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ORIGINAL RESEARCH PAPER

Conservation of *Primula farinosa* in Poland with respect to the genetic structure of populations

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Abstract
The bird's-eye primrose (*Primula farinosa* L.) is an endangered species in Poland. The sole remaining, and critically endangered, population of approximately 300 flowering plants is located in the Beskid Sądecki Mountains (Jaworki, Poland). The genetic investigation was performed using highly variable microsatellite markers on a total of four populations: the Polish population and its three nearest known Slovak counterparts. We hypothesize that the Polish population is a relic of the previously much wider Central European mountain/lowland range and is thus genetically distinct from the rest of the Slovak stock. Clarification of this issue is needed before active protection interventions such as artificial supplementation can be carried out. Our results, particularly those from STRUCTURE Bayesian clustering, showed clear population structure within the dataset: all three Slovak populations were dominated by one genetic group, while the Polish population comprised mostly individuals dominated by the second genetic group. Only limited gene flow was observed between the Polish and Slovak populations. This demonstrates the distinct genetic makeup of the Jaworki population, which is probably the result of prolonged isolation from the rest of the *P. farinosa* range and admixture of genes from various lineages. Their origin could be determined by including plant material from the rest of the *P. farinosa* range, i.e., Scandinavian, Baltic, and Alpine/Carpathian locations, in a future study. The immediate practical application of our results is the recommendation that all supplementation interventions to the Jaworki population must be limited to plants produced from locally collected seeds.

Keywords
diversity; genetic melting pot; microsatellite markers; relict population

Introduction

The genetic diversity of a species is one of the most important factors affecting its ability to respond to changing environmental conditions, therefore the preservation of sufficient genetic variation within protected populations of endangered species is a crucial element in plant conservation [1,2]. However, a conflicting obligation is the need to retain the specific genetic makeup of a population, resulting from its adaption to the local ecological conditions. These opposite principles of plant conservation have an immediate practical implication: the question concerning the source of plant material

used in the restoration (particularly augmentation) process: from the mother population – securing the local genetic resources, or from the foreign population – enlarging genetic variation and plant resilience. This question can be answered only with proper knowledge on the genetic structure of a given population in relation to other populations of the species. Consequently, to ensure proper genetic management, any restoration activity should be preceded by investigation of population genetic structure [3,4].

Primula farinosa L. (bird's-eye primrose) is a hermaphroditic perennial herb [5]. It is distylous with long-styled (pin) and short-styled (thrum) morphs. It flowers in May and its fruits mature in July. Individual plants produce 3 to 15 pink flowers in an umbel at the top of a stem. The flowers are pollinated mainly by butterflies and sometimes by bees and bumble bees [2]. *Primula farinosa* has been described as light-loving [6] and is rarely found at sites with less than 40% natural illumination [7]. It is most commonly found on wet soil in eutrophic mountain marsh on marshy clay loam soil. The marsh sward of *P. farinosa* is not fully compact, having visible gaps with exposed ground. During the plant flowering period the sward is 20 cm high with an additional layer of moss covering 80% of the surface [6]. In Poland, *P. farinosa* is a rare, protected [8], and critically endangered species. It has been included in the *Polish red data book of plants*, the *Polish red list of pteridophytes and flowering plants*, and the *Red Data Book of the Polish Carpathians* [9–11]. In summer, the plants are infected by a parasitic fungus, *Urocystis primulicola* [12], that damages seed heads.

The natural distribution of *P. farinosa* is in Europe, from central Sweden to central Spain. Its distribution range is, however, divided: the northern distribution includes southern Scandinavia, the eastern Baltic lowlands, and the central part of Britain, while the southern, mountain, distribution encompasses mountain ranges of Central and Southern Europe. According to recent phylogeographic reconstructions [13], the species survived the last glacial maximum (LGM) in isolated regions of Southern Europe in the proximity of glaciated mountain systems: the Carpathians, Alps, and Pyrenees. Among populations originating from these three ranges, the Carpathian population is characterized by the highest number of private alleles. All the dispersed populations, located throughout the Middle European Plain between the two main distribution areas (i.e., mountain ranges of Central/Southern Europe and northern populations), catalogued to date are relict, and most of these are now extinct [9,11,14], most probably outcompeted by more expansive species or destroyed by anthropogenic pressure [13].

Primula farinosa no longer exists on any of the nine lowland sites in Poland where it had previously been found [15,16]. The last existing mountain site, discovered in 1959, is in the Radziejowa range of the Beskid Sądecki Mountains in Szczawnica municipality, Lesser Poland Province, southern Poland [16]. This population occurs at the edge of the isolated northernmost part of the southern Carpathian range of *P. farinosa* and is presumably a remnant of the previously much wider Central European range. This population of *P. farinosa* is endangered, as the number of flowering specimens has decreased from 350 in 2004 to only 158 in 2011 [16]. Such a decline in the population size may have resulted from a change in habitat conditions, particularly the deterioration of soil hydrological conditions at the site, spatial isolation, fungal diseases (especially *Urocystis primulicola* infection) and tourism-related anthropogenic pressure [16,17].

As this population is critically small and isolated there is a risk of loss of genetic diversity in the population through the bottleneck effect. Also, its long geographic isolation (surrounded by forests and separated from neighboring populations by a mountain range) most probably resulted in development of a unique genetic makeup shaped independently from the rest of the species' southern distribution range. This makes the population scientifically valuable and particularly worthy of preservation. Yet the same presumed genetic uniqueness makes any active conservation procedures, undertaken without prior knowledge about the genetic relation of the population to its nearest counterparts, a very risky effort. Therefore, the aim of this study was to investigate the genetic structure of the last remaining Polish population of *P. farinosa* and the nearest Slovak populations to provide data necessary to plan active conservation measures, in particular to decide whether the Polish population can be supplemented with plants produced from seeds collected from other populations.

Material and methods

Plant material

Primula farinosa plant material for genetic analyses was collected from four sites at which the species naturally occurs. Material was collected with consent of the Regional Directorate for Environmental Protection (RDOŚ) in Krakow (ST-II.6400.2.2013.IW) under the project: "Active and conservative protection of bird's-eye primrose *Primula farinosa* at the sole Polish occurrence site in Beskid Sądecki, within the area of Natura 2000", in the presence of RDOŚ and Pieniny National Park (PIENAP, Slovakia) employees. Locations of sampling sites are shown in Fig. 1 and detailed information about individual populations is presented in Tab. 1. Geographic coordinates of the sites were determined using a GPS Garmin eTrex Vista C device.

P. farinosa leaf samples were collected from the four sampling sites during the flowering period. Two leaves were collected from each specimen, taking particular care not to cause further damage to the plants. The collected leaves were packed in sealed string bags filled with silica gel, to allow for complete drying of the plant material, and stored in this form until DNA extraction.

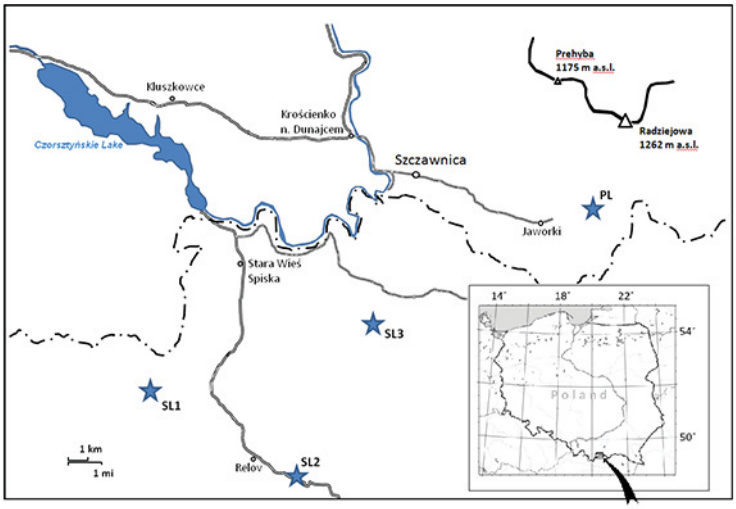

Fig. 1 Geographical locations of the *Primula farinosa* populations. Polish population near Jaworki (PL) and Slovak populations (SL). Population labels as in Tab. 1.

Tab. 1 Location of evaluated *Primula farinosa* populations.

Tested population	Approximate locality	GPS coordinates	No. of tested samples
PL	Jaworki	N49°24′24.4″ E020°33′13.3″	100
SL1	Velka Frankova	N49°20′10.9″ E020°17′51.3″	25
SL2	Relov	N49°17′51.6″ E020°22′49.9″	25
SL3	Haligovce	N49°22′35.0″ E020°26′55.0″	25

Study site

The Polish population (PL) is located within a 700-m^2 midforest marsh, on a slope with an inclination of 10–30°, at 796–810 m a.s.l. in the Radziejowa range. It occurs near to meadow-pasture communities and forests located on marshy ground constantly supplied by several watercourses. Because of the difficult outflow, the area is locally boggy, and the site is quite diverse in terms of the occurring microhabitats. The Velka Frankova (SL1) site is located in a small (approx. 1,800 m^2), flat marsh, surrounded by bushes. On the edge of the clearing, there are water outlets feeding the adjacent stream. In Relov (SL2),

P. farinosa is found in a meadow overgrown by bushes, located on a slope fed by small groundwater seeps above a busy road. In Haligovce (SL3), the plant occupies a narrow, approx. 10 m, strip of wetland marsh, partially overgrown with the reed *Phragmites australis* (mowed). This site is located at the foot of a scrub-covered slope.

DNA extraction, SSR amplification and detection

Approximately 20 mg of dried leaf blade from each plant was placed in an Eppendorf tube with stainless steel beads and pulverized by shaking for 3 minutes at 30 oscillations per second in an oscillating mill MM400 (Retsch, Haan, Germany). DNA was then extracted using a Genomic Mini AX PLANT kit (A&A Biotechnology, Gdynia, Poland) following the manufacturer's instructions. The obtained DNA extracts were stored at −20°C until DNA amplification.

The following eight microsatellite markers were used for SSR fingerprinting: Prifar07017, Prifar11639, Prifar02491, Prifar40580, Prifar70769, Prifar71034, Prifar39067, Prifar55554 [2]. DNA amplifications were performed independently for each locus. Thermo Fisher Scientific reagents were used for 25 µL reaction mixtures containing: 1× TrueStart Buffer, 4 mM $MgCl_2$, 0.12 mM each dNTP, 0.8 µM fluorescently labeled primer F, 0.8 µM unlabeled primer R, 0.16 mg/mL BSA, 0.5 U TrueStart polymerase, and 2.5 µL DNA. PCRs were run in a T100 thermal cycler (Bio-Rad, Hercules, CA, USA) with the following cycling profile: 5 min initial denaturation at 94°C, followed by 25 touchdown cycles with 30 s denaturation at 94°C, 30 s annealing at changing temperature (−0.5°C per cycle, from 67.5°C in the first to 55°C in the 25th cycle), 1 min elongation at 72°C, followed by 20 cycles with 30 s denaturation at 94°C, 30 s annealing at 55°C, 1 min elongation at 72°C, and a final extension step of 10 min at 72°C.

After amplification, the PCR products were combined into three panels for allele determination. Panel 1 consisted of markers Prifar07017, Prifar70769, and Prifar71034; Panel 2 of markers Prifar11639, Prifar02491, and Prifar39067; and Panel 3 of markers Prifar40580 and Prifar55554. One µL of each sample was added to 11.25 µL of HiDi formamide containing 0.2 µL LIZ 600 size standard (Thermo Fisher Scientific, Waltham, MA, USA) and run on a 3500 Genetic Analyzer (Thermo Fisher Scientific).

Data analysis

The STRUCTURE 2.3.4 program [18] was used to identify genetically homogeneous groups of individuals throughout the studied populations. The algorithm places individuals into K clusters characterized by a distinct set of allele frequencies at each locus. The calculations were conducted with 3×10^5 replicates of Markov chain Monte Carlo (MCMC) and 5×10^4 burn-in iterations, in the STRUCTURE 2.3.4 using the admixture ancestry model with sample locations set as priors (LOCPRIOR option, recommended for analysis where weak population structure is expected) and the correlated allele frequencies model. Calculations were made for $K = 1–5$ with ten replications for each value of K. Estimation of the optimal number of groups was based on the likelihood of partitions, examined as a function of increasing K [18] and ΔK [19] values, estimating the change in the likelihood function with respect to K – an indicator of the most reliable clustering structure. Delta K distribution was computed with the STRUCTURE HARVESTER software [20].

Nei's [21] genetic distances were calculated with NTSYSpc ver. 2.11 multivariate analysis package [22]. This genetic distance measures the accumulated allele differences per locus. For a more holistic view, the projection of individuals was calculated with nonmetric dimensional scaling (NMDS) based on Nei's distances, with a minimum spanning tree (MST) [23] superimposed on a NMDS ordination to detect local distortions [24]. NMDS displays a monotone relationship to the distances implied by the original data matrix and obtains a better fit with fewer dimensions compared to other ordination methods [25,26]. The NMDS analysis and stress value (representing the difference between the distance in the reduced dimension compared to the complete multidimensional scale) were calculated with the NTSYSpc ver. 2.11 multivariate analysis package [22].

AMOVA calculations and indices of population genetic variability (expected heterozygosity and number of alleles per locus) were computed using Arlequin 3.5 [27].

Results

We analyzed a total of 175 specimens originating from four sites, one Polish and three Slovak (Tab. 1). A total of 44 alleles were detected across the eight microsatellite loci used, ranging from two for the least variable locus, Prifar07017, to nine for the most variable, Prifar39067 (Tab. 2). Two standard indices of population genetic variability were computed using the microsatellite data: expected heterozygosity (Tab. 3) and mean number of alleles per locus (Tab. 2). Both indices were highest for the Polish population (PL), which had an expected heterozygosity of 0.437 and a mean number of alleles per locus of 4.375. Conversely, the lowest values of both expected heterozygosity (0.372) and mean number of alleles per locus (2.875) were observed in the Slovak population SL1. Thus, based on these indices, the Polish population was the most variable of the four populations studied. The AMOVA results showed that this within-population component accounted for 81.47% of the overall variation observed.

Tab. 2 Number of alleles per locus in populations of *P. farinosa* in Poland (PL) and Slovakia (SL). Abbreviations are those as in Tab. 1.

Locus No.	Number of alleles						
	PL	SL1	SL2	SL3	Mean	*SD*	Tot. number
1	2	1	1	1	1.25	0.500	2
2	6	3	7	5	5.25	1.708	8
3	5	3	3	2	3.25	1.258	6
4	3	3	3	3	3.00	0.000	3
5	2	2	1	2	1.75	0.500	3
6	4	3	2	3	3.00	0.816	7
7	7	5	6	6	6.00	0.816	9
8	6	3	3	3	3.75	1.500	6
Mean	4.375	2.875	3.250	3.125	3.406	0.664	5.500
SD	1.923	1.126	2.188	1.642	1.720	0.454	2.563

Tab. 3 Expected heterozygosity in populations of *Primula farinosa* in Poland (PL) and Slovakia (SL). Abbreviations as in Tab. 1.

Locus No.	Expected heterozygosity						
	PL	SL1	SL2	SL3	Mean	*SD*	Tot. het.
1	0.18090	0.00000	0.00000	0.00000	0.04523	0.09045	0.10791
2	0.74915	0.38857	0.75429	0.67020	0.64055	0.17234	0.74233
3	0.23543	0.51184	0.56898	0.42857	0.43620	0.14574	0.48526
4	0.52116	0.47265	0.56245	0.66041	0.55417	0.07977	0.61803
5	0.02010	0.07837	0.00000	0.04000	0.03462	0.03343	0.02843
6	0.52170	0.15347	0.15020	0.47440	0.32494	0.20082	0.49928
7	0.55457	0.71020	0.75755	0.77388	0.69905	0.10003	0.68635
8	0.71568	0.66041	0.56163	0.31592	0.56341	0.17687	0.71724
Mean	0.43733	0.37194	0.41939	0.42042	0.41227	0.02812	0.48560
SD	0.26273	0.26723	0.31933	0.28792	0.28430	0.02580	0.27497

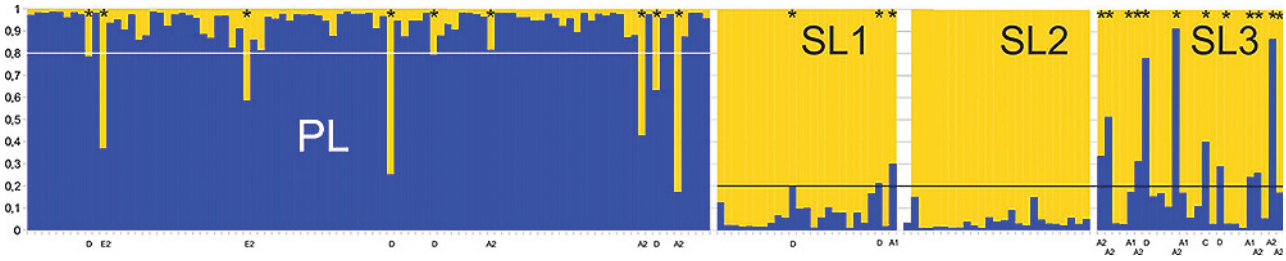

Fig. 2 Results of STRUCTURE clustering of individuals from the Polish (PL) and Slovak (SL) populations of *Primula farinosa*. Each individual is represented by a vertical line. The two detected genetic clusters are color-coded: blue – Cluster 1, and yellow – Cluster 2. The relative share of each color represents the individual's estimated membership fraction in each cluster. Individuals originated from various genetic groups as revealed by multivariate NMDS analysis (see Fig. 3) are marked with asterisks. Population labels as in Tab. 1.

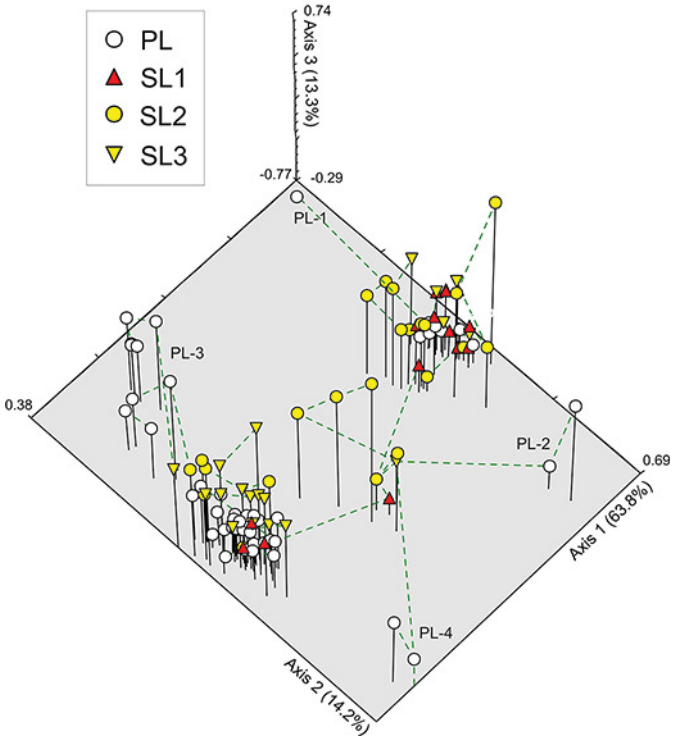

Fig. 3 Results of multivariate NMDS analysis, with a MST classification (NTSYSpc) overlaid (dashed line), for *Primula farinosa* populations in Poland (PL) and Slovakia (SL). Stress: 0.01276. Population labels as in Tab. 1.

In the Bayesian STRUCTURE analysis, the division of individuals according to ΔK was optimal for $K = 2$, with the blue Cluster 1 dominant in the Polish population (PL) and the yellow Cluster 2 prevailing in all three Slovak populations (Fig. 2). Therefore, individuals in the Polish population generally had a different genetic profile to the Slovak populations SL1–SL3. However, all four populations included an admixture of alien genes, with the highest proportion of these occurring in the SL3 population (Fig. 2).

The NMDS ordination method classified most of the individuals into three main groups. Axis 1 explained 63.8% of the total variance, Axis 2 14.2%, and Axis 3 13.8% (Fig. 3). Thirteen individuals of the PL population, grouped in clusters PL-1 to PL-4 of the NMDS analysis, formed separate genetic groups (see Fig. 3). The remaining PL individuals were intermingled with those of the Slovak populations. They formed three distinct clusters distributed along Axis 1; among them was a group without admixture of the PL individuals, located in the center of the diagram. The MST (see dashed line in Fig. 3) showed that individuals from the genetically distinct NMDS groups PL-1 and PL-3 were each related to a different larger NMDS group. These larger NMDS groups were, however, on the opposite side of the Axis 1 spectrum and comprised mixed collections of PL and SL individuals of various origin. In contrast, groups PL-2 and PL-4, also genetically distinct, had links with the pure SL group located in the center of the diagram (Fig. 3).

Discussion

It is likely that the cold-tolerant *P. farinosa*, a poor competitor, extended its range across Europe during past interglacial periods. The opening of an area rich in wet microhabitats clear of dense vegetation would have provided conditions favorable for a cold-adapted but wetland-dependent species. However, during glacial maxima it was unable to maintain its range, becoming restricted to lowland periglacial areas [13]. Population PL seems to be a remnant of the last of these interglacial expansion phases that resulted in the wide geographic range of *P. farinosa*, covering both macroslopes of the Carpathians (SL and PL) and Polish lowlands. The high genetic variability (see below) of the PL population is probably a result of its central location between the Carpathian and Polish lowland populations that in the past formed a practically continuous distribution.

The greater genetic distinctness (measured by the number of private alleles) of the Carpathian population compared with other European populations [13], together with the high genetic variability of the PL population (present results), suggest a genetic melting pot [28], a place where divergent lineages from separate refugia might have met. The northern forelands of the Carpathians and central Poland are known to be such a place, as shown in a phylogeographic study of a forest grass species, *Bromus benekeni* [29].

Based on population modeling, *Primula farinosa* underwent a burst of expansion from its glacial refugia in Southern Europe just after the last glacial retreat [13]. However, colonization of the dry Central European postglacial steppe-tundra was not even and was limited by the availability of wet habitats [13]. This produced a highly fragmented distribution. The wide temperature tolerance of *P. farinosa* [30] allowed it to thrive during the LGM in glacial refugia and expand rapidly afterwards. However, the factor enabling this rapid expansion after glacier withdrawal, the availability of open wet microhabitats, weakened over time as natural succession of habitats progressed, leading to increasing competition from plant species more adaptive at higher temperatures [13]. Indeed, *P. farinosa* is a weak competitor easily crowded out by large herbs, shrubs, and trees [31]. The occurrence of more thermophilic and competitive clonal species (such as *Scirpus sylvaticus* and various species of *Juncus*), and their gradual expansion in recent decades, have also been observed on the PL site [16].

Plant competition along with anthropogenic pressure, particularly drainage of wetlands, are most probably responsible for the recent disappearance of Polish lowland populations of *P. farinosa* and its gradual decrease in most European countries [11,32–35]. Today, the range of *P. farinosa* is highly fragmented and restricted to wet open microhabitats such as calcareous mountain marsh [36].

The overall genetic pattern of our populations is similar to those observed in other studies: most of the observed genetic variation occurred within populations, a situation expected for an outcrossing species. Our between-population and within-population variation proportions, 18.53% and 81.47%, respectively, very closely matched the results of Reisch et al. [34] (20.59% and 79.41%, respectively) for populations located near Obersdorf (northern calcareous Alps, Germany) and were only slightly different from those of Sørensen et al. [35] (33% and 67%, respectively) obtained for populations collected throughout Denmark. Similar results were obtained for other *Primula* species: *P. apennina* [37] and *P. veris* [38]. Both basic genetic indexes used in our study show that the Polish population PL is the most variable of the four studied. The next most variable population was SL3, the population with the strongest PL influence in STRUCTURE clustering. Multivariate NMDS analysis also showed the greatest genetic diversity in the PL population, where 13 individuals had quite different genetic constitution in comparison with the remaining individuals. Conversely, the Slovak population SL1 was the least internally differentiated.

These results indicate that the genetic variability of the PL population, the sole remaining Polish population of *P. farinosa* in Jaworki, is not yet diminished by either the bottleneck effect resulting from low population size or geographic isolation limiting admixture from other populations. The demographic prospect of very small populations of *P. farinosa*, an outcrossing heterostylic species, is even more uncertain because both flower types need to be present in a balanced ratio for effective reproduction. The ratio of flower types in the Jaworki population is currently well balanced [16]. This situation may, however, be changed by further reduction in the number of plants. Therefore, the most imminent conservation issue for this population is maintaining a safe number of plants, if necessary by supplementing it with ex situ produced plants. With supplementation, there is always a question about the most suitable origin of seeds: local, from the protected population itself, vs. foreign, from nearby populations of the same species. This problem is addressed with the microsatellite data in our study and discussed below.

The Polish population of *P. farinosa* PL proved to be genetically distinct from its Slovak counterparts in STRUCTURE Bayesian clustering. The analysis, based on eight independent loci, showed that the most relevant genetic division of the studied dataset is $K = 2$: two independent genetic clusters, whose arrangement revealed clear population structure. Almost all individuals in PL were pure blue Cluster 1, while all Slovak populations were dominated by yellow Cluster 2. Yet in each population, except for

SL2, some individuals were genetically different from their neighbors and resembled individuals from the remote population. In the Polish population, nine individuals with the "Slovak genetic profile" were noted. In the Slovak SL1 and SL3 populations, there were three and 13 individuals, respectively, with a "Polish genetic profile". Thus, the effect was most prominent in the closest Slovak population SL3.

The distinct genetic makeup of the PL population is most probably the result of geographic isolation since the end of the postglacial expansion phase and possibly the admixture of individuals from non-Carpathian provenances, presumably of lowland origin and now mostly extinct. The nearest known population is SL3 in Haligovce, Slovakia, 11.5 km to the southwest. Although the distance does not seem great, the isolation of the PL population depends on two additional factors that hinder gene flow: it is separated from the nearest Slovak population by the Małe Pieniny range (highest peak 1,052 m a.s.l.) and located in a midforest clearing. Therefore, the present genetic isolation of this population is high. However, the Late Glacial vegetation of the Western Carpathians was more open, as they were covered by steppe-tundra or forest-tundra, which were replaced in the Preboreal in higher altitudes by pine-birch and pine forests [39]. Subsequently, meadow-type communities developed, occupying openings in moist forests, accompanied by succession of small, shallow water bodies into swamps and peat bogs [40]. In the cold phase of the Younger Dryas, the tree line border in the Western Carpathians reached 600–800 m a.s.l. [41]. In the Late Glacial/Holocene break, migration of plants between the two slopes of the low mountain range was facilitated by the wet environment and open landscape, in comparison to the forest phases of the Holocene.

It seems that the Polish population could have originated from various genetic stocks, predominantly Carpathian and Polish lowland. The lack of samples from other parts of *P. farinosa*'s distribution range, especially from now mostly extinct Central European lowland populations, makes this scenario hypothetical. Nevertheless, the links between the Polish and Slovak populations, especially SL3, were clearly demonstrated. The mutual genetic similarities observed in some individuals in two Slovak populations (SL2 and, especially, SL3) and the Polish populations could be a result of recent migration/gene transfer. These individuals are most probably recombinants arising from random cross-pollinations.

The results discussed above document the unique genetic makeup of the sole remaining Polish population of *P. farinosa*. This unique genetic composition must be considered when planning active conservation procedures, especially supplementation with ex situ produced plants. The supplementary plants should be limited to those produced from locally collected seeds, as foreign genetic material would disrupt the distinctive genetic profile of the PL population.

Conclusions

Preservation of a sustainable number of individuals in a protected population is the main purpose of any conservation activity. Artificial supplementation is a method used to restore the original number of individuals and to increase, if necessary, the genetic variability of particularly valuable endangered populations. However, foreign genetic stock should not be used to supplement genetically distinct populations as this would disrupt their locally adapted and/or historically unique genetic makeup. The results of our genetic analysis proved that the sole remaining *P. farinosa* population in Jaworki is genetically distinct from its nearest Slovak counterparts, thus all plants used in supplementation interventions should be produced using only locally collected seeds. Moreover, this decision does not pose a risk to the genetic diversity of the Jaworki population as it is genetically still highly variable despite the dwindling number of plants.

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