Genetic variation in lowland and mountain populations of *Tofieldia calyculata* and their ability to survive within low levels of genetic diversity

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Abstract

Loss of genetic diversity is expected to be a common reason for decline of populations of many rare species. To what extent this is true for populations at the range periphery remains to be explored. Alpine species with peripheral lowland populations are ideal but poorly known model system to address this issue. We investigated genetic diversity and structure of populations of *Tofieldia calyculata*, species common in central European mountains but highly endangered in lowlands using 17 microsatellite loci.

We showed that lowland populations have lower genetic diversity than mountain populations and they are not clearly differentiated from mountain populations. Species probably survived the last glaciation in refugia in margins of Alps and western Carpathians. Some lowland populations are probably relict as well and contain unique genetic information. Their low genetic diversity is likely the result of reduction of population sizes, gene flow during the Holocene and selfing. However postglacial colonization is also a case of some lowland populations. Based on data from herbarium specimens from extinct lowland populations, we demonstrated that lowland populations had low genetic diversity also in the past and main part of the genetic diversity was lost due to extinction of whole populations. Within population genetic diversity has not changed since the last century suggesting that these populations are able to survive with low levels of genetic diversity under suitable habitat conditions. This idea is also supported by finding of large viable recent populations with very low genetic diversity. We conclude that lowland populations are unique and deserve adequate conservation.

Introduction

It is generally assumed that genetic diversity is crucial for long-time persistence of plant populations (Frankham et al. 2010). In the centre of distribution range the populations are expected to be more genetically diverse and less differentiated than populations at the range periphery (Eckert et al., 2008; Hardie and Hutchings, 2010). Need to protect peripheral populations, if the species is common in the centre of the distribution, is controversial (Hunter and Hutchinson 1994; Hardie and Hutchings 2010). Recent studies, however, suggested that peripheral populations may contain high genetic diversity (Wagner et al. 2011, Plenk et al. 2017, Kropf et al. 2020) and can be genetically differentiated from central populations (Reisch et al. 2003, Plenk et al. 2017). These peripheral populations may represent sources of genetic diversity allowing species to cope with changing climatic conditions (Hampe and Petit 2005; Gibson et al. 2009) and a source of possible speciation events (Hardie and Hutchings 2010). Understanding the genetic differentiation among central and peripheral populations is thus crucial for setting priorities in species conservation.

An interesting system with potentially strongly differentiated peripheral and central populations are species common in central European mountains (Alps, Carpathians) with peripheral endangered populations in lowlands of central Europe (Reisch et al., 2003; Štěpánková, 2010, Duwe et al., 2017). In lowlands these species are restricted into scarce primary or secondary open habitats (fens, meadows, dry grasslands, rocks) and are often rare and declining. For setting conservation priorities, it is very important

to distinguish if the lowland populations are genetically different and relict or if they are genetically depauperate and not differentiated due to being a product of postglacial colonization from glacial refugia situated on the margins of Alps (Schönswetter et al., 2005) or Carpathians (Mráz et al. 2007; Ronikier et al. 2008; Kolář et al. 2016). Genetic diversity of the species with alpine-lowland distribution is poorly understood. Only a few recent studies focused on species with this type of distribution (*e.g.* Greimler and Dobeš 2000; Lutz et al. 2000; Reisch et al. 2003; Windmaier et al. 2016; Duwe et al. 2017; Duwe et al. 2018; Knotek and Kolář 2018) with mixed results. Genetic diversity of lowland populations is often reduced due to their isolation and small size (Duwe et al. 2017; Duwe et al. 2018). In other species, lowland populations show no genetic erosion and high levels of genetic diversity probably due to high longevity of individuals and population sizes large enough for long time preservation of genetic diversity (Lutz 2000; Reisch et al. 2002; Reisch et al. 2003; Knotek and Kolář 2018). Evidence for relict status of lowland populations was given by either high genetic diversity and presence of rare alleles (Greibler and Dobeš 2000; Knotek and Kolář 2018) or low within population diversity but high between population differentiation due to their high isolation and bottleneck effects (Reisch et al. 2003).

Low genetic diversity found in some of the lowland populations may have originated by two alternative processes. These populations were always depauperate due to being result of long-distance dispersal after the last ice-age (Wroblewska 2008; Windmaier et al., 2016). Alternatively, the populations could be relict and their low genetic diversity may be a result of their recent strong size reduction leading to strong genetic drift. Low within population diversity but high differentiation of populations is expected in this scenario (Reisch et al., 2003; Hensen et al., 2010; Vogler and Reisch, 2013). Distinguishing whether the populations are relict and are able to survive with low genetic diversity is important for decision whether it makes sense to protect these populations. Such knowledge can be obtained by exploring genetic structure of populations within the distribution range. Important knowledge can be also obtained by analysis of older samples from herbaria, which can help us to distinguish whether lowland populations have always been impoverished and their low genetic diversity was not the reason for their decline or their genetic diversity has declined during the last century due to human mediated landscape changes. Despite some difficulties resulting from bad quality and fragmentation of old DNA, analysis of old herbarium samples has been recently successfully used in some endangered species to test how much genetic variability was lost in the last decades (Cozzolino et al. 2007; Frey et al. 2017; reviewed by Bieker and Martin 2018).

Using microsatellite markers on samples both from centre mountain and peripheral lowland populations, we compared genetic diversity and composition of lowland and mountain populations of *Tofieldia calyculata* (L.) Wahlenb., a common plant species in the Alps and western Carpathians, with strongly declining peripheral populations in lowlands of central and north-eastern Europe (Gabrielová et al., 2011; Kaplan et al., 2015). We also analysed herbarium specimens from historical lowland populations since 19th century. These data should help to suggest optimal conservation actions for the species. We ask the following questions:

- 1) What is the genetic structure of mountain and lowland populations and what does it tell about possible origin of the lowland populations?
- 2) Can the differences in genetic diversity among populations be explained by their lowland/mountain location and population size?
- 3) How much genetic variability was lost due to extinction of some lowland populations and the decline in size of the remaining ones?

Methods

Study species

Tofieldia calyculata (L.) Wahlenb. ($2n=30$) is a small diploid perennial plant from family *Tofieldiaceae*. Each individual consists of rosettes with several ensiform leaves. The inflorescence is a raceme with about 40 yellow flowers (Štěpánková, 2010). The fruit is a capsule with several dozens of tiny (0.048 mg) brown seeds. The species has mixed reproductive system with possibility of selfing (personal observation from natural populations, also see S1 in Supplementary Material) and clonality. The species is distributed mainly in mountains of central Europe. Alps and Western Carpathians are the centre of the distribution range of the species. Peripheral populations occur in lowlands of central Europe (Czech Republic, Poland, Austria) with some isolated localities in Pyrenees, Romania and north-eastern Europe (Gotland island and Baltic states). In lowlands, species has disappeared from most of its historical localities and it is highly endangered. It is classified as vulnerable in Poland and Ukraine, endangered in Lithuania, critically endangered in the Czech Republic and Croatia, and extinct in Latvia and Hungary (Gabrielová et al. 2011).

In the Czech Republic, the species used to be quite common in several regions in the past. However, it has disappeared from almost all localities due to drainage, land-use changes and ongoing succession (Kaplan et al., 2015). From more than 50 localities recorded since 19th century, the species currently grows only on 5 of them (Kaplan et al. 2015). The size of the remaining ones has been strongly reduced and currently ranges from 1 to 400 individuals (unpublished data).

The species grows in calcareous fens (*Caricion davallianae*, *Sphagno warnstorffii*-*Tomentypnion nitensis*), wet meadows (*Molinion caeruleae*), calcareous pine and spruce woods (e.g. *Erico carnae-pinion*), on wet slopes and rocks, and in several habitats in alpine and subalpine zone in the mountains (Štěpánková, 2010).

Data collection

We collected samples from 30 recent populations of *T. calyculata* (Table 1, Fig.1A) covering all parts of the distribution range of the species including 13 central (mountain) populations and 17 peripheral (lowland) populations. We collected leaves from at least 20 individuals in every population, if possible. Leaves were collected from individuals equally distributed across the population to avoid sampling

clones. Leaves were dried and stored in silicagel. The population size was estimated by estimating the number of flowering individuals. We were unable to record the number of vegetative plants as these can be easily overlooked in dense vegetation.

We also obtained leaves from 135 individuals from herbarium specimens from 6 herbaria from the Czech Republic and Switzerland (PRC, PR, CB, HR, LIT, BERN, see Table 2 and Table S2 in Supplementary material) collected between 1864 and 2011 and originated mostly from populations from the Czech Republic, but also from Italy, Austria, France, Switzerland, Slovakia and Sweden (Gotland island). Herbaria samples were used in Structure, PcoA and AMOVA analysis (Fig 1, for detail see below). Only subset of herbaria samples (samples from the Czech Republic were also used to explore the loss of genetic diversity in populations of *Tofieldia calyculata* in the Czech Republic since the 19th century.

Altogether, we analysed 577 individuals (442 field samples, 135 herbarium samples).

Genetic analysis

We used 19 previously developed microsatellite markers for the analysis (Vlasta et al., 2020). DNA was extracted from leaf samples using modified CTAB method (Lodhi et al., 1994) with all volumes downscaled 10×. DNA amplification was done in two multiplex reactions (previously described in Vlasta et al., 2020) containing 2.5 µL of QIAGEN Multiplex PCR Master Mix and 20 ng of DNA dissolved in 0.5 µL of distilled water. For multiplex mix I (MM I), the PCR contained 1.94 µL of primer mix (10 µM each in initial volume) and 0.06 µL of H₂O, for MM II the PCR contained 1.875 µL of primer mix (10 µM each in initial volume) and 0.125 µL of H₂O. The following conditions of Thermocycler were used: an initial denaturation step at 95°C for 10 min; followed by 35 cycles of denaturation (95°C for 30 s), annealing (60°C for 40 s), and extension (72°C for 30 s); and a final extension at 72°C for 8 min. PCR products were diluted with ddH₂O 10× (PCR product of MMI) and 20× (PCR product of MMII). Each PCR product (1 µL) was mixed with 12 µL of formamide mixed with 0.1 µL of size standard (GeneScan 500 LIZ; Thermo Fisher Scientific, Waltham, Massachusetts, USA). Fragment lengths were determined by capillary gel electrophoresis with an ABI 3130 Genetic Analyzer using GeneMapper 4.0 (Thermo Fisher Scientific).

For analysis of herbarium specimens, the Thermocycler conditions were modified by prolonging time for denaturation, annealing and extension steps: an initial denaturation step at 95°C for 10 min; followed by 35 cycles of denaturation (95°C for 90 s), annealing (60°C for 120 s), and extension (72°C for 120 s); and a final extension at 72°C for 8 min. PCR products were not diluted (for herbarium specimens from 1864-1939) or were diluted 5× (for herbarium specimens from 1940-2015).

Data analysis

We excluded 2 loci from further analyses due to high proportion of missing alleles. All analyses were thus done with 17 loci. We excluded individuals from herbarium specimens with less than 70% loci amplified (mainly from older herbarium specimens, 35 individuals) for further analyses.

Genetic diversity of lowland and mountain populations

We divided populations into 2 groups – mountain populations (populations from Alps and Carpathians; species is common and it is not endangered, altitude ranging between 650 and 2040 m) and lowland populations (from the Czech Republic, Poland, Estonia, Gotland island and lowlands of Austria; species is not common and it is endangered, altitude between 4 and 580 m).

We used SPAGEDi (Hardy and Vekemans 2002) to calculate number of alleles, observed and expected heterozygosities, allelic richness, F_{ST} and individual inbreeding coefficient (F_I). Additionally, we calculated percentage of polymorphic loci, counted number of unique alleles for each population (alleles present in only one population) and for lowland and mountain populations (alleles found only in one of these two groups). GENEPOP version 4.2 (Rousset, 2008) was used to test deviation of each population from Hardy-Weinberg equilibrium. Individuals from herbarium specimens from the same locality collected in different years were analysed as one population if the time between years did not exceed 20 years. Populations with less than 5 individuals were excluded from these analyses.

We calculated pair-wise correlations among all the genetic characteristics using MS Excel. All except F_I were highly correlated to each other (see table S3 in Supplementary Material). We thus only analysed F_I and allelic richness as representatives of all the other variables and commonly used measures of genetic diversity in similar studies.

We tested the effect of distribution of population (lowland, mountain) and logarithm of population size on genetic characteristics of populations (allelic richness and F_I) using generalized linear model with Gamma distribution (in case of allelic richness) and ANOVA (in case of F_I) using R version 3.6.3 (R development team, 2017). Populations with less than 5 individuals were excluded from these analyses. We also excluded all populations from herbaria for this analysis – we compared only current populations.

Genetic structure of the populations

STRUCTURE analysis (Pritchard et al., 2000) was performed to reveal the structure of the populations. First, we removed all clones from the analysis using AFLPDAT function clone (Ehrich, 2006) in R version 3.6.3 (R development team, 2017). All the remaining samples were used including all samples for herbarium specimens with more than 70% loci amplified (altogether 408 individuals). We excluded historical herbarium samples that duplicated our recent data (herbarium specimens from the same populations from different time periods). The analysis was run with K from 1 to 10 with a burn-in period of 100,000 iterations and run for 1,000,000. Ten individual runs were performed for each K. The admixture model was used. Optimal K was determined by Evanno method (Evanno et al., 2005) based on the ΔK statistic using Structure Harvester (Earl and von Holdt, 2012).

We also performed PcoA analysis based on Jaccard coefficients using PAST version 2.17c (Hammer et al., 2001). To test distribution of genetic variability between lowland and mountain populations, between populations within the groups and within populations, we performed AMOVA using Arlequin (Excoffier

and Lischer 2010). Three analyses were done – one including two geographic groups (lowland, mountain) and two separate analyses of each group.

Results from STRUCTURE showed presence of two genetic clusters (groups) with some mixed populations between them. These two groups were then analysed separately to reveal sub-structure of these 2 groups using both STRUCTURE and PcoA. Mixed populations (populations <0.8 probability of belonging to first or second cluster) were not included in these analyses. In the first group, 18 populations with 169 individuals were analysed, in the second group, 34 populations with 186 individuals were analysed.

Analysis of herbarium samples

We used generalized linear model with Gamma distribution to test the effects of herbarium specimen age and location at which it was stored on the amplification success using linearly transformed percentage of amplified loci as dependent variable. We were not able to test the interaction between the two variables as plants from different herbaria originated from different years. We used all the data from herbaria in this analysis. The analysis was done using R version 3.6.3 (R development team, 2017)

We used only samples from the Czech Republic in the next analysis, both current samples and herbaria historical samples from 1864 to 1986. Samples were divided into six groups based on geographic distribution of the species (Table 2). In four regions (Džbán, Polabí, Českolipsko region and southern Bohemia), where current populations are present, we counted all alleles amplified for all loci in all samples (even in samples where less than 70% loci were amplified) for each region. We compared the alleles from the past with alleles in the current populations. We divided all alleles found in each of the four regions into three groups – 1) alleles present in current populations, 2) alleles which have disappeared from current populations (i.e. present in the historical, but not recent samples, genetic loss on within population level), 3) alleles which have disappeared from extinct populations (genetic loss on between population level) and estimated proportion of alleles in each of the groups.

Results

Genetic diversity of lowland and mountain populations

We found 158 alleles in the 17 scored loci. The percentage of polymorphic loci in a population ranged between 0 and 100%. Allelic richness ranged between 1 and 2.09. Observed and expected heterozygosities ranged between 0 to 0.432 and 0 to 0.512, respectively. The highest genetic diversity was found in mountain population Weißenbach in Eastern Alps. The lowest genetic diversity was found in two lowland populations Ojcowski park narodowy in Poland and Cikánský dolík in the Czech Republic. The highest number of unique alleles was found in population Weißenbach (7 unique alleles). Other unique alleles were found in other mountain populations and few unique alleles were found in lowland populations (see Fig. 2, Table 1 and 2). Fifty alleles (31.6%) were found only in mountain populations, 12 alleles (7.6%) were found only in lowland populations.

Lowland populations had reduced allelic richness compared to mountain populations (Table 3, Fig.2 and 3). Individual inbreeding coefficient (FI) did not differ between lowland and mountain populations (Table 3, Fig 3). Neither allelic richness, nor FI were influenced by population size (Table 3).

We found significant deviance from Hardy-Weinberg equilibrium in almost all populations (Genepop, $p < 0.05$) except two mountain (Le Taillefer, Mount Rax) and two lowland populations (Bagno Serebryskie, Jestřebské slatě). Individual inbreeding coefficient was also highly significantly different from 0 in almost all populations, with exceptions of populations Bagno Serebryskie and Jestřebské slatě (Tables 1 and 2).

Genetic structure of the populations and origin of lowland populations

AMOVA analysis revealed that 7.25% of variability was between lowland and mountain populations, 54.2% of variability between populations within groups and 38.55% within populations. In separate analyses of each group, 44.7% of variability was between populations in mountains whereas 68.2% of the variability was between populations in lowlands (Table 4).

In Structure analysis, the most likely number of clusters was assigned to two groups based on the ΔK statistic ($K=2$, Table S4 in Supplementary Material, Fig 1A). First group consists of populations from eastern Alps, western Carpathians, Estonia and Gotland. Second group contained the remaining populations – populations from northern Bohemia, Poland, central, southern and western Alps and Pyrenes. Populations from lowlands of Austria and from southern Bohemia were mixed. These results were also supported by PcoA analysis (Fig.4). Lowland and mountain populations were not distinguished.

After separate STRUCTURE and PcoA analysis three sub-groups were found within the first group ($K=3$, Table S4, Fig 1B) – populations from Estonia and Gotland, Carpathian populations and populations from Eastern Alps (Fig. 1B and 4). Within the second group, two sub-groups were recognized after separate STRUCTURE analysis ($K=2$, Table S4, Fig. 1B) – populations from central, southern and western Alps together with populations from southern Poland form one sub-group, populations from northern Bohemia and south-eastern Poland (Lublin region) form another group (Figures 1B and 4).

Recent changes in genetic diversity in lowland populations from the Czech Republic

Analysis of herbarium specimens from the Czech Republic showed that 43.4% of alleles observed in herbarium specimens was lost due to extinction of the species from almost all historical localities. Comparison of the four main regions where the species was present in the past shows that more genetic diversity was lost in some regions than in others (Fig.5). In Polabí region and southern Bohemia (where only few individuals are currently present) around half of the variability was lost (59%, 49% respectively). In Českolipsko region and Džbán region, where current populations with hundreds or dozens of individuals still exist, only 21% and 14% of variability was lost respectively. Almost all Czech historical populations had a low within population genetic diversity compared to central mountain populations and the populations were highly differentiated. Main part of the genetic variability was therefore lost due to

extinction of whole populations. Percentage of amplified loci was significantly affected by age of the herbarium specimen (GLM, Explained deviance= 40.83, $p < 0.001$, Fig.6) and also by their storage place (GLM, Explained deviance= 36.06, $p < 0.001$).

Discussion

Genetic diversity of lowland and mountain populations

We found significantly lower genetic diversity in lowland peripheral populations compared to mountain populations. Low genetic diversity (Vogler and Reisch, 2013; Duwe et al., 2017) and high genetic differentiation (Greimler and Dobeš, 2000; Vogler and Reisch, 2013; Duwe et al., 2017) were also found in other species with remnant lowland populations. Other studies showed almost no genetic diversity in lowland populations compared to the mountains (*e.g.* Machon *et al.*, 2001; Kirschner et al. 2011). Our results indicate that despite of lowered genetic diversity of lowland populations, they still contain relatively high amount of genetic variability including some unique alleles and alleles which are not present in mountain populations. This is consistent with results of other studies on species with similar distribution (Reisch et al. 2002; Reisch et al. 2003; Duwe et al. 2018) reporting more genetic variability in mountains, but with enough genetic variability in lowlands.

We did not find any relationship between genetic diversity and population size. Positive relationship between population size and genetic diversity is well-supported in the literature (Tomimatsu and Ohara 2003; Leimu et al. 2006; Šmídová et al. 2011; Dostálek et al. 2014; Münzbergová et al. 2018) . However, other studies showed no relationship (Pluess and Stöcklin 2004; Bachmann and Hensen 2007; Klank et al. 2012). While some lowland populations of *T. calyculata* with very low genetic diversity are very small, we also found no genetic diversity in a large viable (based on our unpublished demographic data) population in Ojcowski park narodowy, where all the plants sampled had the same genotype. We also found very low genetic diversity in one large mountain Carpathian population (Pusté Pole). Similarly, almost no genetic diversity was found in Estonian populations. Estonian populations are located on island of Saaremaa and their low genetic diversity could be consequence of postglacial colonization of this formerly glaciated area. The low genetic diversity in several large viable populations suggests that low genetic diversity may not be a problem for the species. Although there are many studies that showed the importance of genetic diversity for fitness of individuals and plant populations (*e.g.* Luijten et al., 2002; Picó et al., 2007; Ilves et al. 2013), there is evidence from other rare species which are able to survive and form viable populations even with low levels of genetic diversity (Lammi et al., 1999; Noel et al., 2007; Vogler and Reisch, 2013; Plenk et al., 2019). It is thus likely that loss of genetic diversity is not the key reason for the decline of the populations of *T. calyculata*. The small populations are likely small due to unsuitable habitat conditions and not low genetic diversity. *Tofieldia calyculata* is a long-lived perennial species, so it is possible that negative consequences of loss of genetic diversity will appear in the next generations (but see the chapter below about herbaria samples). We also did not study the effect of genetic diversity on fitness of the individuals.

T. calyculata is self-compatible (field observations, also see S1 in Supplementary Material). This is in line with significant deviance from Hardy-Weinberg equilibrium in almost all populations and high F_I . Selfing species are less affected by low genetic diversity than self-incompatible species (Hamrick and Godt 1996; Honnay and Jacquemyn 2007; Segarra-Moragues and Mateu-Andrés 2007). F_I did not differ between lowland and mountain populations indicating selfing probably happens in all populations. Selfing of the species is also supported by results of AMOVA analysis which revealed higher proportion of variability between than within populations with the difference being larger in lowland populations. This indicates higher selfing and isolation of lowland populations possibly explaining their lower genetic diversity.

Genetic structure of the populations and origin of lowland populations

Results of STRUCTURE analysis did not confirm the clear separation of lowland and mountain populations and showed that the situation is more complex. Both lowland and mountain populations form several independent groups. Populations from north-eastern limestone Alps are more closely related to Carpathian populations than to the rest of the populations from Alps. Similar genetic split between populations from western and eastern Alps was described in range of previous studies (Ronikier et al. 2008; Thiel-Egenter et al. 2011; Ronikier et al. 2012; Slovák et al. 2012). While eastern Alps and western Carpathians were not glaciated or hosted only small glacial sheets, central and western Alps were covered by huge glacier (Ivy-ochs, 2015). Eastern Alps and western Carpathians are therefore proposed to be glacial refugia for many cold-adapted species (Schönswetter et al. 2005; Ronikier et al. 2008; Slovák et al. 2012; Dítě et al. 2018). In line with this, the highest number of unique alleles and also the highest values of allelic richness and heterozygosities were found in populations on southern and north-eastern edges of Alps and also in some western Carpathian populations suggesting possible glacial refugia for *T. calyculata*. Suitable habitats including calcareous fens were probably present in non-glaciated parts of Alps and western Carpathians during the last ice-age (Janská et al., 2017). After tree expansion in Holocene, fen species such as *T. calyculata* likely survived on old fens originated in late glacial or at beginning of Holocene (Hájek et al., 2011; Hájková et al., 2020). Indeed, the highest genetic diversity within western Carpathians was found in population in Belianské lúky, biggest Slovak fen more than 10 000 years old hosting many relict species (Hájková et al., 2012). Populations from Western Carpathians or Eastern Alps could be a source of postglacial colonization of northern areas (Estonia, Gotland) as shown by high similarity of these populations in our study. Role of Carpathians as source of postglacial colonization of Baltic area was recently also described in *Arabidopsis arenosa* (Kolář et al., 2016).

Presence of glacial refugia in lowlands is possible. Lowland populations have low within population but high between population genetic diversity. This pattern was previously assigned to relict populations (Reisch et al. 2003; Hensen et al. 2010). While in mountain populations 44.2% of variability was among populations, in lowland populations 68.2% of variation was among the populations indicating restricted gene flow among lowland populations. Populations of *T. calyculata* in lowlands could have existed already during the last glacial period and their low genetic diversity could be due to reduction of

population sizes together with restricted gene flow leading to strong genetic drift after forest expansion during Holocene. Differentiation of populations was also supported by selfing of the species.

Possible lowland occurrences of *T. calyculata* during last ice-age are also supported by palynological records. Pollen of *Tofieldia* sp. was found in late Glacial or at beginning of Holocene in central Bohemia (Pokorný et al., 2015), in Estonia (Niinemets et al. 2002) and in lowlands of Germany (Brande, 1996) and Poland (Ralska-Jasiewiczowa and Rzętkowska 1987). Pollen of *T. calyculata* cannot be distinguished from pollen of *Tofieldia pusilla* which currently co-occurs with *T. calyculata* in Alps and Tatra Mountains, but the distribution of the species does not overlap in lowlands nowadays. It however cannot be excluded that pollen found in the palynological records was pollen of *T. pusilla*.

Relict character of the lowland populations is further supported by presence of unique alleles in some lowland populations. Unique alleles were found in lowland populations in the Czech Republic, Poland, Estonia and in populations from lowlands of Austria. In the Czech Republic, we found some unique alleles in two historical populations (Polabská černava, Cibulka). In the region of Polabská černava (Polabí region), the species grew in old fen localities whose history goes back to late Glacial period (Petr and Novák 2014). The species co-occurred with other fen species considered as relicts such as *Cladium mariscus* (Pokorny et al. 2010) or *Ligularia sibirica* (Hendrych 2003; Šmídová et al. 2011).

Alternative hypothesis of origin of lowland populations of *T. calyculata* can be postglacial colonisation from glacial refugia as previously described in *Primula farinosa* (Theodoridis et al., 2017), the species with quite similar distribution and ecology or in *Polygala chamaebuxus* (Windmaißer et al., 2016). This hypothesis does not contradict the arguments mentioned above. In this scenario, lowland populations could be considered as post-glacial relicts with sufficiently long history to allow occurrence of some new genetic variability. This hypothesis cannot be excluded especially due to lower genetic diversity of lowland populations. The postglacial expansion is probable especially in populations from north-eastern Europe (Estonia, Gotland). To conclude, lowland populations are probably result of both processes – postglacial colonisation from glacial refugia and interglacial contraction of relict lowland populations.

Recent changes in genetic diversity in lowland populations from the Czech Republic

There are still some methodological problems when performing genetic analysis of herbarium samples, such as different sampling of plants, missing data, mistakes in labels and lower quality of DNA due to high age of specimens and conditions of their storage (recently reviewed by Bieker and Martin, 2018). We found that percentage of amplified loci was significantly affected by the age of the herbarium specimens with lower amplification in older samples, although some old herbarium specimens from 19th century were successfully amplified. Using herbarium samples for testing loss of genetic diversity in rare species is still not common (but see Cozzolino et al. 2007; Beatty et al., 2014; Frey et al., 2017). Cozzolino et al. (2007) documented loss of genetic diversity and increased differentiation of populations of *Anacamptis palustris* after human-mediated landscape changes. Shifts in genetic diversity was also documented using herbarium specimens of *Trapa natans* (Frey et al., 2017) and *Saxifraga hirculus* (Beatty et al.

2014). In *T. calyculata*, historical populations had very low genetic diversity, even when corrected for the different sample size of current and historical populations (allelic richness values). Comparison of pool of alleles from herbarium specimens with pool of alleles from present populations suggests that 43.4% of alleles observed in herbarium specimens was lost and the loss was mostly due to extinction of whole populations (Fig.5). The loss was larger in Polabí and southern Bohemia regions with only few remaining plants present at the moment (59 and 49% of variability lost). In contrast, in regions still hosting remaining populations (Českolipsko, Džbán) only 21 and 14% of the variation has been lost. In the largest Czech population – Jestřebské slatě (Českolipsko region), the same alleles were present in 1930s, 1980s and nowadays. Some other alleles were found in this population in 1910s, but low percentage of amplified loci disable us to make conclusions about the genetic diversity before 1930s. In the second biggest population, Cikánský dolík (Džbán region), all plants have the same genotype at the moment. We found few other alleles in herbarium specimens from 19th century in this population, but within population genetic diversity was negligible already in the 19th century. The changes in genetic diversity could have happen before the 20th century. The low number of samples from 19th century and the bad quality of their DNA do not allow us to assess the full diversity of the populations in this period. In addition, no herbarium samples before 1860s are available.

Conservation recommendations

Lowland populations are genetically different from mountain populations although the differentiation is more complicated. They contain some unique alleles and alleles which were not found in mountain populations. We found that a lot of diversity was found between populations, indicating large differentiation of populations of *T. calyculata*. Every population of *T. calyculata* is in a way unique and deserves protection and multiple populations have to be protected to save maximum of genetic diversity.

Existence of large viable populations with very low genetic diversity suggests that populations of *T. calyculata* can be restored even from last individuals remaining. Almost no genetic variability was lost on within population level at least during the time period disponible from herbarium specimens. All this suggest that the recent declines of the populations are not due to loss of genetic diversity.

Mountain populations from Alps and Carpathians are not currently endangered. They can be however endangered by future climate change due to habitat shifts and change of environmental conditions (Dirnböck et al. 2003). Mountain populations containing high portion of genetic diversity thus should not be neglected neither when setting conservation priorities to protect this species.

Declarations

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We obtained all permission to enter to protected areas and for manipulation with the species in countries where the species is protected.

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Tables

Table 1. - List of current populations (from fresh material) used in the study – population genetic statistics included

Population									Unique	Estimated	FI
	Country	Distribution	N	Ho	He	A	Ar(k=4)	P	alleles	population size	
Gosausee	AU	M	20	0.35	0.42	54	1.86	1	0	3000	0.18*
Dachstein	AU	M	20	0.17	0.46	57	1.94	1	1	1500	0.62*
Weissenbach	AU	M	20	0.41	0.51	75	2.09	0.94	7	350	0.20*
Innsbruck	AU	M	5	0.15	0.34	36	1.68	0.82	1	150	0.58*
Mount Rax	AU	M	9	0.43	0.51	51	2.07	0.94	2	-	0.15*
Le Taillefer	FR	M	5	0.04	0.10	20	1.16	0.18	1	-	0.66*
Rotmoss+	SWI	M	3	-	-	23	-	0.38	2	-	-
Romoss+	SWI	M	3	-	-	23	-	0.35	0	-	-
Monkova dolina	SK	M	20	0.17	0.37	47	1.75	0.88	1	150	0.56*
Belianské lúky	SK	M	20	0.35	0.42	48	1.85	0.82	2	750	0.18*
Kláštorská roklina	SK	M	13	0.01	0.19	28	1.35	0.53	0	75	0.93*
Pusté pole	SK	M	20	0.11	0.16	28	1.31	0.47	0	1500	0.35*
Kanon Stratená	SK	M	10	0.10	0.25	31	1.48	0.53	0	25	0.61*
Ibmermoor	AUS	L	20	0.30	0.46	52	1.85	0.88	0	350	0.26*
Grabensee	AUS	L	20	0.16	0.32	43	1.65	0.76	4	350	0.52*
Mautern	AUS	L	15	0.004	0.05	21	1.1	0.18	0	7	0.93*
Młaki nad Pogorą	PL	L	20	0.23	0.28	35	1.55	0.71	0	150	0.18*
Sosnowiec-bory	PL	L	20	0.18	0.31	33	1.59	0.71	0	75	0.44*
Jaworzno-Szczakowa	PL	L	20	0.11	0.32	35	1.61	0.71	1	350	0.66*
Ojcowski park narodowy	PL	L	16	0	0	17	1	0	0	1500	1*
Bagno serebryskie	PL	L	20	0.15	0.18	32	1.33	0.59	0	170	0.13
Brzezno	PL	L	20	0.09	0.20	32	1.37	0.71	0	140	0.56*
Kihelkonna	EST	L	15	0	0.008	18	1.01	0.06	0	350	1*
Paatsa	EST	L	17	0.003	0.004	18	1.01	0.06	0	350	0
Viidumäe	EST	L	19	0.06	0.14	30	1.27	0.41	1	150	0.60*
Jestřebské slate - Baronský rybník	CZ	L	23	0.12	0.14	23	1.24	0.29	0	100	0.14
V Bahnách	CZ	L	3	-	-	17	-	0	0	1	-
Cikánský dolík	CZ	L	21	0	0	17	1	0	0	25	-
Loučeň	CZ	L	1	-	-	17	-	0	0	1	-
Kralovické louky	CZ	L	4	-	-	17	-	0.06	0	4	-

Notes: +from Bern botanical garden, AU= Austria, FR=France, SWI=Switzerland, SK=Slovakia, PL=Poland, EST=Estonia, CZ=the Czech Republic, M=mountain populations, L=lowland populations, N=number of samples analysed, Ho=observed heterozygosity, He=expected heterozygosity, A= number of alleles in population, Ar=allelic richness, k=number of gene copies, P= % of polymorphic loci, FI= individual inbreeding coefficient, m * p<0.05

Table 2 - list of populations from herbaria used in this study, (populations and samples with unsuccessful amplification and herbaria accession numbers not included in the table - see table S2 in Supplementary Material)

Locality	Herbarium				Ar				Unique				
	Country	Region	Year	code	Distribution	N	Ho	He	A	(k=4)	P	alleles	FI
Jestřebské slatě	CZ	CL	1935-	PRC	L	5	0.01	0.13	22	1.23	0.29	0	0.92*
			1936										
Jestřebské slatě	CZ	CL	1980-	LIT	L	2	-	-	19	-	0.12	0	-
			1982										
Grumichova rokle+	CZ	CL	1944	PRC	L	1	-	-	14	-	0	0	-
Tetčíněves+	CZ	CL	1963-	LIT	L	2	-	-	17	-	0	0	-
			1964										
Tetčíněves+	CZ	CL	1986	LIT	L	1	-	-	15	-	0	0	-
Třtice	CZ	DZ	1946-	PR	L	5	0.01	0.05	19	1.08	0.12	0	0.77
			1956										
Třtice	CZ	DZ	1970	LIT	L	1	-	-	13	-	0	0	-
Bilíčov	CZ	DZ	1910	PRC	L	1	-	-	14	-	0	0	-
Drnek+	CZ	DZ	1953	CB	L	1	-	-	15	-	0	0	-
Loučeň	CZ	PB	1881	PRC	L	1	-	-	15	-	0.07	0	-
Polabská černava+	CZ	PB	1915	PR	L	3	-	-	24	-	0.44	1	-
Polabská černava+	CZ	PB	1943-	PR	L	6	0.07	0.20	25	1.36	0.41	1	0.70*
			1954										
Polabská černava+	CZ	PB	1977	LIT	L	1	-	-	18	-	0.06	1	-
Hrabanovská	CZ	PB	1927	PR	L	2	-	-	18	-	0.13	0	-
černava+													
Čertův důl+	CZ	PB	1906	PR,PRC	L	6	0.06	0.09	21	1.16	0.24	0	0.38
Cibulka+	CZ	PR	1866-	PR, LIT	L	4			37	-	0.82	1	0.95*
			1885										
Zbytka+	CZ	EAS	1942	PRC, HR	L	5	0.04	0.03	18	1.05	0.06	0	-0.33
Stráž nad	CZ	SOU	1935	PRC	L	1	-	-	16	-	0	0	-
Nežárkou+													
Todenská hora+	CZ	SOU	1962	CB	L	3	-	-	19	-	0.19	0	-
Lhenice+	CZ	SOU	1925	PRC	L	1	-	-	13	-	0.08	0	-
Brloh+	CZ	SOU	1969	CB	L	1	-	-	17	-	0.06	0	-
Moravský kras+	CZ	MOR	1968	PR	L	1	-	-	19	-	0.19	0	-
Pyrenees, Ariège	FR	-	1940	PRC	M	3	-	-	18	-	0.06	0	-0.33
Gotland	SW	-	1930	PRC	L	2	-	-	18	-	0.06	0	0.14
Fernpassstrase	AUS	-	2011	CB	M	3	-	-	31	-	0.65	3	0.15*
Weissenbach am	AUS	-	2011	CB	M	3	-	-	28	-	0.35	0	0.13
Lech													
Weissenbach am	AUS	-	2011	CB	M	3	-	-	23	-	0.35	0	-0.17
Lech II													
Schlern	IT	-	2000	PRC	M	2	-	-	21	-	0.31	2	0.20
Coal santo	IT	-	1991	PRC	M	3	-	-	30	-	0.65	3	-
Welschenrohr,	SWI	-	1993	BERN	M	3	-	-	17	-	0	0	-

Malsenberg												
Hasenmatt, Selzach	SWI	-	1970	BERN	M	3	-	-	20	-	0.12	0 -0.09
Graubünder,												
Fruolcla Crap Alv	SWI	-	2015	PR	M	5	-	-	19	-	0.19	0 -
Graubünder, Val												
Sinestra	SWI	-	2015	PR	M	2	-	-	21	-	0	0 -
Uri, Realp	SWI	-	2015	PR	M	3	-	-	16	-	0.31	0 -
Selva di Val												
Gardena	IT	-	1985	PR	M	3	-	-	23	-	0.29	1 0.6*
Friuli, Tarcento	IT	-	1967	PR	L	3	-	-	32	-	0.69	0 -
			1968,									
Sulovské skály	SK	-	1983	HR,LIT	M	1	-	-	17	-	0.06	0 -
Malá Fatra, Chleeb	SK	-	1962	LIT	M	2	-	-	28	-	0.53	0 0.05
			1968,									
Harmanec, jeskyně	SK	-	1976	HR	M	2	-	-	27	-	0.47	0 0.71*

Notes: +extinct population, CZ= Czech Republic, FR=France, SW=Sweden, SWI=Switzerland, AUS=Austria, CL= Českolipsko, DZ=Džbán, PB=Polabí, PR=Prague, EAS=Eastern Bohemia, SOU= Southern Bohemia, MOR=Moravia, L=Lowland populations, M=Mountain populations, N=number of samples, Ho=Observed heterozygosity, H=expected heterozygosity, A=number of alleles in population, Ar= allelic richness, P=percentage of polymorphic loci, FI= individual inbreeding coefficient, * p<0.05

Table 3 – Relationship between allelic richness (Ar) and individuals inbreeding coefficient (FI) and distribution (lowland/mountain) and population size, GLM with gamma distribution was used in case of Allelic richness and ANOVA was used in case of FI

	Ar			FI		
	Df	Explained deviance	p-value	Df	F	p-value
Distribution	1	0.27	0.007*	1	0.04	0.95
Log(Population size)	1	0.09	0.13	1	1.74	0.20
Residuals	20			20		

* significant value

Table 4 – Results from Analysis of molecular variance (AMOVA) – populations divided into lowland and mountain group (A) and separate analysis of each group (B,C)

Source of variation	Sum of squares	Variance components	Percentage variation
A) Lowland and mountain group			
Among groups	269.85	0.38	7.25
Among populations within groups	2649.37	2.84	54.2
Within populations	1869.39	2.02	38.55
Total	4786.62	5.25	
B) Lowland populations			
Among populations	1771.22	3.17	68.17
Within populations	832.64	1.48	31.83
Total	2603.86	4.65	
C) Mountain populations			
Among populations	878.16	2.32	44.71
Within populations	1034.76	2.86	55.29
Total	1912.91	5.18	

Figures

Figure 1

a) Two main genetic groups of populations of *T. calyculata* based on STRUCTURE (k=2), b) Substructure of the two main genetic groups depicted at Fig. 1a based on separate Structure analysis of the two main groups. The first (blue) group was optimally divided into three (K=3), the second (green) group into two clusters (K=2). Black line indicates border between mountain and lowland populations. Note: The designations employed and the presentation of the material on this map do not imply the expression of

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Figure 2

Distribution of a) allelic richness and b) number of unique alleles across the species range. Larger circles indicate populations with larger allelic richness and number of unique alleles (populations with 0 unique

alleles are not shown) Black line indicates border between mountain and lowland populations. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

Figure 3

Comparison of A) allelic richness (Ar) and B) individuals inbreeding coefficient (FI) between lowland and mountain populations

Figure 4

Results of PCoA of whole dataset (A) and separate PCoA analysis of two main groups distinguished by STRUCTURE (B, C, Fig. 3)

Figure 5

Loss of genetic diversity in lowland populations from the four regions from the Czech Republic. Bars indicate how many alleles survived in current populations, how many alleles have been lost on within population level and how many alleles have been lost on between populations level (due to extinction of whole populations)

Figure 6

Relationship between age of herbaria samples and percentage of amplified loci (GLM, Explained deviance= 40.83, $p < 0.001$)

Supplementary Files

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