Phylogeographical structure of a narrow-endemic plant in an isolated high-mountain range: the case of Cochlearia tatrae in the Tatra Mts (Western Carpathians)

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Phylogeographical analyses of alpine species in temperate Europe, distributed in island-like habitats in high-mountain ranges, generally focus on widely distributed species at wide geographical scales. However, genetic diversity and population differentiation in the alpine zone is strongly associated not only with patterns in large-scale isolation, but also local topographic structure of habitats. Regionally endemic species offer the possibility of a realistic overview of genetic diversity in relation to local scale history without the effect of unrecognized external gene flow. Here, we focus on Cochlearia tatrae, a narrow endemic species occurring only within an isolated high-mountain area in the Tatra Mts. Based on population sampling across its entire range, AFLP genotyping and DNA sequencing (non-coding plastid DNA and nrITS) this species’ genetic structure was assessed in the spatial context of its distribution and discussed in terms of its Late Pleistocene history. Pattern of genetic structure in C. tatrae populations did not include strongly divergent genetic lineages with high levels of unique genetic markers. In the PCoA and Neighbour-Net analyses of AFLP data, individuals formed a genetically coherent complex. However, despite the lack of discontinuities, the general tendency was for them to cluster in a way that reflects individual populations and geographical provenance. Despite the small area of distribution of this species (~80 x 20 km), the Bayesian analysis of population structure revealed four genetic groups, with a latitudinal (east–west) distribution across the Tatra Mts. CpDNA and ITS sequences varied little but localized distribution of several closely related plastid haplotypes mostly supported the delimitation of the genetic groups. Based on this phylogeographical structure it is assumed that the Last Glacial history of C. tatrae was characterized by vertical movements and isolation in peripheral, periglacial microrefugia where the conditions were cold and moist. Subsequent postglacial upslope movements, together with poor dispersal and little gene flow resulted in several genetic lineages distributed longitudinally along the Tatra Mts.

Keywords: alpine landscape, Carpathians, conservation, endemism, phylogeography, spatial genetic structure

Introduction

Mountainous areas in temperate Europe provide terrestrial island-like ecological systems, both at a large spatial scale (isolation of high-altitude vegetation belts in major

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mountain ranges) and at a local spatial scale (environmental gradients defined by topographical complexity within the mountains). The residence, persistence and distribution of cold-adapted species in temperate mountains is strongly influenced by the Quaternary climatic oscillations (Hewitt 1996, Comes & Kadereit 1998, 2003, Stehlik 2000, 2003, Kadereit et al. 2004). In principle, the cooler phases of the Pleistocene (ice ages) glaciation reduced the availability of habitats in high-mountain environments (Schönswetter et al. 2005) but, in the same time, increased the availability of climatic niches for cold-adapted plants. The latter potentially induced altitudinal shifts and range expansion of such species toward lower altitudes, which enabled them to survive, move and increase gene exchange both within and among mountain regions (Kropf et al. 2006, 2008, Birks & Willis 2008, Ronikier et al. 2012). During warm phases, cold-adapted species were induced to occupy higher altitudes, which resulted in increased fragmentation and reduced gene flow among regions (see Hewitt 1999, Birks & Willis 2008, Gentili et al. 2015 for overview).

The island-like situation in alpine areas results in isolation and allopatric speciation, making mountain areas important centers of endemism (Médail & Verlaque 1997, Kier et al. 2009). Endemism provides a unique contribution to biodiversity and endemics are often used as flagship taxa for nature protection initiatives. Information on genetic structure, diversity and differentiation is one of the main prerequisites for understanding a species’ long-term responses to environmental changes and potential resilience. It is also important for efficient conservation planning. This can be especially so for alpine species, which occur in discrete localities, well delimited and isolated within a high-mountain landscape, which constrains dispersal, pollen flow and establishment of individuals. The question is to what extent are such species limited by gene flow and highly structured genetic diversity, which is fundamental to understanding their condition and the potential consequences of global climate change-driven processes in mountain environments (Diaz et al. 2003, Blanco-Pastor et al. 2013).

The genetic structure of species is shaped by current population resources and habitat constraints (such as local topographic constitution of the high-mountain zone including relief, steepness, relative altitude, etc.) on the one hand and influence of past environmental changes on the other. Past processes, which shaped the extant ranges and diversity of populations and thus mountain biodiversity, can to some extent be inferred using molecular-genetic tools (e.g. Kropf et al. 2006, Schönswetter et al. 2006, Ronikier et al. 2012) and are essential for understanding extant genetic diversity (Taberlet et al. 2012). Narrow-endemic species provide peculiar yet very suitable case studies for molecular biogeography. Such species, with small and well-delimited total geographical distributions, provide a unique possibility of determining the historical processes influencing genetic diversity based on an entire distribution not influenced by unrecognized external gene flow. However, while the Quaternary history of widely distributed alpine species in Europe, at a large spatial scale, has been quite widely addressed (see reviews by Schönswetter et al. 2005, Ronikier 2011, Schmitt 2017), history of locally distributed, narrow-range species remains poorly understood, even in the most intensely investigated European Alps (Pittet et al. 2020). The studies on such alpine plants show that extant genetic diversity and differentiation of populations is influenced, in addition to the biological traits of species, by local topographic features of the alpine zone, including the degree of insularity of respective habitats, directly related to gene flow and past altitudinal shifts in range (Bettin et al. 2007, Blanco-Pastor et al. 2013, Casazza et al. 2013, García-Fernández et al. 2013).
The Carpathians are one of the major parts of the European Alpine system and an important hotspot of biodiversity and endemism in central Europe (e.g. Pawłowski 1970, Tasenkevich 1998, Hurdu et al. 2016, Mráz & Ronikier 2016). While being of comparable geographical extent to the Alps, they are much lower (both in terms of average and maximum altitude) and display a significantly different distribution of alpine habitats characterized by their smaller area and greater spatial isolation (Pawłowski 1970, Ronikier 2011). The Tatra Mts, the highest range in the entire Carpathians, are situated in their northernmost part (49°10’N) in Poland and Slovakia. They form an outstanding topographic culmination in the Western Carpathians, extending mostly longitudinally (~80 × 20 km) and reaching an altitude of 2655 m a.s.l. They belong to the few mountain ranges in the Carpathians that locally were significantly glaciated during the Quaternary ice ages (Zasadni & Kłapyta 2014, Kłapyta & Zasadni 2017/2018), which is reflected in their distinct postglacial geomorphology. The Tatra Mts constitute a compact, isolated high-mountain island, mostly surrounded by much lower ranges and with a large altitudinal gradient reaching almost 2000 m. As such, they offer an excellent context in which to study the genetic structure of alpine plants and possible past biogeographic scenarios at a local scale.

Some recent studies outlined basic phylogeographical patterns at the scale of the Carpathians and their historical floristic connections with adjacent chains (e.g. Mráz et al. 2008, Puşcaş et al. 2008, Ronikier et al. 2008, Ronikier 2011, Stachurska-Swakoń et al. 2012, 2020, Mráz & Ronikier 2016, Wąsowicz et al. 2016, Šrámková-Fuxová et al. 2017). However, Carpathian phylogeographical studies attempting to understand the perspective of the history of populations in terms of local-scale factors and processes are lacking despite the importance of this region and its conservation value.

Here, we studied *Cochlearia tatrae* Borbás (*Brassicaceae*), one of the narrow-endemic species in the Tatra Mts. It belongs to a genus that is one of the important models for studying the evolutionary processes and transitions that influenced the increase in biological complexity in Europe in the Quaternary (Koch et al. 1996, 1998, Koch 2012). Radiation within this genus took place during the Pleistocene with the deepest split observed 0.7 mya (Hohmann et al. 2015). Section *Cochlearia*, to which *C. tatrae* belongs, contains a polyploid complex, which includes several lineages with taxa with different ploidy levels, different adaptations to coastal and inland environmental conditions and different distributions in lowland and mountain regions (Koch et al. 1998, 2003, Abs 1999, Koch 2002, 2012, Cieślak et al. 2007, 2010, Cires et al. 2011). In central Europe, two taxa, namely *C. tatrae*, an allopolyploid from the Carpathians, and *C. excelsa*, a diploid from the Alps, are found exclusively in high-mountain habitats. Phylogenetic analyses indicate they are independent evolutionary lineages (Koch et al. 1998, Wolf 2017). The distribution of *C. tatrae* is strictly limited to the Tatra Mts (Fig. 1A). It is a rare and endangered species, included in the Red Book in Poland (Mirek & Delimat 2014), the Red List of vascular plants in the Carpathian part of Slovakia (Turis et al. 2014) and legally protected in both countries. At the European level, it is also listed in the IUCN Red List of Threatened Species as ‘Vulnerable’ (Feráková et al. 2011) and included as a priority species in the Annex II of the European Union Habitats Directive (Council Directive 92/43/EEC).
Fig. 1. – Distribution and genetic structure of *Cochlearia tatrae* in the Tatra Mts. (A) Sampled populations (white circles, numbers refer to the number assigned to each of the populations in Table 1), distribution of *C. tatrae* (light grey areas), borders of the Tatra Mts and its internal units (white dashed lines). (B) Results of the Bayesian population structure analysis of all individuals of *C. tatrae* based on AFLP markers, inferred using TESS. Bar graphs of individuals for $K = 4$; populations are separated by vertical lines. (C) Probability distribution of the membership of particular geographical areas for four genetic groups based on the TESS analysis (see text for details; colours correspond to those used in B). Population locations in geographical space are indicated by black circles.
The main goal of the present study was to investigate the genetic structure of *C. tatrae* populations in order to obtain an insight into their Pleistocene history. To this end, we used high-resolution AFLP genotyping (Vos et al. 1995) supported by sequence analysis of selected non-coding DNA regions. In a preliminary analysis, we determined the position of *C. tatrae* populations in relation to those of other *Cochlearia* species in adjacent geographical regions to confirm the distinctiveness of the *C. tatrae* gene pool and its evolutionary relationships within sect. *Cochlearia*. Then, we determined the extant genetic diversity and spatial genetic structure of *C. tatrae* in order to identify the presence of potentially distinct lineages and location of possible genetic discontinuities across the high-mountain landscape. Based on the above, we infer the likely response of *C. tatrae* to the Quaternary climatic oscillations: (i) Did this species survive the last glacial period in many isolated areas (microrefugia), involving notably today’s disjunct distribution, or (ii) one area of the range served as a refugium and source of the postglacial spread? Although the legacy of historical factors and recent population genetic processes are difficult to disentangle, we expect the presence of a significant, spatially explicit AFLP structure and geographically segregating haplotypes to most likely reflect past isolation patterns caused by the Last Glacial Maximum glaciation, thus supporting the first scenario. In contrast, lack of distinct genetic lineages would suggest postglacial recolonization from a single area (the second scenario). Finally, we also briefly discuss the genetic structure of *C. tatrae* in the context of nature conservation and priorities of the high-mountain flora in the Tatra Mts.

**Materials and methods**

**Study species**

*Cochlearia tatrae* is a narrow-endemic species restricted to the Tatra Mts (Fig. 1A). Its distribution covers mainly the eastern part of the area (the High Tatra Mts), with the highest concentration of populations, but also includes a small, disjunct part in the western part of the area (the Western Tatra Mts), ~20 km apart. The species’ total distribution area encompasses ~700 km² and a few dozen scattered, isolated and mostly small populations (Paclová 1977, Feráková et al. 2011). The size of the populations ranges from a few to several dozen individuals (Paclová 1977, Mirek 2004). The largest populations occur in the central part of the distribution area, for example on the northern slopes of the Miegszowieckie Szczeczy peaks (Miegszowiecki Kocioł) and the upper parts of the Lomnický štit peak (E. Cieślak, M. Ronikier, personal observations). The whole distribution of this species and its habitats are protected within the borders of national parks in Poland and Slovakia.

*Cochlearia tatrae* is an allopolyploid, hexaploid species with 2n = 42 (Kochjarová et al. 2006, Kiefer et al. 2013) and a neoendemic of Pleistocene origin (Koch et al. 1996, Kliment 1999). It is a biennial plant, (5) 10–20 (30) cm high, which reproduces generatively. It is outcrossing, insect-pollinated and does not have any special adaptation for dispersal. Seed is produced in large amounts but only dispersed locally, often by the flow of water (Mirek 2004). The altitudinal ecological optimum of *C. tatrae* extends over the alpine and subnival belts of the Tatra Mts (Pawlowski 1956, Paclová 1977). It grows only on weathered, mineral soils, on ground close to springs, on banks of stream and other
water sources, or on moist granite rocks, gravels or screes. It is a characteristic species of the scree vegetation community *Oxyrio digynae-Saxifragetum carpaticae* (Pawłowski 1956, Matuszkiewicz 2006). Its habitat is listed in the European Habitats Directive as Habitat 8110 – Siliceous scree of the montane to snow levels (*Androsacetalia alpinae* and *Galeopsietalia ladani*).  

**Sampling**

Seven populations of *C. tatrae* were sampled across its whole distribution, with 8–25 samples per population depending on its size (Table 1, Fig. 1A). One population represents a minor and naturally disjunct part of this species distribution in the the Western Tatra Mts (Plačlive, population no. 1) and the other populations constitute the core of the distribution in the High Tatra Mts. The total sample consisted of 133 plants. Each plant sampled consisted of 1–3 leaves, which were placed in a tube with silica gel immediately after collecting, dried and stored at room temperature until DNA isolation. In the case of...
the largest population on the Mięguszowieckie Szczyty peaks, which consists of two altitudinally separated subgroups on the northern slope at ~1850–2000 and 2200–2350 m a.s.l, respectively (nos. 3a and 3b in Table 1, Fig. 2), the exact locations of all individuals were recorded using a field GPS. Herbarium material (vouchers) was collected only from large populations, for conservation reasons and lack of taxonomic ambiguities, and deposited in the Herbarium of the W. Szafer Institute of Botany, Polish Academy of Sciences in Kraków (KRAM).

In the first step of the analyses, populations of other central-European species of Cochlearia: *C. borzaeana* (Coman et Nyár.) Pobed. (Eastern Carpathians), *C. excelsa* Zahlbr. ex Fritsch (Eastern Alps), *C. macrorrhiza* (Schur) Pobed. (Lower Austria), *C. polonica* Frohl. (Kraków-Częstochowa Upland) and *C. pyrenaica* DC. (Eastern Alps, Western Carpathians and adjacent areas) were included so that the *C. tatrae* AFLP data can be seen in the enlarged phylogenetic context (see Electronic Appendix 1, 2).

All samples were used in the AFLP genotyping, while a subset of three plants per population of *C. tatrae* was additionally selected for amplification and sequencing of fragments of plastid and nuclear DNA.
**DNA isolation and AFLP fingerprinting**

Total DNA was isolated using approximately 15–20 mg of dried leaf tissue per sample and the DNeasy Plant Mini Kit system (Qiagen, Hilden, Germany), according to the manufacturer’s protocol.

AFLP analysis was performed according to Vos et al. (1995), as described in detail by Cieślak et al. (2007). We tested ten selective primer combinations using four individuals from geographically distant populations. Final analyses were carried out on 225 samples (including 133 samples of *C. tatrae*), using four combinations of primers that gave clear, unambiguous and polymorphic profiles: EcoRI-AAG/MseI-CTA, EcoRI-AAT/MseI-CAC, EcoRI-ACT/MseI-CAC and EcoRI-AGC/MseI-CAT (Table 1). Genotyping reproducibility was tested by including within- and between-plate duplicates of ~5% of the samples (Bonin et al. 2004); reproducibility of our AFLP markers reached 98%. Amplification products were separated using an internal size standard (GeneScan ROX-500) on an ABI Prism 3100 Avant automated sequencer using POP-4 polymer (Applied Biosystems, Foster City, CA, USA).

Since the ploidy level might be proportional to the number of AFLP fragments (Kardolus et al. 1998) and influence the distinctiveness of concluded groups, the average numbers of bands (per individual) of analysed species were compiled along with their ploidy. The differences between mean fragment numbers of particular species were found to be less than 16%. Especially for *Cochlearia tatrae* in which the average band number (92.2) was even smaller than the average value for all individuals (97.0). On that basis, we assumed that the probability of an effect on our AFLP analyses due to the difference in ploidy levels is very small.

**DNA sequencing**

The nuclear ribosomal Internal Transcribed Spacer (ITS) region (White et al. 1990, Blattner 1999) and non-coding plastid DNA (cpDNA) regions rpl20-rps12 (Shaw et al. 2005) and rps16-trnK (Shaw et al. 2007) (selected based on screening six cpDNA regions) were used for sequencing. The composition of the PCR mixture and thermal cycling profile for ITS was used as described in detail by Stachurska-Swakoń et al. (2020). For rpl20-rps12 and rps16-trnK, the PCR mixture, in a total volume of 25 μL, contained: 1 U AmpliTaq360 DNA, 1× PCR Buffer supplied with the enzyme, 2.5 mM MgCl₂, 0.2 μM of each primer, 0.12 mM dNTP (Sigma-Aldrich Co., St. Louis, MO, USA), 0.8% BSA at a concentration of 1 mg/ml (New England BioLabs Inc., Ipswitch, MA, USA) and 1 μL of DNA template. The following PCR cycling profile was used: 5 min at 94°C; 35 cycles of 45 s at 94°C, 1 min at 53°C, 2 min at 72°C; final extension step of 10 min at 72°C; cooling to 4 °C. Enzymatic purification of PCR products and sequencing were conducted as described by Stachurska-Swakoń et al. (2020).

**Data analysis**

AFLP markers were sized against the ROX-500 standard in GeneScan Analysis Software, ver. 3.7 (Applied Biosystems). Obtained marker sets were imported to Genographer Software (ver. 1.6.0; J. Benham, Montana State University; current version of the software available at https://sourceforge.net/projects/genographer), which was
used to score clear and reproducible fragments in the range of 50–500 bp (the binary data
matrix used in subsequent analyses available from the authors on request).

Relationships between our samples of *C. tatrae* and the other species of *Cochlearia*
from central Europe were assessed using Principal Coordinates Analysis (PCoA) based
on Jaccard’s coefficient in FAMD software (Schlüter & Harris 2006) and by Neighbour-
Net analysis based on a Nei-Li distance matrix implemented in SPLITStree4 (Huson &
Bryant 2006), where branch support was estimated by bootstrapping based on 1000 repli-
cates. The analysis of molecular variance (AMOVA) used groups defined a priori in
a hierarchical system: species – populations. Significance levels were determined based
on 1023 permutations. AMOVA analysis and derived $F_{ST}$, $F_{SC}$ and $F_{CT}$ values were calcu-
lated using ARLEQUIN ver. 3.5 (Excoffier & Lischer 2010).

More detailed analyses were carried out on the main, intraspecific data set for
*C. tatrae*. Genetic diversity of populations was characterized using the following para-
ters: number (P) and percentage (%poly) of polymorphic fragments; discriminating frag-
ments, Nd (markers present in all samples from the population and absent elsewhere);
private fragments, Np (markers present only in one population, but not necessarily in all
its individuals); Nei’s index as a measure of the average gene diversity (He; Nei 1987);
frequency down-weighted marker, in order to quantify the genetic “uniqueness” of popu-
lations (DW; Schönswetter & Tribsch 2005). Parameters of genetic diversity and differ-
etiation of populations were calculated using POPGENE ver. 1.32 (Yeh et al. 1999),
except for DW values, which were calculated using R-script AFLPdat (Ehrich 2006).

General relationships of the individuals studied were assessed using a Principal Coor-
dinates Analysis (PCoA) of the whole dataset based on Jaccard’s coefficient, calculated
using FAMD software (Schlüter & Harris 2006), and Neighbour-Net implemented in
SPLITStree4 (Huson & Bryant 2006), with branch support estimated by bootstrapping
with 100,000 replicates. Spearman rank test was used to check for correlations between
numbers of individuals and numbers of polymorphic fragments (STATISTICA ver. 5.1,
G software StatSoft Inc.).

Historical gene flow was roughly estimated using POPGENE ver. 1.32 (Yeh et al.
1999) as $Nm = 0.25(1-F_{ST})/F_{ST}$ (Slatkin & Barton 1989), where $F_{ST}$ was calculated using
the method of Wright (1978). The analysis of molecular variance (AMOVA) was calcu-
lated as described above, and based on groups defined a priori in a hierarchical system:
population – geographical area (the Western Tatra Mts vs the High Tatra Mts).

Isolation by distance (IBD) model was examined by assignment of correlations
between genetic ($F_{ST}$) and geographical (km) distance between all pairs of populations
using Mantel test (Mantel 1967) with 40,000 permutations in ARLEQUIN ver. 3.5
(Excoffier & Lischer 2010). Since population Plačlive (no. 1) is located furthest from all
the others, the Mantel test was used for two data sets: with all populations included and
with the abovementioned population excluded (thus, only for populations from the High
Tatra Mts).

In order to investigate the spatial genetic structure of populations of *C. tatrae*,
a Bayesian analysis using model-based clustering as implemented in the TESS program
was used (Chen et al. 2007). We performed this analysis for all the populations sampled
over the whole species’ range in the Tatra Mts. Spatial coordinates were available for all
localities (single value for each locality), except the populations in the Mięguszowieckie
Szczyty massif (3a, 3b), where exact coordinates were available for individuals. Since the
computational method worked best when the coordinates for every sample are different, geographic positions in these populations were randomized (except the two above mentioned populations). The standard deviation of X and Y coordinates for each population after randomization was lower than 0.0003 [deg], which means that distances between individuals with such modified coordinates were smaller than 1 m. The calculations were carried out assuming an admixture model and for two fixed values of the TESS interaction parameter, \( \psi_1 = 0.60 \) and \( \psi_2 = 0.99 \) as well as for the case, where this parameter was optimized by the program during the fitting procedure. In the latter, it was settled in the range of 0.13–0.18, and had no meaningful influence on the calculation results. The \( \psi \) parameter indicates the relative importance given to spatial connectivity. Relatively small values indicate that the obtained cluster structure stems from the genetic data to a much higher degree than the spatial, geographical arrangement of the populations studied.

The effective number of clusters, K, is always less or equal to the maximum number of clusters, \( K_{\text{MAX}} \), which should be set by the operator. In our case the K value was determined by the sequential increasing of the \( K_{\text{MAX}} \) value and running the program until the final inferred number of clusters (K) became less than \( K_{\text{MAX}} \). The calculations were carried out using 50000 cycles, where the first 20000 was regarded as a burn-in period. Each set of cycles was repeated 150 times for each \( K_{\text{MAX}} \) value. The measure of goodness of fit of the data with the model, so called deviance information criterion (DIC), was computed for each run of the program. Low DIC values indicate a good fit. For populations studied DIC values decreased with increasing \( K_{\text{MAX}} \), but the differences between subsequent K-values for \( K_{\text{MAX}} > 4 \) were nearly three times smaller. For that reason, we decided to choose K = 4 for the detailed description of the data. For that value, we averaged the estimated admixture coefficients over 15 runs using the smallest DIC values. Clustering outputs from different runs were handled using software CLUMPP (Jakobsson & Rosenberg 2007).

Based on the resulting coefficients, cluster membership of each point in the investigated area were also estimated to assess the extent of genetic groups in geographical space (the area occupied by this species). This was done using a spatial interpolation of admixture coefficients and applied here using the krigging method as implemented in R packages ‘spatial’ and ‘fields’.

An additional TESS analysis was carried out for populations sampled on the Miegużowieckie Szczty peaks (3a and 3b), which form the largest and spatially/altitudinally most extensive assemblage of individuals and provide an insight into the fine-scale structure of genetic diversity across a landscape. All computational assumptions of the analysis of the full data set (see above) were kept except the effective number of clusters, K, which, based on the DIC analysis, was reduced to 3.

DNA sequence data were aligned and analysed using the Geneious Pro 6.0.2 program (Drummond et al. 2011). Each sequence was reviewed manually for uncalled and mis-called bases, and all variable positions were confirmed by comparing sequences from the forward and reverse strands. ITS and cpDNA regions were analysed separately and statistical parsimony networks were obtained using the 95% connection limit approach as implemented in TCS (Clement et al. 2000).
Results

Genetic relationships between Cochlearia tatrae and other species of Cochlearia in central Europe

The AFLP analysis of the large sample set that included 225 samples from central-European populations of six species of Cochlearia (see Electronic Appendix 1, 2) yielded 238 fragments. In the PCoA diagram individuals were segregated into several groups mainly according to their taxonomic affinity, but also the geographical provenance of populations (Electronic Appendix 3). Distribution of groups along the first axis generally reflected the longitudinal location with all the westernmost (Alps and vicinity) samples located on the left part of the diagram. While other species formed single groups there are two divergent geographical lineages (western and eastern) for the most widely distributed C. pyrenaica. This is corroborated by splits in the Neighbour-Net, which additionally support the split between the C. pyrenaica lineages, separated by the narrow-endemic C. macrorrhiza and branches of C. excelsa (Electronic Appendix 3). In both analyses, C. tatrae is the most separated and internally compact group. In particular, the Neighbour-Net revealed a clear split from the other taxa analysed, which is supported by high bootstrap values and no traces of reticulation involving C. tatrae. Result of the hierarchical AMOVA of the six species clearly indicate the highest percentage of variation (44.1%) was between species and 33.0% was assigned to within-population level, with $F_{ST} = 0.67$ (Electronic Appendix 5).

Genetic variation in Cochlearia tatrae

The AFLP analysis at the intraspecific level (only C. tatrae populations) yielded 208 DNA fragments, of which 202 (98%) were polymorphic. There were no identical genotypes among the individuals studied. The number of polymorphic fragments in populations ranged from 53 (Plačlive, population no. 1) to 114 (Mieguiszowiecki Kocioł, no. 3a), with 83 fragments on average (SD = 20.3). Private fragments were found in the two westernmost populations, Plačlive (no. 1) and Hrubý vrch (no. 2) and no diagnostic fragments were recorded (Table 1). Nei’s gene diversity index ($H_e$) ranged from 0.08 (no. 1) to 0.17 (no. 3a), with a mean value of 0.12 (SD = 0.02); the frequency-down-weighted values (DW) ranged from 13.72 (no. 7) to 70.42 (no. 3b) with a mean of 41.86 (SD = 21.66). Spearman’s rank test revealed no correlation between the number of individuals and the number of polymorphic fragments ($R = 0.81$, $P < 0.01$).

In the PCoA diagrams (explaining 13.27%, 12.27% and 6.94% of variability, respectively, for the first three axes; Fig. 3A) individuals are generally arranged in homogeneous clusters representing populations. In addition, their spatial distribution (relative to axis 1) reflected the geographical location of the populations, in a west–east gradient, despite lacking clear discontinuities between the groups. For axes 2 and 3 (Fig. 3A) the separation of the geographically isolated population (Plačlive – no. 1, the only population from the Western Tatra Mts) is clear. The clusters of individuals indicated by PCoA are corroborated by the Neighbour-Net network and generally reflect spatially isolated populations although mostly with low bootstrap support (< 50%) except for the two westernmost populations: Plačlive (no. 1) and Hrubý vrch (no. 2) (Fig. 3B). In addition, the Neighbour-Net revealed that there was internal variation in the individuals in the largest
Fig. 3. – Analysis of *Cochlearia tatrae* based on 208 AFLP fragments of the 133 individuals studied. (A) Principal Coordinate Analysis diagram based on Jaccard’s coefficient: ordination at 1 vs. 2, 1 vs. 3 and 2 vs. 3 axes. (B) Neighbour-Net network based on Nei and Li’s (1979) genetic distances. Numbers along the branches are bootstrap values based on $10^5$ replicates.
and spatially extended group on the Mięguszowieckie Szczyty massif (nos. 3a and 3b), but again with low bootstrap values.

Analysis of the genetic variation in *C. tatrae* based on AMOVA indicates that most of the variation can be attributed to within-populations, i.e. 71.2%, relative to 28.8% for among-population variation (P < 0.001, $F_{ST} = 0.29$; Table 2). When defining the most isolated population at Plačlive (no. 1, Western Tatra Mts) as separate from other populations in the High Tatra Mts, within-population variation still dominates, 64.3%, P < 0.001, relative to 23.02% for within groups ($F_{SC} = 0.26$, P < 0.001), but the distribution of variation between groups is not negligible and accounts for 12.7% (P < 0.14 and $F_{CT} = 0.13$; Table 2). Accordingly, higher pairwise $F_{ST}$ values were recorded among the populations from the Western Tatra Mts and the High Tatra Mts (0.21–0.18) than among single populations from within the High Tatra Mts (0.01–0.07). Mantel test confirmed a significant, positive correlation between genetic differentiation and geographic distance for the total data set ($r = 0.708$, P = 0.05, Fig. 4) and only for populations from the High Tatra Mts ($r = 0.41$, P = 0.05, Fig. 4). This result is consistent with the IBD model.

**Geographical relationships**

Results of the TESS Bayesian analyses are presented in Fig. 1B, where admixture coefficients attributing each of 133 individuals to the genetic clusters detected are indicated by various colours. A spatial interpolation of the cluster assignments to populations using the krigging method, which extended the distribution of genetic groups from the populations studied to the entire geographical range of *C. tatrae*, is presented in Fig. 1C. The analysis delimited four separate groups of individuals. Locations with similar longitudes were mainly associated with genetic clusters, independently of their latitudes. Accordingly, one cluster mainly consisted of western populations: Plačlive (no. 1) in the Western Tatra Mts, and Hrubý vrch (no. 2), the westernmost population sampled in the High Tatra Mts (with minor admixture on the Mięguszowieckie Szczyty peaks in the central part of the range). The second cluster mainly consisted of populations from the central part of the High Tatra Mts and included populations from the Velická Dolina valley (no. 4) and Prielom pass (no. 5), with small admixture eastwards of population no. 6 on the

| Source of variation                      | df | Sum of squares | Variance components | Percentage variation | F index |
|-----------------------------------------|----|----------------|---------------------|----------------------|---------|
| Among populations                       | 7  | 798.359        | 6.056 Va            | 28.85                |         |
| Within populations                      | 125| 1866.588       | 14.932 Vb           | 71.15                |         |
| Total                                   | 132| 2664.947       | 20.989              |                      | $F_{ST}$: 0.29 |
| Among geographical groups              | 1  | 132.976        | 3.022 Va            | 12.69                |         |
| (Western Tatra Mts vs High Tatra Mts)   |    |                |                     |                      |         |
| Among populations within groups         | 5  | 603.081        | 5.480 Vb            | 23.02                |         |
| Within populations                      | 126| 1928.891       | 15.309 Vc           | 64.29                |         |
| Total                                   | 132| 2664.947       | 23.811              |                      | $F_{SC}$: 0.26 |
|                                         |    |                |                     |                      | $F_{ST}$: 0.35 |
|                                         |    |                |                     |                      | $F_{CT}$: 0.13 |

Table 2. – AMOVA analysis based on AFLP data for the populations of *Cochlearia tatrae* studied, calculated for all populations and with a priori delimitation of two geographical groups encompassing disjunct parts of the range. Significance tests based on 1023 permutations.
Lomnický štít peak. The third, minor cluster was geographically restricted, as an admixture, to populations on the Mięguszowieckie Szczyty massif (nos. 3a and 3b). Finally, the fourth cluster was centered in the easternmost part of the area, i.e. the Lomnický štít peak (no. 6) and Čierne sedlo pass (no. 7), but extending more to the west by an admixture with population no. 3a, as is also indicated by the Neighbour-Net network (Fig. 3B).

The separate, fine-scale Bayesian analysis of populations on the Mięguszowieckie Szczyty peaks (nos. 3a and 3b) indicated three genetic clusters (Fig. 2). One group contained individuals from both subpopulations and thus not affected by the altitudinal gap separating them. Then, some of the individuals were assigned to two other clusters, which were present at either lower- or higher-altitudes and were strictly spatially segregated within these subpopulations. Presence of three clusters for the Mięguszowieckie Szczyty populations confirmed the three genetic clusters previously recorded in the full analysis (Fig. 1B, see also above) in which one group was local and two others constituted admixtures from groups of more distant populations.

Analysis of gene flow between populations revealed the presence of two groups, which is in accordance with the other analyses. One is the spatially most isolated population at Plačlive (no. 1, Western Tatra Mts), with very low gene flow rates with all other populations (Nm from 0.5 to 0.8). The second group consisted of the remaining populations, where the Nm for pairs of populations was clearly higher (most of them in the 2.2–6.5 range, Electronic Appendix 6).

**DNA sequence variability in populations**

We were able to obtain good quality sequences of the DNA regions studied for only some of the samples and the final data set was reduced, especially for ITS. In the case of cpDNA, it was possible to align sequences for both regions for 19 individuals (three pairs in five populations and two pairs in two populations) and this concatenated data set was used for haplotype inference. Sequences used for analyses were submitted to GenBank.
under the following numbers: ITS for 10 individuals (MT635845–MT635854), rpl20/rps12 and rps16/trnK for 19 individuals (rpl20/rps12: MT675055–MT675062, MT675066–MT675068, MT675070–MT675074, MT675076–MT675078 and rps16/trnK: MT675079–MT675084, MT675086–MT675089, MT675091–MT675099).

The ITS alignment was 660 bp long with two nucleotide substitutions (C/T and A/G). The rpl20-rps12 alignment was 730 bp long with two nucleotide substitutions (A/C and A/T) and one 2-bp insertion/deletion (TT); in addition, one ambiguous site (Y) was noted. The rps16-trnK alignment was 660 bp long with two nucleotide substitutions (T/G and A/C).

In total, three closely related ITS ribotypes (R1–R3) and seven cpDNA haplotypes (H1–H7) were identified, of which two minor variants (H6, H7) were found each only in a single individual (Table 1, Fig. 5A, B). Sequence variants partly distinguished populations or groups of populations. Three plastid haplotypes were characteristic of single populations: H1 – Plačlive (population no. 1), H4 – Lomnický štit (no. 6) and H5 – Velická Dolina (no. 4). Two haplotypes were found in two populations: H2 at Hrubý vrch (no. 2) and at Mięguszowieckie Szczyty (no. 3) and H3 at Prielom (no. 5) and Čierne sedlo (no. 7), and two in single individuals in populations: H6 at Mięguszowieckie Szczyty (no. 3) and H7 at Velická Dolina (no. 4).

Ribotype R1 characterized three populations from the High Tatra Mts (Hrubý vrch, no. 2, Mięguszowieckie Szczyty, no. 3 and Lomnický štit, no. 6). The most common ribotype R2 was recorded across the range both in the High Tatra Mts (Prielom, no. 5, and Čierne sedlo, no. 7) and the Western Tatra Mts (Plačlive, no. 1). Finally, ribotype R3 was detected in populations Velická Dolina (no. 4) and Prielom (no. 5).
Discussion

Genetic diversity and gene flow between populations of Cochlearia tatrae

Our analysis is the first attempt to describe the genetic variability and differentiation between natural populations of the rare, narrow-endemic Cochlearia tatrae. Our first-step analysis of populations of C. tatrae in a large taxonomic context revealed no recent gene exchange with other taxa of Cochlearia currently present in central Europe. Based on AFLP data, while the eastern populations (in the Carpathians and their vicinity) are segregated from the western ones (the Alps and their vicinity; see also Koch et al. 2003), populations of C. tatrae formed a genetically distinct group clearly different from all the remaining taxa including the Carpathian populations of C. borzaeana and C. pyrenaica (Electronic Appendix 3, 4), which is in accordance with earlier cytological analyses (Kochjarová et al. 2006) and the recent comprehensive insight into the evolutionary history of the genus Cochlearia based on several pieces of evidence (Wolf 2017).

The level of genetic variation in populations of C. tatrae, reaching up to He = 0.17 (average He value 0.12), is relatively high compared to the diversified mean genetic diversity values reported for other alpine species, for example in a study of 22 taxa in the Alps and the Carpathians (0.03–0.24; Thiel-Egenter et al. 2009). It is higher than the average value for all species studied in the Carpathians (0.10, SD = 0.06), notably with the wind-pollinated taxa having the highest values (Thiel-Egenter et al. 2009). Overall, the diversity values of C. tatrae populations confirm that narrow endemism is not necessarily more linked with a lower genetic diversity than in widespread species (García-Fernández et al. 2013, Cieślak et al. 2015, Forrest et al. 2017) and in general intraspecific genetic diversity should mainly be associated, apart from life history traits, with post-glacial history of the species rather than with habitat diversity or extent of distribution (Taberlet et al. 2012). It has also been suggested that the high level of genetic variation in C. tatrae might be attributed to a hybrid origin of this species, whose putative parent species are C. officinalis and C. pyrenaica (Koch et al. 1998), or possibly even one of the arctic diploid taxa currently absent from the area, because an association between C. tatrae and C. groenlandica is suggested based on a plastid DNA analysis (Koch et al. 1996). Despite comprehensive analyses based on complete plastome data, no final conclusions on the evolutionary origin of the three central-European polyploid inland taxa (including C. tatrae) can be drawn and a complex legacy of early biogeographical events may have influenced the gene pool of these species (Wolf 2017).

A moderate FST value (0.29), and general lack of discontinuities in the PCoA diagram indicate no deep genetic breaks among populations. A continuous distribution of genetic variation in space, reflecting gene flow between populations, is expected given the small geographic distribution (see Materials and methods). However, the level of gene flow (based on Nm values) and pairwise FST values clearly depended on geographical distance and especially the isolation of the naturally remote population in the Western Tatra Mts (Plačlive, population no. 1) in relation to the core range within the High Tatra Mts (Fig. 1A). Accordingly, small-scale topographical barriers (such as valleys or crests) seem to have had a lower effect than geographical distance. Nevertheless, Mantel tests based on pairwise FST values indicated significant correlations even when the detached population no. 1 was not included, which confirms the existence of constraints on dispersal even over short geographical distances in high-mountain landscapes. A small-scale spatial
structure was revealed even in an analysis of the largest population studied that extended along an altitudinal gradient (3a and 3b; Fig. 2), in which the spatial structure of three genetic groups is maintained and possibly so for a long period of time previously. Populations can thus be considered to be more or less discrete units in a landscape, with constraints on gene exchange among them, linked with both local, gravity-mediated seed dispersal and small-scale gene flow via pollen due to limited pollinator activity, visitation rate and migration in the high-mountain conditions (García-Camacho & Totland 2009, Scheepens et al. 2012).

The genetic structure of C. tatrae in its topographically complex habitat seems more strongly affected by the landscape, than are plants with similar spatial extents but occurring in a more homogeneous alpine landscape (Blanco-Pastor et al. 2013). The genetic structure of C. tatrae is also affected by greater spatial disruption of its specific habitats as is suggested for Saxifraga stellaris, another alpine species of patchy, moist habitats, compared with its pioneering congener Saxifraga oppositifolia (Kropf et al. 2008).

For many alpine species, isolation by distance and genetic divergence also reflect a legacy of glacial survival in spatially isolated refugia and thus historical interruption of gene flow between populations (e.g. Tribsch & Schönswetter 2003, Schönswetter et al. 2005, Kropf et al. 2006, Ronikier 2011, Schmitt 2017). This is most obvious at large spatial scales where allopatric lineages occur across mountain systems (e.g. Ronikier et al. 2012) but historical isolation also affects the extant genetic structure of narrow-endemic taxa (Bettin et al. 2007, Casazza et al. 2013).

Geographical structure and range history of Cochlearia tatrae

Populations of C. tatrae form distinct groups in Neighbour-Net analyses although with low bootstrap support (Fig. 3B) and are more or less segregated in PCoA space in congruence with their geographical location (Fig. 3A). At the scale of the whole range, within the small area of the High Tatra Mts, several groups are defined by the Bayesian analyses, mostly distributed along a longitudinal gradient, in accordance with the IBD model. They are well-delimited, although they also show some admixture among neighbouring populations, most pronounced in the central part of the area (the Mięguszowieckie Szczyty massif, nos. 3a, 3b – although a high DW value, likely linked with the unique genetic group revealed in this population, supports its past isolation).

A recent phylogenetic analysis based on whole plastid genomes indicates an early adaptation to arctic habitats in Cochlearia and further recurrent, temporally separated colonization and adaptation to high-mountain regions in central Europe (Wolf 2017). C. tatrae belongs to a relatively old lineage in the genus, which diverged about 229 kya, during the penultimate glaciation, with a further internal diversification at about 86 kya. Thus, it is assumed that the formation of this taxon predates the last glaciation (Wolf 2017) and it survived the latest glacial episode within its extant distribution area.

Delimitation of coherent genetic groups across its distribution based on Bayesian inference certainly reflects a historical signal of past isolation and post-glacial formation of the current C. tatrae range. Hence, it supports the first scenario assumed by us (survival in many isolated areas) rather than postglacial recolonization from a single source. The Tatra Mts, in contrast to most of the Western Carpathians, were significantly glaciated during the Pleistocene glacial periods (Zasadni & Kłapyta 2014, and literature cited
Currently, there are no glaciers in the Tatra Mts, but they bear well-marked traces of these former glaciations. The LGM orographic permanent snow line in the Tatra Mts is estimated to be at 1500–1600 m a.s.l. (Klimaszewski 1988) and LGM reconstruction indicates that the Tatra Mts were strongly but not evenly covered by glaciers (Zasadni & Klapyta 2014, Zasadni et al. 2018). Due to the occurrence of extensive glaciers in the valleys, in some cases descending beyond the mountains to the piedmont area (Kłapyta et al. 2016), available glacial refugia were physically isolated. Within the mountains, they were generally distributed along steep, uncovered rocky crests at the highest altitudes and lower crests below the snowline. Large areas with a mosaic of habitats potentially suitable for high-mountain plants were also available in adjacent low-altitude locations along the entire range. Although survival on high-mountain slopes is not excluded because of today’s occurrence at the highest altitudes, lower, periglacial habitats appear the most appropriate for *C. tatrae* adapted to moist and cold gravelly habitats.

While nowadays appropriate conditions generally occur high within mountainous areas, during cold glacial periods they were temporarily available in the foothills along glacier moraines, in glacier ablation zones. Most of the populations of *C. tatrae* studied are located within previously glacier-covered sites or concave gullies with permanent snow cover during the LGM (potentially except for those located on crests, populations no. 2 and 6; Zasadni & Klapyta 2014). We therefore assume that following downslope shifts induced by glaciation, the species occupied suitable habitats among the mosaic of alpine and steppe tundra elements and tree patches in well protected and relatively humid valleys (Jankovská & Pokorný 2008, Zasadni & Klapyta 2014). In such an altitudinal shift model, postglacial genetic structure is affected by the degree of connectivity of glacial low-altitude populations (García-Fernández et al. 2013). In the Tatra Mts, the varied extent of glaciers could have shifted some populations further inland or allowed their survival in mountain valleys. Accordingly, sites located along the mountains and harbouring isolated population groups with limited exchange among them, acted as a system of proximal glacial microrefugia at the periphery of the mountains (Rull 2009). They subsequently served as recolonization sources for high-mountain habitats in the postglacial period through upslope movement following deglaciation, paraglacial processes and rising of the snowline (see also Kropf et al. 2003). This scenario explains the observed population structure, which is maintained by limited dispersal capacities.

It is interesting to note that the isolated genetic group in the Western Tatra Mts extends to the westernmost populations in the High Tatra Mts beyond the current distribution gap. This corresponds to the less glaciated area and higher potential glacial- and early postglacial habitat connectivity in the western part of the range (Zasadni & Klapyta 2014). Thus, the two westernmost populations (Plačlive, no. 1 and Hrubý vrch, no. 2) are likely to have the same postglacial provenance. The isolation of the western part of the range is further supported by the presence of private fragments only in these populations and the high DW value of the westernmost population.

Glacial isolation in local refugia, such as alpine nunataks, can be supported by genetic markers not prone to homogenization and thus potentially better conserving genetic variants evolved locally, such as plastid DNA (Bettin et al. 2007, Schönswetter & Schneeweiss 2019). Non-coding DNA sequences displayed a low variation in *C. tatrae* and only a few closely related variants were found. However, while variation in ITS was too little to allow any insight, the narrow distribution of cpDNA haplotypes, confined to single or
neighbouring populations, generally supports the AFLP-based inference of the longitudinal isolation pattern (east–west) and several last glacial microrefugia (Fig. 5). Distribution of haplotypes in part directly reflects the extent of the AFLP groups, but partly indicates an even higher fragmentation. In one case, a plastid haplotype (H3) is recorded in populations no. 5 and 7, which belong to different AFLP groups (cf. Fig. 1B). This can be attributed to different dispersal mode of nuclear and plastid DNA vectors, which may cause distributional shifts, as is often the case between nuclear- and plastid-based patterns (Ronikier et al. 2012).

Conclusions regarding the conservation of high-mountain landscapes

*Cochlearia tatrae* is genetically coherent and does not include strongly divergent genetic lineages, which would be characterized, for instance, by high levels of unique genetic variation. However, the assumed Last Glacial history of this species inferred from phylogeographical data, characterized by vertical movements, together with poor dispersal, resulted in the maintenance of several genetic groups distributed longitudinally along the Tatra Mts range. Intuitively, the small and disjunct part of the range in the Western Tatra Mts is considered to be of special conservation value and our data indicates it is the most divergent and most isolated in terms of gene flow, even though the western phylogeographical group extends close to the populations in the main part of this species’ range in the High Tatra Mts. Although not characterized by unique gene pools, these genetic groups can be considered natural conservation units that maintain the historical legacy of the species and can thus be informative for spatial conservation strategies within the Tatra Mts, one of the most important natural protected areas in central Europe. Further analyses of other species with both endemic and more widespread geographical elements will verify to what extent the pattern found in *C. tatrae* can be generalized.

See www.preslia.cz for Electronic Appendices 1–6

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**Souhrn**

Dosavadní fylogeografické analýzy alpínských druhů disjunktně rozšířených ve vysokých pohořích mírného pásu Evropy byly zaměřeny převážně na široce rozšířené druhy a studovány na velké geografické škále. Genetická diverzita a diferenciace populací v alpínském stupni jsou však silně ovlivněny nejen faktory, které se projevují na velkém měřítku, ale také místní topografickou strukturou stanovišť. Endemické druhy nabízejí možnost posouzení genetické rozmanitosti ve vztahu k místní historii, aniž by došlo k možnému ovlivnění studovánoho vzorku nerozpoznánym tokem genů z okolních území. V naší studii jsme se zaměřili na druh *Cochlearia tatrae*, který je endemitem Tater s areálem o velikosti přibližně 80 × 20 km na hranicích Slovenska a Polska. Populační vzorky z celého areálu druhu jsme analyzovali metodou AFLP a sekvenováním DNA, abychom vyhodnotili genetickou strukturu druhu v kontextu rozšíření druhu a jeho historie v období pozdního pleistocénu. Populace *C. tatrae* tvoří jednu souvislou genetickou skupinu bez výrazných divergentních linii podporěných
unikátními znaky. V mnohorozměrných statistických analýzách se však jednotlivé vzorky přesto shlukovaly podle geografického původu a příslušnosti k populacím. Návzdory malému areálu druha odhalily Bayesovské analýzy čtyř genetických skupin, uspořádaných v pohoří v západovýchodním směru. Na základě této fylogeografické struktury předpokládáme, že během poslední doby ledové docházelo k vertikálním migracím a izolaci jednotlivých populací *C. tatrae* v periferních, periglaciálních mikrorefugiích, která poskytovala příhodnější mikroklimatické podmínky pro jejich přežití. Následné postglaciální migrace do vyšších poloh spolu s omezeným tokem genů vedly k rozlišení na několik genetických linii, které jsou nyní rozšířené podél pohoří.

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