Parallel Alpine Differentiation in
Arabidopsis arenosa

Adam Knotek1,2, Veronika Konečná1,2, Guillaume Wos1, Doubravka Požárová1, Gabriela Šrámková1, Magdalena Bohutinská1,2, Vojtěch Zeisek1,2, Karol Marhold1,3 and Filip Kolář1,2,*

1 Department of Botany, Charles University, Prague, Czechia, 2 Institute of Botany, The Czech Academy of Sciences, Průhonice, Czechia, 3 Institute of Botany, Slovak Academy of Sciences, Bratislava, Slovakia, 4 Department of Botany, University of Innsbruck, Innsbruck, Austria

Parallel evolution provides powerful natural experiments for studying repeatability of evolution and genomic basis of adaptation. Well-documented examples from plants are, however, still rare, as are inquiries of mechanisms driving convergence in some traits while divergence in others. Arabidopsis arenosa, a predominantly foothill species with scattered morphologically distinct alpine occurrences is a promising candidate. Yet, the hypothesis of parallelism remained untested. We sampled foothill and alpine populations in all regions known to harbor the alpine ecotype and used SNP genotyping to test for repeated alpine colonization. Then, we combined field surveys and a common garden experiment to quantify phenotypic parallelism. Genetic clustering by region but not elevation and coalescent simulations demonstrated parallel origin of alpine ecotype in four mountain regions. Alpine populations exhibited parallelism in height and floral traits which persisted after two generations in cultivation. In contrast, leaf traits were distinctive only in certain region(s), reflecting a mixture of plasticity and genetically determined non-parallelism. We demonstrate varying degrees and causes of parallelism and non-parallelism across populations and traits within a plant species. Parallel divergence along a sharp elevation gradient makes A. arenosa a promising candidate for studying genomic basis of adaptation.

Keywords: adaptation, alpine environments, Arabidopsis, convergence, parallel evolution, phenotypic parallelism

INTRODUCTION

When and why does evolution result in a predictable outcome remain challenging questions in evolutionary biology. Parallel emergence of identical phenotypes in similar environments brings one of the most intuitive examples of the action of natural selection and provides independent biological replicates allowing for identifying the genetic basis and phenotypic outcomes of adaptation (Elmer and Meyer, 2011; Losos, 2011). In particular, parallel evolution of genetically determined phenotypically distinct entities within a species, so-called ecotypes, provide valuable insights into processes of local adaptation and incipient ecological speciation in nature (Bolnick et al., 2018; Thompson et al., 2019). In contrast to well-studied examples of ecotypic parallelism from animals such as fishes (Rundle et al., 2000; Jones et al., 2012; Reid et al., 2016; Stuart et al., 2017), snails (Butlin et al., 2014; Ravinet et al., 2016), and stick insects (Nosil et al., 2002; Soria-Carrasco et al., 2014), mechanisms driving parallel evolution in plants are largely unknown (but see Roda et al., 2013b; Bertel et al., 2018).
While directional selection driven by similar environment shall lead to convergence, alternative neutral and selective forces may counteract it, leading to larger genetic and phenotypic divergence among the derived ecotypes (reviewed in Bolnick et al., 2018). Specifically, both stochastic processes such as genetic drift, varying intensity of gene flow and different pools of initial variation available for selection as well as deterministic processes such as selection in response to locally distinct micro-environments and genetic architecture of the traits can lead to non-parallel patterns in phenotypic variation (Elmer et al., 2014; Langerhans, 2018). Consequently, there is no clear-cut border between parallel and non-parallel phenotypes, and the extent of parallelism may vary over traits or particular sets of compared populations (Stuart et al., 2017). Finally, apparent similarity across populations can reflect short-term environmentally induced traits (phenotypic plasticity), however, the relative contribution of the heritable vs plastic forces in the manifestation of phenotypic parallelism remains unknown (Pfennig et al., 2010).

Multiple (N > 2) independent environmental transitions within a species provide powerful naturally replicated systems to infer the mechanisms driving parallel phenotypic evolution as well as the extent of variation in parallel vs non-parallel response. Such systems allow multiple pairwise comparisons, in contrast to systems comparing only two parallel transitions. Indeed, independently evolved animal ecotypes documented that a combination of both stochastic and deterministic processes led to a varying extent of parallelism (Bolnick et al., 2018). In contrast, such detailed inquiries in plants are scarce due to paucity of well-established systems that involve multiple replicates of environmental transitions within a species. The limited evidence from the so far investigated systems, Heliosperma pusillum (Trucchi et al., 2017; Caryophyllaceae; five pairs of alpine-foothill ecotypes, Bertel et al., 2018) and Senecio lautus (Rodá et al., 2013a, Asteraceae; seven pairs of dune-heathland ecotypes, Rodá et al., 2013b), suggest considerable morphological divergence among the independently formed ecotypes despite similar environmental triggers, perhaps as a result of genetic drift (Trucchi et al., 2017) or gene flow (Rodá et al., 2013a). However, to infer mechanisms driving parallel evolution in plants in general and its genetic basis in particular, additional genetically well-tractable plant systems are needed.

Alpine populations of Arabidopsis arenosa represent a promising system for addressing drivers and consequences of parallel evolution in a genomic and molecular genetic context of the well-researched Arabidopsis genus. The species thrives at >1,000 m a.s.l.) of Central and Eastern Europe, sometimes treated as a separate species “A. neglecta”: Eastern Alps (Melzer, 1960), Eastern (Pachschwöll and Pachschwöll, 2019), Southern (Bartok et al., 2016), and Western Carpathians (Měšiček and Golašová, 2002). While only tetraploid populations colonized the alpine stands in the former three regions, both diploid and tetraploid populations reached the alpine belt in the Western Carpathians. As the two cytotypes still occupy distinct alpine sub-regions there (diploids in Vysoké Tatry Mts. and tetraploids in Západné Tatry Mts.; Wos et al., 2019), we kept the ploidies as separate units for emergence of distinct alpine phenotypic “syndromes” (e.g., contracted rosette plants, dense cushions, and giant rosettes) that have been recurrently formed in distinct mountain ranges throughout the world (Hedberg and Hedberg, 1979; Körner, 2003; Halbritter et al., 2018; Konečná et al., 2019). Indeed, alpine A. arenosa constitutively exhibits a distinct morphotype characterized by lower stature, less-lobed and thicker leaves, larger flowers and wider siliques (Měšiček and Golašová, 2002). On the other hand, the island-like distribution of alpine habitats promotes parallel colonization of individual alpine “islands” by the spatially closest foothill populations (Levin, 2001). In line with this, a recent genomic study of A. arenosa (Monnahan et al., 2019) demonstrated geographical structuring of its range-wide genetic diversity, suggesting that the alpine ecotype might be of a polytopic origin. This hypothesis, as well as the extent of phenotypic parallelism and its plastic vs. genetic basis, however, remains untested.

In this study, we sampled multiple alpine and adjacent foothill A. arenosa populations covering all mountain regions known to harbor the alpine ecotype. Using genome-wide SNP genotyping we tested our main hypothesis that alpine environment within each mountain region has been colonized independently (parallel origin scenario) as opposed to clustering of the alpine populations together (single origin scenario). Indeed, a combination of genetic structure analyses and modeling revealed multi-parallel origin of the alpine populations. Thus, we combined analyses of field-sampled phenotypic data and a common garden experiment to assess whether the independent alpine transitions are also associated with phenotypic parallelism. Specifically, we ask: (1) Had independent alpine colonization triggered similar phenotypic transitions in distinct mountain regions? (2) Does the alpine phenotype remain divergent from the “typical” foothill form in standardized conditions and, if so, which characters contribute to the genetically determined alpine syndrome? (3) In contrast, are there traits exhibiting a rather opposite, non-parallel response?

**MATERIALS AND METHODS**

**Field Sampling**

Arabidopsis arenosa is a perennial outcrosser encompassing diploid and autotetraploid populations (Kolář et al., 2016b; Monnahan et al., 2019) that is widespread in foothill (colline to sub-montane) elevations across Central and Eastern Europe. Scattered occurrences of morphologically distinct populations have been recorded from four distinct mountain regions in Europe, sometimes treated as a separate species “A. neglecta”: Eastern Alps (Melzer, 1960), Eastern (Pachschwöll and Pachschwöll, 2019), Southern (Bartok et al., 2016), and Western Carpathians (Měšiček and Golašová, 2002). While only tetraploid populations colonized the alpine stands in the former three regions, both diploid and tetraploid populations reached the alpine belt in the Western Carpathians. As the two cytotypes still occupy distinct alpine sub-regions there (diploids in Vysoké Tatry Mts. and tetraploids in Západné Tatry Mts.; Wos et al., 2019), we kept the ploidies as separate units for...
the sake of clarity. In sum, we hereafter refer to five regions: Niedere Tauern and surrounding foothills of the Eastern Alps (NT region, tetraploid), Rodna Mts. and adjacent regions of Eastern Carpathians (RD, tetraploid), Făgăraș Mts. in Southern Carpathians (FG, tetraploid), Vysoké Tatry Mts. and adjacent foothill diploid populations in Western Carpathians (VT, diploid), Západné Tatry Mts. and adjacent foothill tetraploid populations in Western Carpathians (ZT, tetraploid).

In each mountain region we sampled multiple populations from foothill habitats (semi-shaded rocky outcrops, screes and steep slopes; “foothill ecotype”) and from alpine sites (screes and rocky outcrops above the timberline; “alpine ecotype”). As this heliophilous species occurs on “islands” of environmentally suitable conditions (rocky outcrops in lower elevations, rocks and screes in isolated glacial cirques in the alpine zone), the population was defined as a set of individuals occurring in a spatially discrete area in a homogeneous vegetation type surrounded by habitats with unsuitable conditions for the species (e.g., forests, dense grasslands, and arable land). Both ecotypes are separated by a clear distribution gap spanning at least 500 m of elevation which also corresponds with the timberline. We avoided sampling plants in riverbeds immediately below the alpine populations that could represent recent colonizers germinated from washed seeds of the originally alpine plants. We sampled adult individuals from a total of 58 populations – 30 from foothill and 28 from alpine habitats. Within each population, we collected on average 17 individuals at the full-flowering stage: a small part of fresh tissue for ploidy determination, leaf tissue desiccated in silica gel for genotyping and vouchers for morphometrics. We selected largest rosette leaf, second stem leaf from the base and one random flower from the terminal inflorescence and fixed them onto paper with transparent tape for detailed measurements of organ sizes; the entire individual was then press-dried. For each population we also sampled the following local environmental parameters at a microsite with abundant occurrence of A. arenosa: (i) vegetation samples (phytosociological relevés, each covering an area of 3 \times 3 m) recording percentage of the area covered by herb layer and listing all vascular plant species and (ii) mixed rhizosphere soil samples from five microsites within the vegetation sample.

Ploidy level of each sampled individual was determined using flow cytometry as described by Kolář et al. (2016b).

**Experimental Cultivation**

In addition to field sampling, we established a common garden experiment to test whether the plants of alpine origin keep their distinct appearance when cultivated in the foothill-like conditions. We used seeds of A. arenosa collected from 16 natural populations overlapping with those sampled for field phenotyping and genotyping (except for pop. AA254 that was used in the experiment as a replacement of spatially close, <10 km, pop. AA145 which was included in the field dataset). The populations represent four regions (NT, VT, ZT, and FG) and two distinct elevations (two foothill and two alpine populations per region; see Supplementary Table S1 for locality details). To minimize maternal effect of the original localities, we firstly raised one generation in growth chambers under constant conditions. To simulate outcrossing in natural populations, each plant was hand-pollinated by a mixture of pollen from the same population (~14 individuals) for a period of one week; the offspring thus represented a genetically variable mixture of full- and half-sibs. The plants used for phenotyping were raised in the Botanical Garden of the University of Innsbruck (Austria) situated in the Alpine valley, i.e., in conditions resembling the foothill habitat. Phenotypic traits were collected on all plants in the full-flowering stage in an identical way as for the field sampling. For details on cultivation conditions, see Supplementary Methods S1.

**Inference of Parallel Origins**

We genotyped 156 individuals from 46 populations (2–8 individuals per population, 3.5 on average) using the double-digest RADseq protocol of Arnold et al. (2015). For an additional 44 individuals from 11 populations genome-wide SNP data were already available from a genome resequencing study (Monnahan et al., 2019). Raw reads processing and filtration generally followed our earlier study (31); for details see the Supplementary Methods S1.

We inferred population grouping using several complementary approaches. Firstly, we ran K-means clustering, a non-parametric method with no assumption on ploidy, in adegenet v1.4-2 using 1000 random starts for each K between 1 and 20 and selected the partition with the lowest Bayesian Information Criterion (BIC) value (Jombart, 2008). Secondly, we used model-based method FastStructure (Raj et al., 2014). We randomly sampled two alleles per tetraploid individual (using a custom script) – this approach has been demonstrated not to lead to biased clustering in autotetraploid samples in general (Stift et al., 2019) and *Arabidopsis* in particular (Monnahan et al., 2019). We ran the FastStructure with 10 replicates under K = 5 (the same number as for K-means clustering, representing the number of the regions) and additionally only for tetraploid individuals under K = 4. Finally, we displayed genetic distances among individuals using principal component analysis (PCA) as implemented in adegenet v2.1.1. For clustering analyses, we used random thinning over 150 kb windows (length of our RAD-locus) to reduce the effect of linkage and removed singletons resulting in a dataset of 4,341 SNPs. PCA, AMOVA and genetic distances (see below) were calculated using the full set of 103,928 filtered SNPs.

Additionally, we tested for parallel origin of alpine populations using coalescent simulations in fastsimcoal v.26 (Excoffier et al., 2013) – an approach that is also suitable for autotetraploids and mixed-ploidy systems (Arnold et al., 2012). We constructed unfolded (polarization following Monnahan et al., 2019) four-dimensional joint allele frequency spectra from genome-wide SNPs from a subset of sufficiently sampled populations, one alpine and one foothill per region (~ 176–417 k SNPs per population, see Supplementary Table S2 and Supplementary Methods S3 for details; one-dimensional AFS of the populations are published in Monnahan et al., 2019). We compared all regions occupied by tetraploid populations (NT, RD, FG, and ZT) in a pairwise manner. Taking into account single origin of the widespread A. arenosa tetraploid cytotype (Arnold et al., 2013; Monnahan et al., 2019), we had not compared all
diploid-tetraploid pairs but only the spatially closest diploid and tetraploid populations from Western Carpathians (VT and ZT) for which a complex reticulation relationships was suggested previously (Wos et al., 2019). Briefly, for each pair of regions, we compared the fit of our data with two competing topologies: (i) sister position of alpine and foothill populations from the same region (i.e., parallel origin) and (ii) sister position of populations from distinct regions but belonging to the same ecotype (i.e., single origin of each ecotype). For each topology we either assumed or not assumed secondary contact (between-ecotype gene flow) within each region, what resulted in a total of four scenarios per regional pair (Figure 1C). As the main aim of the study was testing for single vs parallel origin of the ecotypes (i.e., model comparison) we had not followed with additional analyses quantifying population divergence (i.e., parameter estimates) within each region.

We used hierarchical analysis of molecular variance (AMOVA) implemented in the R package pegas to test for genetic differentiation (i) among regions and among populations within regions and (ii) among ecotypes and among populations within each ecotype (both in the total dataset and in each region separately). We calculated nucleotide diversity ($\pi$) and Tajima’s D for each population with at least four individuals ($N = 26$) and pairwise differentiation ($F_{ST}$) between these populations using custom python3 scripts (available at https://github.com/mbohutinska/ScanTools_ProtEvol).

Ecological Data
To assess environmental differences among the populations, we combined broad-scale climatic parameters acquired from a database (SolarGIS, version 1.9, operated by GeoModel Solar, Bratislava, Slovakia) with local conditions sampled at the original site of each population. First, we estimated the average values of three climatic variables: Precipitation, Temperature and Photosynthetic Active Radiation (PAR), over April, May, and June, which correspond to the main growth period of Arabidopsis arenosa. Second, we measured soil pH of each site by thermo-corrected electrode (WTW Multilab 540, Ionalyzer, pH/mV meter) at the Analytical Laboratory of the Institute of Botany (Průhonice, Czech Republic). Finally, we inferred local environmental conditions from the vegetation samples: (i) total vegetation cover of herb layer and (ii) Ellenberg Indicators Values (EIVs) calculated from the species list using JUICE (Tichý, 2002). EIVs provide estimates of environmental characteristics of the sites inferred from species composition based on expert knowledge (Ellenberg, 1992). In total, we recorded 671 plant species of which 76% have EIVs. We used only EIVs for Light, Nutrients and Moisture as the remaining EIVs (for Temperature, Continentality, and Soil Reaction) overlapped with
the above-described climatic and soil variables. In sum, our
dataset of environmental parameters comprised the following
eight variables: Precipitation, Temperature, PAR, soil_pH,
Vegetation_cover, EIV_Light, EIV_Nutrients and EIV_Moisture.
No pair of the parameters exhibited > 0.8 Pearson correlation
(Supplementary Table S3).

Phenotypic Traits
To test for parallel phenotypic response to alpine environment,
we described the morphology of each plant sampled using
16 traits (999/223 vouchers in total scored for the field
and common garden datasets, respectively). On each plant
we measured 12 phenotypic characters describing the overall
shape and size of both vegetative and reproductive organs
(except for siliques which were not fully developed in the
full-flowering stage which our sampling aimed at). To assess
variation in shape of the organs independent of absolute size,
we further derived four ratios (all characters are described
and listed in Supplementary Table S4). Missing values in
the field dataset (1.5% in total) were replaced by population
means. For statistical analyses all characters except four (PL,
PW, SL and SW), were log-transformed to approach normal
distribution. No pair of traits was very strongly correlated (> 0.8,
Supplementary Table S3).

Statistical Analyses of Ecological and
Morphological Data
We used PCA calculated by base R function `prcomp` to visualize
ecological differences among populations and morphological
differences among individuals. Separate PCAs have been
calculated on standardized (zero mean and unit variance) sets
of (i) the eight climatic and local environmental variables and
the 16 morphological traits recorded on the individuals collected
(ii) in field and (iii) in a common garden experiment. Overall
differentiation in environmental conditions and morphology of
the ecotypes were tested by permutation multivariate analyses
of variance (permanova). We first calculated Euclidean distances
among population (environmental data) or individual (field
and common garden morphological data) values using `dist`
function and then ran a permanova test with `adonis2` function
(number of permutations = 30,000) in R package vegan 2.5–
4. In addition, we quantified the range of morphological
variation of each ecotype by calculating disparity as the
median distance between each individual and centroid of their
respective ecotype in the ordination space using the R
package disprity.

To quantify morphological differentiation between pre-
defined groups (ecotypes and regions) across all traits we ran
classificatory discriminant analysis with cross-validation as
implemented in Morphotools 1.1 (Koutecký, 2014). To assess
relative contribution of individual morphological characters
of the between foothill-alpine differentiation individuals we
calculated a constrained ordination (linear discriminant analysis,
LDA) in Morphotools. We calculated the discriminant analyses
and permanova tests (i) for complete datasets and then (ii)
separately for each region with ecotype as a factor to assess

major drivers of foothill-alpine phenotypic differentiation within
each region and (iii) separately for each ecotype with region
as a factor to quantify variation in between-region phenotypic
differentiation within each ecotype.

Finally, we ran generalized linear mixed models (GLM)
for each of the 16 phenotypic traits to test for the effects
of ecotype, region and their interaction (individual-based data
with population as a random factor, lme function) in nlme
package v3.1-137. Parallelism in foothill-alpine differentiation
was considered for traits with significant effect of ecotype
but non-significant ecotype × region interaction (lack of
regionally specific differences between ecotypes) that was
revealed consistently in both field and common garden datasets.
In turn, a trait with significant effect of ecotype × region
interaction was indicative of non-parallelism (evidence for
region-specific differences). Finally, traits exhibiting significant
ecotypic effect in the field dataset but not in common garden
were considered as plastic with respect to alpine differentiation.

To assess the degree of parallelism and non-parallelism in a more
quantitative way, effect sizes of each factor and their interaction
were estimated from a linear model using a partial eta-squared
criterion, K = 5 (partition supported by the lowest Bayesian information
criterion, Supplementary Figure S1) according to the geographic
regions disregarding their alpine or foothill origin (i.e., the
ecotype; Figure 1). Populations from the spatially close VT and
ZT regions, which differed by ploidy, were the single exception:
here, the majority of the alpine diploid populations (from VT
region) and some alpine tetraploid (ZT) populations formed a
separate cluster distinct from the remaining Western Carpathian
samples, regardless of ploidy. FastStructure run under K = 5
supported this clustering and revealed clear separation of all
groups but the VT and ZT groups, which were remarkably
admixed (Supplementary Figure S2). Principal component
analysis confirmed spatial, not ecotypic, clustering and revealed
three main clusters: populations from the RD and FG regions
were separated from the VT and ZT cluster along the first axis,
while NT populations differentiated from the rest along the
second axis (Figure 1B).

Coalescent simulations demonstrated that parallel origin
scenarios are more likely than scenarios assuming single
origin of alpine ecotype and this result was consistent across all combinations of tetraploid populations (Akaike weights supporting parallel origin ranged 0.99–1 across the pairwise comparisons of regions; Supplementary Table S5, see also Supplementary Figure S3 for the distribution of AIC values). In contrast, for the spatially close diploid (VT) and tetraploid (ZT) populations the scenario involving sister position of alpine populations of both ploidies was preferred (Akaike weight for the scenario assuming single origin followed by migration was 1; Supplementary Table S5). In summary, genetic analysis coherently demonstrated polytropic origin of the alpine ecotype in four distinct mountain ranges (Alps – NT, Eastern Carpathians – RD, Southern Carpathians – FG, and Western Carpathians – VT and ZT; Figure 1C). In order to account for the ploidy difference among the Western Carpathian populations, we kept the VT and ZT populations separate in the following analyses. Additional analyses when the VT and ZT populations were merged into a single unit (Supplementary Table S6), demonstrated that such alternative grouping does not lead to qualitatively different results with respect to the patterns in alpine-foothill differentiation.

Ecotypic differentiation explained a negligible proportion of genetic variance (3%) while the five regions accounted for 20% of variation (AMOVA analysis, see Supplementary Table S7). Consequently, genetic differentiation between foothill and alpine populations was non-significant within any of the mountain regions in separate AMOVA analyses (Table 1, see also pairwise FST among populations, Supplementary Table S8). These results suggest there is overall low inter-population divergence within each region, and such observation is consistent across regions. The alpine colonization was not accompanied by a reduction in genetic diversity, as populations belonging to both ecotypes exhibited similar nucleotide diversity overall (Wilcoxon test, W = 77, p = 0.93) as well as within each mountain region separately and also Tajima’s D values were close to neutrality (i.e., zero) in both ecotypes (Table 1).

**Habitat Differentiation**

Environmental conditions of foothill and alpine sites significantly differed (permutational multivariate analysis of variance of population, permanova, F = 16.92, p < 0.001; Figures 2A,B). In contrast, populations from different mountain regions did not differ based on the environmental parameters recorded (F = 1.21, p = 0.30).

**Morphological Differentiation in Natural Populations**

Field sampled alpine individuals were overall morphologically differentiated from their foothill counterparts as revealed by their separation in PCA (Figures 2C,D and Supplementary Figure S4), high (87%) classification success in the classificatory discriminant analysis and significant effect of ecotype in permanova (F = 49.25, p < 0.001). Morphological differentiation by ecotype was also significant for each region separately, with high classification success (89–100% across regions, Table 1). Overall, morphological differentiation measured by disparity was significantly higher among the foothill than among the alpine individuals (F1,997 = 113.4, p < 0.001) suggesting that alpine individuals were morphologically more similar to each other than were their foothill counterparts.

Fourteen of the 16 scored characters significantly differentiated between the ecotypes, however, the level of parallelism remarkably differed across traits (Figure 3). Stem height and traits reflecting flower size consistently varied between ecotypes across regions as demonstrated by significant effect of ecotype but non-significant ecotype × region interaction (GLM, Figure 3, Supplementary Table S9, and Supplementary Figure S5) and strong contribution to the ordination constrained by ecotype (highest loadings to the discriminant axis in LDA, Supplementary Table S10). Overall, alpine plants, regardless of their region of origin, were shorter with larger calyces and petals (Supplementary Table S4). In contrast, traits describing leaf size and shape were those with the strongest ecotype × region interaction indicating region-specific (i.e., non-parallel) morphological differentiation between ecotypes (Figure 3). LDAs run separately for each region revealed such non-parallel response reflected distinct foothill-alpine differentiation in the FG region which was, apart from the height, most strongly driven by traits on leaves (LDA, Supplementary Table S10). Such regionally specific effect was primarily driven by the foothill FG morphotypes which is apparent from their very distinct position in the ordination space (Figure 2C) and higher distinctness of the foothill-FG than the alpine-FG populations when contrasted to populations from other regions belonging to the same ecotype (classification success as an FG group was 86% vs 77% for the foothill vs. alpine populations, respectively).

**Phenotypic Variation in Common Garden**

Overall phenotypic differentiation between plants originating from alpine and foothill environment remained highly significant even under two generations of cultivation in a common garden (a subset of 16 populations from four regions; Table 1, Figures 2E,F). Differences between originally foothill and alpine populations were also significant within each region studied (permanova, Table 1), however, with markedly varying strength among the four regions examined, being weakest for NT plants (63% classification success) and strongest for plants from FG region (95%, Table 1). This implies that while alpine populations from some regions (FG and ZT in particular) keep their high morphological distinctness regardless of growth conditions, foothill and alpine populations from the NT region became more similar to each other when grown in a common garden. Morphological disparity of the originally foothill and alpine individuals was still significantly higher than that of their alpine counterparts (F1,221 = 30.96, p < 0.001).

Similarly to the field data, traits describing plant height and floral size contributed most strongly to the ecotypic differentiation across all regions (strong effect of ecotype but no ecotype × region interaction; Figure 3 and Supplementary Table S9). On the other hand, ecotypic differentiation
disappeared under common garden cultivation for nearly all leaf traits, with the exception of leaf lobe characters. However, leaf lobe traits also exhibited significant region × ecotype interaction due to their strong discriminative power between ecotypes in the FG region but not elsewhere (LDA, Supplementary Table S10).

In summary, reduced stem height and larger floral organs showed the strongest parallelism in the genetic component of morphological differentiation across regions (significant effect of ecotype but non-significant ecotype × region interaction both in field and in the common garden; Figure 3). On the other hand, leaf traits generally showed utmost regionally specific discriminative power in the field (no effect of ecotype and/or non-significant ecotype × region interaction) and such regionally specific discriminative effect mostly disappeared in a common garden, demonstrating plasticity in alpine-foothill differentiation for the majority of leaf traits. The only exception were leaf lobe traits that strongly discriminated foothill and alpine ecotype in one particular region (FG) constantly under both field and common garden conditions, demonstrating genetically determined non-parallelism.

**DISCUSSION**

Here, we combined a survey of genetic, ecological and morphological variation in natural populations with a common garden experiment to demonstrate multi-parallel ecotypic differentiation in a wild *Arabidopsis*. Replicated emergence of similar genetically based traits associated with alpine colonization suggests selection triggered by the challenging alpine environment shaped morphological variation of the ancestrally foothill *Arabidopsis* species.

**Parallel Origin of the Alpine Ecotype**

A mosaic distribution of ecotypes within a species’ phylogeny, with genetic diversity rather structured by geographical proximity than by environment (the ecotypes), serves as evidence of repeated ecological divergence (Nosil et al., 2008; Johannesson et al., 2010). The clustering, distance-based and coalescent analyses of genome-wide SNPs congruently demonstrated that the sampled *Arabidopsis arenosa* populations exhibit distinct regional clustering regardless of their alpine vs foothill origin in four regions, i.e., supporting the parallel origin scenario. In line with biogeography (Pawlowski, 1970; Mráz and Ronikier, 2016), the major genetic groups corresponded to the four spatially well-defined and floristically distinct mountain regions: Eastern Alps (NT group) and Southern (FG), Eastern (RD) and Western Carpathians (VT + ZT groups). Genetic similarity of Western Carpathian diploids (VT) and tetraploids (ZT) has been detected previously and probably reflects recent origin of the tetraploid cytotype in the area and/or subsequent interploidy gene flow (Arnold et al., 2015; Monnahan et al., 2019). However, due to distinct ploidy and spatial arrangement reducing the chance for extant gene flow among the VT-alpine and ZT-alpine populations, we considered the two regions as separate units in the following discussion for the sake of clarity.

Parallel origin of the alpine *A. arenosa* ecotype is consistent with previous range-wide studies of the species’ genetic structure (Arnold et al., 2015; Monnahan et al., 2019). Taking into account spatial arrangement of populations and overall phylogeny of the species, colonization of alpine stands from foothill populations likely underlies the observed foothill-alpine differentiation. Firstly, the foothill morphotype represents an ancestral state for the entire species; all three early diverged

### TABLE 1 | Genetic and morphological diversity and differentiation of foothill and alpine populations of *A. arenosa* from the five mountain regions sampled in Central Europe.

| Grouping by region | N | AMOVA (%) | Genetic diversity | Pairwise Fst | Tajima’s D | N | CDA (%) | Differentiation | N | CDA (%) | Differentiation |
|--------------------|---|------------|-------------------|-------------|-----------|---|---------|-----------------|---|---------|-----------------|
| All populations    | 200 | 20 | 0.048 | 0.132 | −0.096 | 999 | 43 | 6.43*** | 223 | 47 | 4.00* |
| Foothill           | 109 | 23 | 0.049 | 0.120 | −0.148 | 559 | 65 | 14.1*** | 117 | 62 | 6.58*** |
| Alpine             | 91  | 30 | 0.048 | 0.139 | −0.025 | 440 | 48 | 10.75*** | 106 | 75 | 19.94*** |

| Grouping by ecotype | N | AMOVA (%) | Genetic diversity | Pairwise Fst | Tajima’s D | N | CDA (%) | Differentiation | N | CDA (%) | Differentiation |
|---------------------|---|------------|-------------------|-------------|-----------|---|---------|-----------------|---|---------|-----------------|
| All regions         | 200 | 3 | 0.049/0.048 | −0.148/−0.025 | 999 | 89 | 49.25*** | 223 | 83 | 108.5*** |
| NT (Niedere Tauern) | 41  | n.s. | 0.047/0.045 | 0.105/0.089 | 999 | 89 | 49.25*** | 223 | 83 | 108.5*** |
| VT (Vysoké Tatry)  | 73  | 3 | 0.047/0.047 | 0.066/0.068 | 232 | 100 | 8.83** | 60 | 63 | 6.52** |
| ZT (Západné Tatry) | 42  | 2 | 0.046/0.051 | 0.049/0.045 | 380 | 89 | 17.15*** | 48 | 83 | 60.91*** |
| RD (Rudna)         | 22  | n.s. | 0.058/0.060 | −/− | 204 | 90 | 16.36*** | 59 | 93 | 17.00*** |
| FG (Fagărăș)      | 22  | 2 | 0.055/0.042 | 0.062/0.119 | 85 | 93 | 3.42* | – | – | – |

1) N RAD-sequenced/phenotyped individuals.
2) Among-group genetic variation component (% in AMOVA).
3) Pairwise nucleotide diversity (n) and Tajima’s D averaged over populations with ≥ 4 individuals (foothill/alpine ecotypes, respectively).
4) Pairwise F₂ averaged over populations with ≥ 4 individuals (foothill/alpine ecotypes, respectively).
5) % of correct classification into ecotype/regional group as inferred by classificatory discriminant analysis of the 16 morphological characters.
6) F-values and significance (*P < 0.05, **P < 0.01, and ***P < 0.001) of permanova analysis of the 16 morphological characters. Parallel origin of alpine ecotype.
diploid lineages of *A. arenosa* comprise only foothill populations (Kolář et al., 2016a). Secondly, foothill populations are spread over the entire area of Central and Eastern Europe wherever suitable rocky habitats are available (Kolář et al., 2016b) while the alpine populations are generally rare and confined to isolated spots within the five mountain regions investigated. Presence of such morphologically distinct populations in other areas is highly unlikely given the floristic knowledge of European mountains (Měšíček and Goliašová, 2002; Fischer, 2008; Bartok et al., 2016).

In summary, spatial distribution of genetic variation demonstrated parallel colonization of the alpine habitats by prevalently foothill *Arabidopsis* species in four biogeographically distinct mountain ranges of Central and Eastern Europe. 

**Parallel Ecotypic Differentiation**

Independent colonization of alpine stands followed by environmentally driven selection should lead to similar phenotypic changes across the alpine populations. Alternatively, drift, divergent selection to locally specific conditions and/or limited variation in the source populations would lead to regionally specific patterns of foothill-alpine differentiation (Bolnick et al., 2018). Independently of the region of origin, alpine *A. arenosa* populations exhibited consistently shorter stems and larger flowers demonstrating parallelism in typical traits associated with “alpine syndrome” that are considered adaptive in alpine environments (Galen, 1989; Halbritter et al., 2018). Elevation gradients belong to the most frequently studied environmental gradients in plant evolutionary ecology since
the rise of this field (Turesson, 1930; Clausen et al., 1940), however, cases of phenotypic parallelism demonstrated by a combination of genetic and experimental data are still very rare in plant literature (Fustier et al., 2017). Interestingly, elevational differentiation has been revealed also in other Arabidopsis species (Fischer et al., 2013; Günther et al., 2016), often replicated across the studied populations (Kubota et al., 2015; Srámková-Fuxová et al., 2017; Hämälä et al., 2018), making this genus also an attractive model for the study of alpine adaptation. However, except for European A. halleri (Srámková-Fuxová et al., 2017) we lack a systematic quantitative assessment of morphological variation, leaving mostly unclear to which extent has parallel colonization been accompanied by parallel phenotypic shifts in other Arabidopsis species.

Alternatively, morphological similarities among the alpine populations may also reflect plastic response toward similar environmental conditions. To separate both effects, we grew plants of alpine and foothill origin from four regions in a common garden. Originally alpine plants kept their overall distinct appearance even in standard conditions, thus corresponding to the original definition of an ecotype sensu Turesson (1922). Importantly, the traits exhibiting the strongest parallelism in foothill-alpine divergence in the field were also highly distinctive in the common garden (stem height and flower size), suggesting the hypothesis of parallel selection over phenotypic plasticity. It shall be noted, however, that alternative genetic yet non-adaptive mechanisms such as developmental constraints or mere chance may still stand behind such parallelism (Losos, 2011; Bolnick et al., 2018). In our case, however, we consider the adaptive scenario most likely given the power of four independent transitions ruling out pure chance, very similar direction of the environmental differentiation across regions, and correspondence of our traits with typical “alpine syndrome” observed across floras worldwide (Körner, 2003; Halbritter et al., 2018). In contrast, the regionally specific ecotypic differentiation detected in the field samples disappeared for the majority of such traits in cultivation, suggesting they rather manifested a plastic response to regionally specific conditions. Such plastic response may still represent a way how to cope with the local nuances of the challenging alpine environments (e.g., Anderson and Gezon, 2015), such a hypothesis would, however, require follow-up experimental tests.

**The Degree and Sources of Non-parallelism**

Even in systems exhibiting generally strong and genetically determined parallelism, both neutral and selective processes may cause significant non-parallel deviations in particular traits or populations (Stuart et al., 2017; Thompson et al., 2019). Although we observed significant non-parallelism within our set of traits, alpine A. arenosa populations showed overall phenotypic homogeneity that was relatively higher than that of their foothill ancestors (as indicated by relatively lower disparity...
of the alpine ecotype). This contrasts to remarkably divergent phenotypic outcomes of independent shifts along the gradient of elevation in the other multi-parallel plant system, Heliosperma (Bertel et al., 2018).

For most of the traits exhibiting non-parallelism in the field data, ecotypic differentiation was no longer present under cultivation in the common garden, demonstrating phenotypic plasticity is the likely major driver of non-parallelism in our data. Only in one case (FG region) an additional trait (leaf lobes) discriminated foothill and alpine populations both in the field and in the common garden suggesting genetic determination of this component of non-parallelism. We consider a neutral scenario that initially divergent variation of the source (foothill) populations is likely responsible for this difference. Notably, the FG-foothill populations were more strongly phenotypically divergent from the other foothill regions than were their FG-alpine counterparts from the other alpine populations. Emergence of non-parallel traits in response to local adaptation is less likely due to overall lower phenotypic variation among the alpine populations and very similar environmental conditions across alpine sites, speaking against strong selection triggered by locally specific conditions. As parallelism informs about the role of selection, its detection is usually the prime aim of most studies (Sackton and Clark, 2019), although the regionally specific deviations provide valuable information on additional evolutionary processes affecting the predictability of evolution (Stuart et al., 2017).

CONCLUSION

Our study demonstrated that spatially isolated alpine environments harbor populations exhibiting remarkable phenotypic parallelism that is manifested by traits that are associated with alpine adaptation in general. Replicated phenotypic shifts in response to similar environmental pressures that are stable over two generations in cultivation suggest the role of selection triggered by the stressful alpine environment. Our findings open new avenues for studying convergent genetic underpinnings of phenotypic parallelism currently being addressed by a follow-up study (Bochutinská et al., 2020). Indeed, alpine A. arenosa is a particularly well-suited model for following such questions due to negligible neutral divergence between ecotypes within each region and lack of strong bottleneck, both of which mitigate potentially confounding signals of past demographic events. This, complemented by the genomic and genetic tractability of the Arabidopsis genus in general and this species in particular (highly variable outcrosser) make Arabidopsis arenosa a highly promising model for studying the genomic basis of parallel adaptation in plants.

REFERENCES

Anderson, J. T., and Gezon, Z. J. (2015). Plasticity in functional traits in the context of climate change: a case study of the subalpine forb Boechera stricta (Brassicaceae). Glob. Chang. Biol. 21, 1689–1703. doi: 10.1111/gcb.12770

DATA AVAILABILITY STATEMENT

The datasets presented in this study are provided as Supplementary Datasets (environmental parameters and morphological traits) and in online repositories (DNA sequences): NCBI BioProjects, accession nos: PRJNA484107 and PRJNA633005.

AUTHOR CONTRIBUTIONS

FK and KM designed the study. AK, MB, GŠ, DP, FK, and KM collected the data. AK, VK, GW, and VZ analyzed the data. FK and AK drafted the manuscript with contribution of all authors. All authors contributed to the article and approved the submitted version.

FUNDING

The project was funded by Czech Science Foundation (project 19-06632S to KM and 17-20357Y to FK), Charles University (GAUK project 708216 to AK), and Research Council of Norway (FRIPRO Mobility fellowship 262033 to FK). Additional support was provided by Ministry of Education Youth and Sports of the Czech Republic (7AMB18AT022 to GW). This work was also supported by the long-term research development project No. RVO 67985939 of the Czech Academy of Sciences.

ACKNOWLEDGMENTS

We thank Eliška Záveská, Magdalena Lučanová, Jana Mořkovská, Jana Smatanová, Peter Schönswetter, Karl Hübler, Stanislav Španiel, Jakub Hojka, Daniel Bohutinský, Jindřich Chrtěk, Klára Kabátová, Frederick Rooks, and Martin Kolník for help with fieldwork. Erwann Arc, Dominik Kaplenig, and Ilse Kraner kindly provided plants cultivated in common garden. Computational resources were provided by the CESNET LM2015042 and the CERIT Scientific Cloud LM2015085. This manuscript has been released as a pre-print at BioRxiv, https://doi.org/10.1101/2020.02.13.948158 (Knotek et al., 2020).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.561526/full#supplementary-material

Arnold, B., Bomblies, K., and Wakeley, J. (2012). Extending coalescent theory to autotetraploids. Genetics 192, 195–204. doi: 10.1534/genetics.112.140582

Arnold, B., Kim, S.-T., and Bomblies, K. (2015). Single geographic origin of a widespread autotetraploid Arabidopsis arenosa lineage followed by interploidy admixture. Mol. Biol. Evol. 32, 1382–1395. doi: 10.1093/molbev/msv089
Bartok, A., Burdi, B., Szatmari, P.-M., Ronikier, M., Puçaş, M., Novikov, A., et al. (2016). New records for the high-mountain flora of the Făgărăș Mts. (southern Carpathians) with discussion on ecological preferences and distribution of studied taxa in the Carpathians. Contr. Bot. 51, 77–153.

Bertel, C., Reietnik, I., Frazier, B., Erschbamer, B., Hübl, K., and Schönswetter, P. (2011). Natural selection drives parallel diversification in the mountain plant Heliosperma pusillum s.l. Oikos 127, 1355–1367. doi: 10.1111/j.0030-1299.2009.12139.x

Bohutinská, M., Vlcek, J., Yair, S., Laenen, B., Konecná, V., Fracassetti, M., et al. (2020). Genomic basis of parallel adaptation varies with divergence in Arabidopsis and its relatives. bioRxiv [Preprint]. doi: 10.1101/2020.03.24.005397

Bolnick, D. I., Barnett, R. D. H., Oke, K. B., Rennison, D. J., and Stuart, Y. E. (2018). (Non) parallel evolution. Annu. Rev. Ecol. Evol. Syst. 49, 303–330. doi: 10.1146/annurev-ecosys-110617-062240

Butlin, R. K., Saura, M., Charrier, G., Jackson, B., André, C., Caballero, A., et al. (2015). Parallel evolution of local adaptation and reproductive isolation in the face of gene flow. Evolution 69, 935–949. doi: 10.1111/evol.12329

Clausen, J., Keck, D. D., and Hiesey, W. M. (1940). Experimental Studies on the Adaptive Differences Between the Albino and Normal Forms of Arabidopsis thaliana (C. A. Mey.) Hayek, “in” his book “Annu. Rev. Ecol. Syst.” 11:e1005361. doi: 10.1111/mec.13721

Kolář, F., Lučanová, M., Záveská, E., Fuxová, G., Mandáková, T., Španiel, S., et al. (2016b). Ecological segregation does not drive the intricate parapatric distribution of diploid and tetraploid cytotypes of the Arabidopsis arenosa group (Brassicaceae). Biol. J. Linn. Soc. Lond. 119, 673–688. doi: 10.1111/bij.12479

Konecná, V., Nowak, M. D., and Kolář, F. (2019). Parallel colonization of subalpine habitats in the central European mountains by Primula elatior. Sci. Rep. 9:3294. doi: 10.1101/137171

Körner, C. (2003). Alpine Plant Life: Functional Plant Ecology of High Mountain Ecosystems. Heidelberg: Springer.

Kottek, M., Kusche, H., Lautenbacher, S., Gedney, N., Frank, C., Gulev, S., et al. (2006). New climate divisions in Europe. Phys. Chem. Earth 31, 723–741. doi: 10.1016/j.pce.2005.10.009

Koutroumpa, F., Makris, G., Ballesta, J., Lozano, M., and Moczek, A. P. (2010). Phenotypic plasticity’s impacts on diversification and speciation. Trends Ecol. Evol. 25, 459–467. doi: 10.1016/j.tree.2010.05.006

Langerhans, R. B. (2018). Predictability and parallelism of multistrait adaptation. J. Hered. 109, 59–70. doi: 10.1093/jhered/esso43

Levin, D. A. (2001). The recurrent origin of plant races and species. Syst. Bot. 26, 197–204. doi: 10.1036/0036-6445-26.2.197

Losos, J. B. (2011). Convergence, adaptation, and constraint. Evolution 65, 1827–1840. doi: 10.1111/j.1558-5646.2011.01289.x

Melzer, H. (1960). Neues und kritisches zur flora der Steiermark und angrenzenden Burgenlandes. Mitt. Naturwiss. Ver. Steiermark 90, 85–102. doi: 10.1111/j.1558-5646.2011.01289.x

Mitros, T., Robinson, M. D., and Moczek, A. P. (2010). Phenotypic plasticity’s impacts on diversification and speciation. Trends Ecol. Evol. 25, 459–467. doi: 10.1016/j.tree.2010.05.006

Matz, V. M., Chowdhary, S., Lipp, W., Schmutz, J., and Guttman, S. D. (2009). Next-generation sequencing and the future of molecular ecology. Trends Ecol. Evol. 24, 643–651. doi: 10.1016/j.tree.2009.04.002

Pfennig, D. W., Wund, M. A., Snell-Rood, E. C., Cruickshank, T., Schlichting, C. D., and Moczek, A. P. (2010). Phenotypic plasticity’s impacts on diversification and speciation. Trends Ecol. Evol. 25, 459–467. doi: 10.1016/j.tree.2010.05.006

Raj, A., Stephens, M., and Pritchard, J. K. (2014). fastSTRUCTURE: variational inference of population structure in large SNP data sets. Genetics 197, 573–589. doi: 10.1534/genetics.114.164350

Ravenet, M., Westram, A., Johannesson, K., Butlin, R., André, C., and Panova, M. (2016). Shared and nonshared genomic divergence in parallel cytotypes of Littorina saxatilis at a local scale. Mol. Ecol. 25, 287–305. doi: 10.1111/mec.13332

Reid, N. M., Proestou, D. A., Clark, B. W., Warren, W. C., Colbourne, J. K., Shaw, J. R., et al. (2016). The genomic landscape of rapid repeated evolutionary adaptation to toxic pollution in wild fish. Science 354, 1305–1308. doi: 10.1126/science.aah4993

Rodan, P., Ambrose, L., Walter, G. M., Liu, H. L., Schaul, A., Lowe, A., et al. (2013a). Genomic evidence for the parallel evolution of coastal forms in the Senecio jacobaea complex. Mol. Ecol. 22, 2941–2952. doi: 10.1111/mec.13231

Rodan, P., Liu, H., Wilkinson, M. J., Walter, G. M., James, M. E., Bernal, D. M., et al. (2013b). Convergence and divergence during the adaptation to similar environments by an Australian groundsel. Evolution 67, 2515–2529. doi: 10.1111/evol.12136

Wen, G., Požárová, D., Konecná, V., Šramkůvá, G., Bohutinská, M., et al. (2020). Parallel alpine differentiation in Arabidopsis arenosa. bioRxiv [Preprint]. doi: 10.1101/2020.02.13.948158

Kolář, F., Fuxová, G., Záveská, E., Nagano, A. J., Hyouklová, L., Lučanová, M., et al. (2016a). Northern glacial refugia and altitudinal niche divergence shape
Knotek et al. Parallel Origin of Alpine Ecotype

Rundle, H. D., Nagel, L., Boughman, J. W., and Schluter, D. (2000). Natural selection and parallel speciation in sympatric sticklebacks. Science 287, 306–308. doi: 10.1126/science.287.5451.306

Sackton, T. B., and Clark, N. (2019). Convergent evolution in the genomics era: new insights and directions. Philos. Trans. R. Soc. Lond. B Biol. Sci. 374:20190102. doi: 10.1098/rstb.2019.0102

Soria-Carrasco, V., Gompert, Z., Comeault, A. A., Farkas, T. E., Parchman, T. L., Johnston, J. S., et al. (2014). Stick insect genomes reveal natural selection’s role in parallel speciation. Science 344, 738–742. doi: 10.1126/science.1252136

Šrámková-Fuxová, G., Záveská, E., Kolář, F., Lučanová, M., Španiel, S., and Marhold, K. (2017). Range-wide genetic structure of Arabidopsis halleri (Brassicaceae): glacial persistence in multiple refugia and origin of the Northern Hemisphere disjunction. Bot. J. Linn. Soc. 185, 321–342. doi: 10.1093/botlinnean/box064

Stift, M., Kolář, F., and Meirmans, P. G. (2019). Structure is more robust than other clustering methods in simulated mixed-ploidy populations. Heredity 123, 429–441. doi: 10.1038/s41437-019-0247-6

Stuart, Y. E., Veen, T., Weber, J. N., Hanson, D., Ravinet, M., Lohman, B. K., et al. (2017). Contrastting effects of environment and genetics generate a continuum of parallel evolution. Nat. Ecol. Evol. 1:0158. doi: 10.1038/s41559-017-0158

Thompson, K. A., Osmond, M. M., and Schluter, D. (2019). Parallel genetic evolution and speciation from standing variation. Evol. Lett. 3, 129–141. doi: 10.1002/evl3.106

Tichý, L. (2002). JUICE, software for vegetation classification. J. Veg. Sci. 13, 451–453. doi: 10.1111/j.1654-1103.2002.tb02069.x

Trucchi, E., Frajman, B., Haverkamp, T. H. A., Schönswetter, P., and Paun, O. (2017). Genomic analyses suggest parallel ecological divergence in Heliosperma pusillum (Caryophyllaceae). New Phytol. 216, 267–278. doi: 10.1111/nph.14722

Turesson, G. (1922). The species and the variety as ecological units. Hereditas 3, 100–113. doi: 10.1111/j.1601-5223.1922.tb02727.x

Turesson, G. (1930). The selective effect of climate upon the plant species. Hereditas 14, 99–152. doi: 10.1111/j.1601-5223.1930.tb02531.x

Wos, G., Mořkovská, J., Bohutínská, M., Šrámková, G., Knotek, A., Lučanová, M., et al. (2019). Role of ploidy in colonization of alpine habitats in natural populations of Arabidopsis arenosa. Ann. Bot. 124, 255–268. doi: 10.1093/aob/mct070

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2020 Knotek, Končná, Wos, Požárková, Šrámková, Bohutínská, Zeisek, Marhold and Kolář. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.