; vs. $p_{(L)} = 0.630$, $p_{(L/W)} = 0.999$). In addition, CI was more similar to I than to C for these two traits ($p_{(L)} = 0.938$, $p_{(L/W)} = 0.662$; vs. $p_{(L)} = 0.132$, $p_{(L/W)} = 0.405$).

Intrapopulation diversity and selection

Out of all iPBS loci, 50.9% were polymorphic in both ssp. *nana* and, on average, in ssp. *communis* (36.2–73.3%). Similar values were observed across the studied locations, except for I/KS, where *PPL* reached 82.8%. No such variations were noted in the *Hj* and *F* parameters. The average inbreeding level ranged from − 0.086 in I/KS to

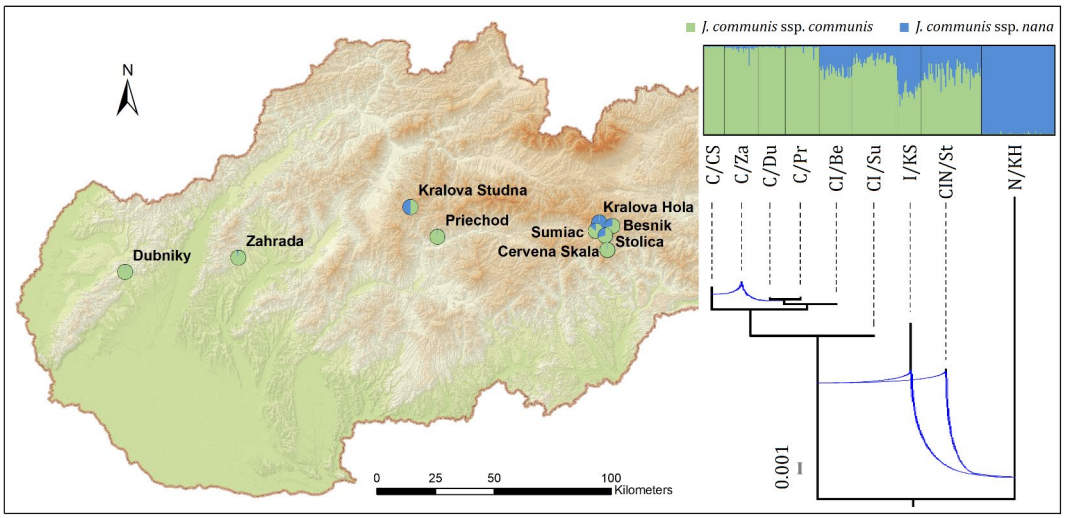

Fig. 4. Genetic structure of juniper in Slovakia as inferred by galled network and STRUCTURE analysis. The galled network shows a generalization of Neighbor-Joining gene trees estimated for eight ancestry informative iPBS markers ($D_J > 0.3$) using Cavalli-Sforza and Edwards's chord distances. The STRUCTURE plot shows individual admixture proportions for the uppermost structure detected by the ΔK approach. For samples information, see Table 1. The map was created in ArcMap 9.3 (https://www.arcgeo.sk/produkty/gis-pre-desktop/).

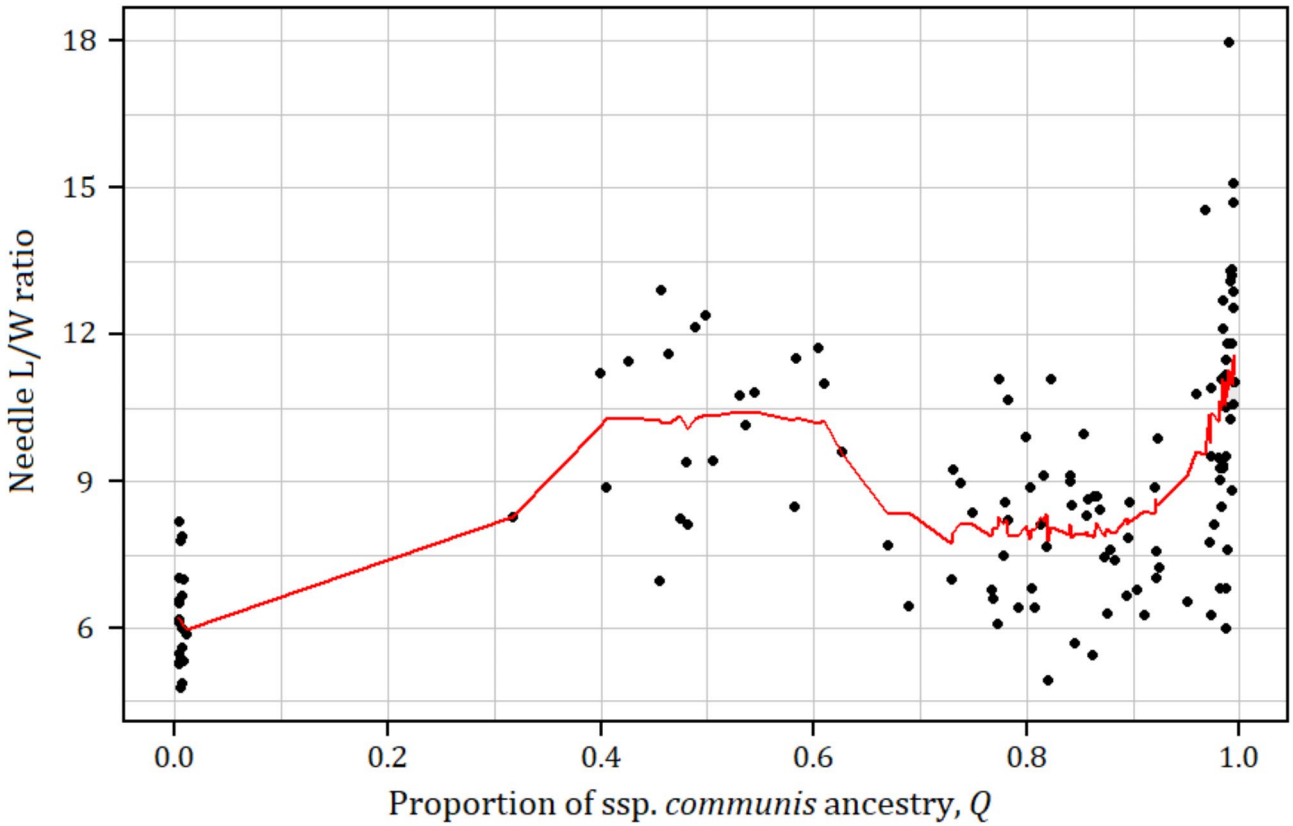

Fig. 5. Admixture regression of needle length/width ratio in two juniper taxa and their hybrid. The parental forms can be defined by the intervals of 0.0–0.2 (ssp. *nana*) and 0.8–1.0 (ssp. *communis*), while the rest corresponds to nothovar. *intermedia*. The red line represents predicted values of the trait based on a partially linear semiparametric model.

Location	*N*	*n*	*PPL*	*Hj* ± SE	*F* ± SE
C/Za	< 150	29	47.4	0.191 ± 0.015	− 0.041 ± 0.052
C/CS	< 200	17	73.3	0.202 ± 0.017	0.031 ± 0.075
C/Pr	< 800	29	46.6	0.199 ± 0.016	0.087 ± 0.064
C/Du	< 120	23	36.2	0.172 ± 0.015	− 0.051 ± 0.036
CI/Su	< 500	38	44.8	0.187 ± 0.015	− 0.007 ± 0.039
CI/Be	< 200	28	45.7	0.200 ± 0.014	− 0.055 ± 0.026
CIN/St	< 500	52	49.1	0.184 ± 0.015	− 0.047 ± 0.022
I/KS	< 150	20	82.8	0.204 ± 0.015	− 0.086 ± 0.035
N/KH	> 1000	62	50.9	0.198 ± 0.015	− 0.005 ± 0.025
Mean		33	53.0	0.193 ± 0.003	− 0.019 ± 0.018

Table 2. Intrapopulation genetic diversity estimated for several juniper locations in Slovakia. *N*—the estimated census subpopulation size; *n*—the number of individuals sampled; *PPL*—the percentage of polymorphic loci at the 5% level corrected for sample size; *Hj* ± SE—the average Nei's gene diversity across loci and its standard error; *F* ± SE—the average inbreeding coefficient across individuals and its standard error; For samples information, see Table 1.

Location	*D*	$p_{(D)}$*	F_S	$p_{(Fs)}$**
C/Za	− 0.086	0.528	− 16.517	0.000
C/CS	0.255	0.659	− 6.325	0.007
C/Pr	0.020	0.571	− 16.360	0.000
C/Du	0.229	0.651	− 12.253	0.001
CI/Su	0.451	0.737	− 24.022	0.000
CI/Be	0.311	0.684	− 14.487	0.000
CIN/St	0.597	0.783	− 24.196	0.000
I/KS	0.266	0.663	− 8.116	0.003
N/KH	0.909	0.861	− 24.137	0.000
Total	0.961	0.861	− 23.584	0.009

Table 3. Tajima's and Fu's neutrality tests for individual juniper locations and the total population. *Significant at $p_{(D)} < 0.05$; **Significant at $p_{(Fs)} < 0.02$.

0.087 in C/Pr (Table 2). We found no significant correlation between any of these variables and subpopulation size ($p_{(PPL)} = 0.746$, $p_{(Hj)} = 0.948$; but $p_{(F)} = 0.063$ for $r(7) = 0.641$).

Tajima's *D* provided no evidence of selection or demographic nonstationarity ($p_{(D)} = 0.528–0.861$). In contrast, Fu's F_S values, which are more powerful statistics[57], were all negative and significant ($p_{(Fs)} < 0.02$). The most negative values were found in N/KH, CI/Su, and, unexpectedly, in CIN/St, a location characterized by the most even admixture distribution ($F_S = -24.137, -24.022, -24.196$). For these subpopulations, the census size was estimated at 500 (CI/Su, CIN/St) or 1,000 individuals (N/KH). Smaller subpopulations (C/CS, C/Du, CI/Be, I/KS) had the least negative F_S values (from $F_S = -6.325$ to -14.487) (Table 3).

In conclusion, the tests indicate demographic expansion and/or selective sweeps. Locus-specific Φ_{PT} analysis revealed 13 outliers that deviated from the other 103 loci (Shapiro–Wilk test: $W(116) = 0.65$, $p < 0.001$). The outliers varied between $\Phi_{PT} = 0.183–0.645$, with eight corresponding to the AIMs identified earlier (Fig. 2).

Apart from the population genetic approach, evidence for adaptive genetic differentiation also comes from the Φ_{PT}–Q_{ST} comparison (Fig. 6). In these plots, we compared the neutral Φ_{PT} (Supplementary Table S3) with the P_{ST} calculated for the three needle traits, each of which was presented as a function of c/h^2 (rather than just a single value; this is because how well P_{ST} approximates Q_{ST} depends on accurate heritability estimates, which are often difficult to obtain in natural environments). The plots illustrate the potential Q_{ST} values and 95% confidence intervals, depending on different assumptions of c/h^2. All traits, especially the needle length and the L/W ratio, were found to be genetically highly differentiated relative to the neutral Φ_{PT} baseline. The $P_{ST(L)}$ and $P_{ST(L/W)}$ were significantly high across almost all the plotted c/h^2 values (from ≈ 0.1; according to our rrBLUP heritability estimates, $c/h^2_{(L)} = 0.972/0.459$, $c/h^2_{(W)} = 1.000/0.522$, and $c/h^2_{(L/W)} = 0.951/0.436$) (Fig. 6).

Discussion

Population structure and differentiation

Small fragmented populations are generally assumed to have restricted gene flow and, consequently, *high* genetic differentiation[1–4]. For *J. communis*, it was first reported in Great Britain[58] based on amplified fragment length polymorphism (AFLP) data. Although no summary statistics were provided (e.g., F_{ST}, Φ_{PT}), the authors suggested 'clear structuring' and restricted gene flow between the studied populations. The same conclusion was drawn by

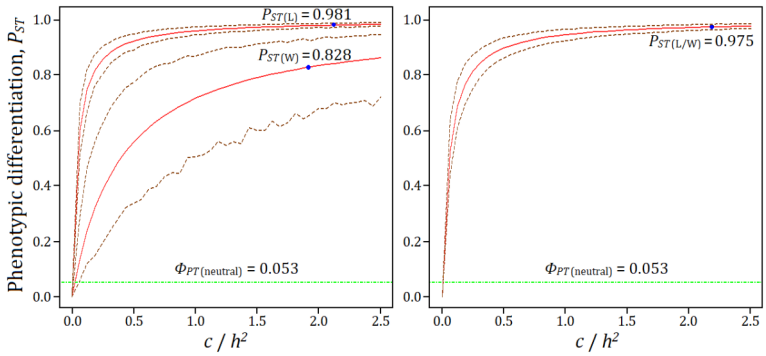

Fig. 6. Comparison of phenotypic and neutral genetic differentiation in juniper subpopulations. Needle length ($P_{ST(L)}$), needle width ($P_{ST(W)}$), and the needle L/W ratio ($P_{ST(L/W)}$) were measured. The green line represents neutral Φ_{PT}, the red line represents P_{ST} as a function of the between-subpopulation/within-subpopulation heritability ratio (c/h^2), and the brown lines depict 95% confidence intervals. The blue dots show the values corresponding to the assumed c/h^2 estimated by ridge regression-Best Linear Unbiased Prediction (rrBLUP), thus the approximations of Q_{ST}.

Provan et al.[59], who used nuclear microsatellite (nSSR) markers and found 'significant differences' between *J. communis* populations in Ireland. However, the level of differentiation was only *moderate* ($\Phi_{ST} = 0.096$), which is relatively comparable with the average estimate for biparentally inherited markers in conifers ($F_{ST} = 0.116$[60]). A similar structure was also found in our study of *J. communis* sensu lato from Slovakia ($\Phi_{PT} = 0.115$). For *J.* ssp. *communis* subpopulations, there was *low* or *no* differentiation ($\Phi_{PT} = 0.014–0.053$, $p = 0.001–0.115$), and only two genetic populations were detected with STRUCTURE: one corresponding to ssp. *communis* (with a minor cluster within) and the other to ssp. *nana*. These results are consistent with other nuclear marker data on fragmented populations from continental Europe.

For instance, low to moderate differentiation was observed in the Netherlands (allozymes $\Phi_{PT} = 0.026$[13]), Germany (nSSR $\Phi_{PT} = 0.025$[15]), Belgium (nSSR $\Phi_{PT} = 0.031$[16]), Northwestern Europe (AFLP $\Phi_{PT} = 0.100$[14]), and between Slovakia, Italy, and Norway (nSSR $F_{ST} = 0.021–0.031$[15]). Using the same markers, moderate differentiation was found even across the entire Eurasian distribution of *J. communis* sensu lato from Middle Europe to the far east of Russia and Alaska (nSSR $\Phi_{PT} = 0.098$[61]). Thus, there seems to be no indication of increased isolation among fragmented European populations compared to large populations of Asia. In fact, it is this wide scale where genetic distances were found to be significantly correlated with geography ($r = 0.574$, $p = 0.01$[61]). We found no spatial structure of ssp. *communis* in Slovakia, and there is also no isolation by distance according to geographically more extensive studies from continental Europe[13–16]. The same applies to the Irish study, where the authors nonetheless concluded restricted gene flow[59].

Gene flow between populations

In population genetics, the common rule of thumb predicts that the average level of gene flow necessary for maintaining intrapopulation diversity while allowing divergence among populations (i.e., drift-migration equilibrium) is one migrant per generation[62]. However, even though the model does not hold for real-world populations and much higher values are suggested to be appropriate for conservation management purposes (e.g., $Nm = 1–10$[63]), the connectivity in *J. c.* ssp. *communis* appears to be still *high*, despite fragmentation. In Germany, for example, Reim et al.[15] reported the average *Nm* to be 9.9. Similarly, our estimates between individual ssp. *communis* populations were 4.5–17.6 migrants per generation. These values are also comparable with the average norm estimated for conifers[64], $Nm > 3$. On the other hand, the population group of *J. communis* sensu lato (i.e., shrub-like, procumbent, and intermediate forms included) is much less connected, as indicated by the $Nm = 1.9$. The actual connection is probably even lower given that these estimates are indirect and do not separate between historical and contemporary gene flow. Consequently, because the F_{ST} approach assumes many unrealistic conditions, such as constant population size, random migration, no selection, mutation, or spatial structure, the *Nm* values are advised to be interpreted with caution[65,66].

A more direct approach is the population assignment test considering individuals' age. In their study, Vanden-Broeck et al.[14] detected from $3\%_{(MLD=2)}$ to $14\%_{(MLD=1)}$ migrants, with the majority having a shrub height smaller than 2 m, suggesting a quite high rate of recent dispersal in northwestern Europe (ssp. *communis*). According to our estimates, we may postulate a similar gene flow intensity for populations in Slovakia (up to 11.2%), but it is approximately half that value in *J. communis* sensu lato (0.7–8.1%). It is worth mentioning that 0.7–5.4% of migrants in this larger group represent dispersal events with some contact zone, and none of them directly connects the two different allopatric stands. In other words, most gene flow occurs within the taxonomic boundaries of these groups or, when hybridizing, in specific habitats.

Intrapopulation diversity

Genetic variation within populations also seems relatively high. Microsatellite estimates of allelic/genotypic diversities (i.e., H_E/H_O) in Europe are, on average, directly comparable to those in Asia, where most populations do not experience anthropogenic fragmentation. This trend also holds for the inbreeding coefficient, which

is high in all cases ($H_E/H_O/F_{IS}$ = 0.602/?/?[59], 0.84/0.59/0.29[15], 0.751/0.451/0.386[16]; vs. 0.708/0.440/0.355 in Eurasia[61]). Interestingly, Hantemirova and Bessonova[61] reported high F values even for the most numerous, undisturbed populations of Yamal (0.337) and Polar Urals (0.313), suggesting the presence of null alleles and/or the Wahlund effect. However, we believe that all SSR-based inbreeding values are likely overestimated by null alleles only, as the Wahlund effect relates to F_{ST} or F_{IT}, not to F calculated for individual sampling sites. For instance, Vanden-Broeck et al.[14] estimated the average Nei's gene diversity (Hj) and inbreeding (F_{IS}) for AFLP markers to be 0.361 and 0.172 in Northwestern Europe, which are about half the values mentioned above. In Slovakia, we observed similar diversity (Hj = 0.193) and no inbreeding (F_{IS} = −0.019) using iPBS. These findings indicate that dominant-marker based F_{IS} may be even more informative than those from SSR studies, despite certain limitations in their estimation (such as violation of FAFLPcalc assumptions, potential band scoring errors, or non-independence between loci; see[50]).

Natural selection and the causes of population decline

Overall, most studies provide no support for genomic erosion in *J. communis*, and the species, due to its adaptability and large global population, is not included on the IUCN Red List[11]. However, recent population fragmentation in many European countries (e.g., Britain[67,68], the Netherlands[13], Germany[70], Spain[71]) has led to its inclusion in Annex I of the EU Habitat Directive (code 5130). The species is now considered locally threatened, which is frequently attributed to various factors such as climate change[72], urbanisation, and intensification of agriculture[68,73]. These factors are assumed to be directly responsible for population fragmentation, resulting in a highly skewed age structure toward old individuals in some areas[74]. Like many other conifers, juniper produces a large portion of empty seeds, which requires an appropriate quantity[75] and diversity of pollen[76] for successful fertilization and cone development. With a decrease in the size and number of young individuals, populations may further suffer from limited sexual regeneration[75]. There were only 1.73–18.98% filled seeds and 0.10–5.49% seed viability reported from northwestern Europe[14]. In Belgium, seed viability ranges from 0 to 30%, with the highest germination rate of only 8.9%[16]. It is believed that overgrazing by livestock combined with limited sexual reproduction has worsened the impacts on juniper[68].

Apart from the short-term threats discussed above, fragmented juniper populations may suffer from genomic erosion due to inbreeding and drift in subsequent generations. However, the process usually takes many generations and may not be apparent for a long time. According to a pink pigeon simulation study[17], this time lag of decline in neutral diversity was estimated to be approximately 100 years, and changes in the genetic load took even twice as long to register. The average generation time of this bird was assumed to be 3.6 years. Common juniper, by contrast, has an average generation span of 20 years[11], suggesting a delay of over 550 and 1,100 years, respectively. Worth noting is that junipers are dioecious and wind-pollinated species with a tendency toward high gene flow and intrapopulation diversity[4,9,77]. It is therefore not surprising that research on common juniper has not shown the genetic impact of habitat fragmentation yet. Our data also indicate no correlation between population size and genetic diversity, as evidenced by other studies as well[13,14,16]. Only one study revealed a significant positive correlation between population size and inbreeding[16]. Moreover, both genetic diversity and inbreeding appear unrelated to the seed germination rate[16] or seed viability[14], i.e., fitness, indicating no 'realized' load of deleterious mutations.

Indeed, the situation in common juniper can best be explained by a time lag of evolutionary genetic effects of habitat fragmentation, sometimes referred to as 'drift debt'[78]. Basically, this process leads recently bottlenecked populations to lose genetic variation due to the absence of mutation-drift-selection equilibrium. Because the level of diversity at balancing selection genes unlikely reflects the actual selection regime in such populations, this variation will be partially lost while moving toward a new equilibrium under a 'no-change' scenario. This is likely why our F_S values are all negative and significant ($p < 0.02$), suggesting demographic expansion and/or selective sweeps (i.e., selection removing genetic diversity). We conclude that the drift-debt phenomenon is the most plausible explanation for these seemingly contradicting results in the fragmentation context.

Conservation strategies and management

A good understanding of the genetic dynamics of declining populations is of particular interest to conservation biologists. The growing awareness of time-delayed extinction (extinction debt) associated with habitat fragmentation also highlights the urgency of various conservation measures[79,80]. The consensus is to push against drift and inbreeding in a process called genetic rescue. For successful rehabilitation of common juniper, several authors have suggested bridging large distances between stands by 'stepping stone' populations or individuals in favorable open areas using different source populations[15,16]. These measures are intended for both the short- and long-term issues but should only be considered 'first aid'. It would be more ideal to use genomics-informed management, that is, to identify individuals with the lowest masked load for translocations and help populations counteract both inbreeding depression (realized load) and potential inbreeding depression (masked load)[81].

With respect to assisted gene flow interventions, we further suggest considering both genetic admixture and local adaptation in natural *J. communis* populations. According to our study, adaptive differentiation may play an important role in the evolution of species' infraspecifics, including ssp. *communis* and ssp. *nana*. We found no direct gene flow between the pure reference stands, and there is also evidence for directional or divergent selection according to both population (the F_S values) and quantitative genetic analyses. In woody plants, needle morphology is crucial for differentiating congeneric taxa[82] and is widely recognized as an altitudinal adaptation associated with more compact leaves in harsher environments[83]; however, there is little information on how much this variation in *J. communis* is explained by genetic ancestry[20]. Our study revealed a substantial genetic component of needle morphometry variation and provided evidence for high genetic differentiation for these traits relative to neutral differentiation. Consequently, we propose that intentional admixture of ssp. *communis*

and ssp. *nana* would be suitable only in the typical habitat of nothovar. *intermedia* to avoid potential outbreeding depression.

Taxonomic Aspect

Finally, we would like to address a few concluding remarks regarding taxonomy. While the main goal of the study was to contribute to the discussion on the conservation status of common juniper from the perspective of the Slovakian territory, delineating species boundaries is a key component of wildlife conservation[84]. Not only the listing of what deserves the attention but also the very management decisions depend on taxonomy–for example, whether gene flow should be prevented or facilitated[85,86]. This question fully relies on the accepted taxonomy, as intraspecific gene flow is often regarded as beneficial in contrast to the interspecific. Unfortunately, species boundaries are arbitrary, and because there is no unique species definition or delineation procedure, different conservationists may come to different recommendations based on the same data[87]. Therefore, we feel the taxonomic aspect requires a comment.

Juniperus communis L. is a variable species with not very consensual infraspecifics. According to Thomas et al.[12], it includes the following:

1. *J. communis* ssp. *communis*—a spreading shrub or small tree, rarely procumbent, needles mostly 8–20 × 1–1.5 mm, loosely set, sharp-pointed; usually occurring on calcareous soils but largely indifferent to the soil type
2. *J. communis* ssp. *nana*—a procumbent shrub (ca. 10 cm high), needles mostly 4–12 × c. 1.5 mm, closely set, blunt-pointed; restricted to well-drained bogs or rocky outcrops in higher altitudes
3. *J. communis* ssp. *hemisphaerica* (J. & C. Presl) Nyman—intermediate; Mediterranean and North-West African mountains
4. *J. communis* ssp. *depressa* (Pursh) Franco—intermediate; North America

Some authors also refer to ssp. *nana* by its original name, *J. sibirica*, separating it as a distinct species. This name was first mentioned by Burgsdoff in 1787 but was later accepted as a synonym of the subspecies *nana* (Hook.) Syme 1868[88]. In addition, substantial overlap between ssp. *communis* and ssp. *nana* in both phenotypic traits (wood anatomy[89], needle tip[90], monoterpene content[91], volatile distillates[92]) and genetic variation (chloroplast DNA[91], allozymes[93], RAPD[94]) led some specialists to further downgrade all infraspecific taxa into varieties. Plants of the World Online database, for instance, introduces *J. communis* var. *saxatilis* Pall. as the correct name for ssp. *nana*; however, some differentiation from the nominate was also observed[95], and *Flora Europaea* lists the taxon as *J. communis* ssp. *alpina* (Suter) Čelak.

So, which taxonomic treatment should we follow? Galtier[87] suggested that one way toward a more robust, objective, and reproducible taxonomy could be a kind of reference system. Species delineation might be achieved by comparing a particular group with reference taxa, where species boundaries are consensual and large amounts of data are available. In conifers, this might be *Pinus sylvestris* and *Pinus mugo* sensu lato, which have allways attracted the attention of European taxonomists. For instance, Łabiszak and Wachowiak[96] sequenced 48 nuclear loci (794 SNPs) and reported that the level of neutral divergence between *P. sylvestris* and *P. mugo* sensu stricto, which are closely related but distinct species, was $\Phi_{PT}=0.260$ (the authors found no signatures of selection or reproductive isolation at these markers). Between even closer entities, this value reached $\Phi_{PT}=0.068–0.088$ for *P. mugo* vs. *P. uncinata* and $\Phi_{PT}=0.056$ for *P. uncinata* ssp. *uncinata* vs. *P. uncinata* ssp. *uliginosa*. The population-level differentiation, calculated for a wide distribution of *P. sylvestris* using nSSR, varied between 0.028 and 0.033[97].

Therefore, our estimates suggest that *J. communis* ssp. *communis* and *J. communis* ssp. *nana* indeed correspond to the subspecies ranks, rather than mere varieties (neutral $\Phi_{PT}=0.081–0.103$, Supplementary Table S3). Our data also confirms that *J. communis* nothovar. *intermedia* (e.g., the populations Kralova Studna and Stolica) is a hybrid between these taxa and should be considered an evolutionarily significant unit (ESU). This is supported by the STRUCTURE results and the galled network that illustrates differences in selection in the absence of neutral differentiation. Generally speaking, gene flow appears sufficient to prevent genetic differentiation at neutral loci but insufficient to prevent genetic differentiation for adaptive phenotypic traits. However, common garden or reciprocal transplant experiments would be critical for further examination of this observation.

Conclusions

To our knowledge, no genetic study has yet analyzed the population structure and hybridization of *J. communis* var. *communis*, *J. communis* var. *saxatilis*, and *J. communis* nothovar. *intermedia*. Therefore, this study represents the first investigation into these important biological aspects of the species for conservation management. Consistent with other European studies, we found that var. *communis* is likely well connected by gene flow in Slovakia, and the populations are not genetically eroded compared to large, undisturbed populations in Asia. The time since habitat fragmentation began was probably too brief to have any genetic consequences for the species; however, the genetic load may still compromise population viability in the future. Furthermore, our findings provide the first evidence for both adaptive and non-adaptive divergence between var. *communis* and var. *saxatilis* (or rather ssp. *communis* and ssp. *nana*), including the support for the hybrid nature of nothovar. *intermedia*. It is, therefore, essential to adopt appropriate conservation measures that consider both local adaptation and taxonomic boundaries. The question of source material for plant translocation can be addressed through genomics-informed management, by identifying specific individuals for interbreeding, such as those with the lowest genetic load. Since only SSR and PCR-based markers have been employed thus far, further research utilizing population genomics is imperative.

Data availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author contributions

M. G. and A. K. harvested the plant material and isolated the DNA samples. J. J. conducted the measurement of needle characteristics. M. K. helped with the DNA isolation, conceived the idea, designed and performed the analyses, and wrote the manuscript. All authors read and approved the final version of the manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Approval for collecting samples

The *Juniperus communis* L. samples were collected with the necessary permissions from the District Office Banská Bystrica under the file number OU-BB-OSZP1-2022/015915-010. All sampling procedures complied with relevant regulations and guidelines.

Additional information

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Correspondence and requests for materials should be addressed to M.K.