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Is genetic diversity congruent with morphological diversity across the distributional range of the *Melampyrum subalpinum* group (Orobanchaceae)?

RNDr. Thesis

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Annotation:

Allozymes were used to assess the genetic structure of 27 populations of *Melampyrum subalpinum* group and an artificial pollination experiment was carried out to examine the possibility of autogamy. Genetic variation was generally congruent with the known morphological variation of the group. The results corresponded with the central-marginal concept. Allelic enrichment due to hybridization with *M. nemorosum* was observed in some Austrian populations. Czech and Slovak populations do not differ from Austrian populations. The high inbreeding coefficient and the pollination experiment do not contradict the possibility of autogamy.

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Statment about the author's contribution to the study:

Jan Chlumský was the first and corresponding author of this study. J. Ch. participated in field sampling, was responsible for the majority of laboratory work and data analysis and wrote the manuscript.

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Is genetic diversity congruent with morphological diversity across the distributional range of the *Melampyrum subalpinum* group (Orobanchaceae)?

Abstract

The *Melampyrum subalpinum* group consists of morphologically diverse populations traditionally treated as closely related taxa with patchy distribution limited to Central Europe. The centre of the morphological variability and geographical distribution of the group lies on the north-eastern edge of the Alps in the Vienna Forest, while marginal, morphologically uniform populations occur in the Czech Republic and Slovakia. Genetic variation and population structure within the distribution range of the group remains unknown; we hypothesise that the marginal populations are genetically depauperate. Allozymes were used to assess the genetic structure of 27 populations present throughout the distribution area; four *Melampyrum nemorosum* populations from the Vienna Forest were also analysed because of the presumed hybridization. An artificial pollination experiment was carried out to examine the possibility of autogamy. Four enzyme systems were clearly resolved and scored for one polymorphic locus each with a total of 20 alleles.

Seven out of 49 flowers with preserved stamens developed seeds after self-pollination. Genetic variation was generally congruent with the known pattern of the morphological variation of the group. The allelic richness was higher in the Austrian populations than in marginal Czech and Slovak populations. Some wide-leaved populations from the Vienna Forest had a rather high number of alleles which may be caused by allelic enrichment due to former hybridization with *M. nemorosum*. Czech and Slovak populations are genetically derived from Austrian populations. The high differentiation among populations suggests that the current gene flow between populations is limited. The high inbreeding coefficient in some populations indicates that there is a certain level of selfing within the populations. The pollination experiment does not contradict the possibility of autogamy. In general, our data are congruent with the central-marginal model with more variable Austrian populations and less variable isolated and probably partly inbreeding Czech and Slovak populations.

Keywords: allozymes, population genetics, hemiparasites, marginal populations

Introduction

Genetic variation of a species can reveal historical events connected with postglacial colonization (Wróblewska, 2008; Chung et al., 2013), historical and present population size changes (Leimu et al., 2006), the impact of environmental factors and anthropogenic disturbances, as well as the species breeding system (Aparicio et al., 2002; Leimu and Mutikainen, 2005), hybridization (Phillipp and Siegismund, 2003), and polyploidization (Rosenbaumová et al. 2004). High levels of variability are seen as healthy, conferring the ability to respond to threats such as disease, parasites and predators, and environmental changes (Amos and Hardwood, 1998). A number of studies has stated that small and scattered peripheral populations tend to be less variable than populations from the centre of distribution of the particular species. The peripheral populations can face the negative effects of inbreeding, genetic drift and the lowered genetic variation caused by bottleneck and founder effects (Lynch et al., 1995; Young et al., 1996; Tomimatsu and Ohara, 2003; Leimu et al., 2006; Chung et al., 2013). However, in some cases small limited populations do not seem to be influenced by these negative effects (Gitzendanner and Soltis, 2000; Mandák et al., 2005; Wróblewska, 2008).

The *Melampyrum subalpinum* group consists of morphologically diverse populations of annual hemiparasitic plants traditionally treated as closely related taxa with a distribution area reaching from the Eastern Alpine foothills to the north of the Czech Republic and the south-western part of Slovakia (cf. Fig. 1). Following the conventional taxonomic concept, at least four taxa are recognized. The centre of morphological diversity lies in the Vienna Forest (Wienerwald), Austria (Štech, 2006). Plants from this region, traditionally designated as *M. subalpinum* (Jur.) A. Kern. s. str. (e.g., Beck, 1882, 1893), have rather wide leaves and bracts, intensively blue-coloured bracts, and relatively dense indumentum on calyx and bracts. These morphotypes are labelled as *M. subalpinum* var. *thermale* in the current Austrian literature (Fischer et al., 2008; Staudinger, 2009). Less variable populations with narrow leaves and bracts and sparse indumentum occur in an area reaching from the Vienna Forest towards the Lower Austria/Styria Alps. Traditionally they are recognized as *M. angustissimum* Beck but in recent Austrian literature the name *M. subalpinum* var. *subalpinum* is used (Fischer et al., 2008; Staudinger, 2009). Morphologically very similar and very uniform marginal populations known from the Czech Republic and Slovakia are traditionally labelled as *M. bohemicum* A. Kern. (Hadač, 1966). However, based on a comprehensive investigation of morphological variation, Štech (2006) considers these populations conspecific with *M. angustissimum*. The early flowering morphotypes of *M. angustissimum* restricted to the higher altitudes of the Alps are described as *M. grandiflorum* A. Kern. Seasonality is a common phenomenon in the genus *Melampyrum* as well as in related hemiparasitic species and genera (Soó, 1926 Soó, 1926-1927; Zopfi, 1993a,b, 1997) and it does not seem to be systematically important because the early flowering plants are quite rare and it is not possible to analyse them separately. Therefore, in this study one early flowering *M. grandiflorum* population (MarsA) is included in the narrow-leaved group together with *M. angustissimum*.

Genetic variation and population structure within the whole distribution range of the group remains unknown. According to the group's morphological variability and geographical distribution, Štech (2006) presumes that the centre of genetic diversity ofthe group lies on the north-eastern edge of the Austrian Alps in the Vienna Forest. The marginal morphologically uniform populations from the Czech Republic and Slovakia are expected to be genetically less variable. Some of the marginal populations are at present declining; whether this is due to a loss of genetic diversity in these small isolated populations remains unknown.

Allozymes are reliable and often used markers for the study of population genetic structure (e.g., Hamrick et al., 1981; Phillipp and Siegismund, 2003; Chrtek and Plačková, 2005; Chung et al., 2013). They are easily detected codominant markers which allow (compared to dominant markers, such as AFLP) to calculate for example allelic frequencies or standard population genetic parameters based on heterozygosity (F-statistics, deviations from the Hardy-Weinberg equilibrium). No previous genetic knowledge of the species is required, large numbers of individuals can be analysed at one time for multiple enzymes, and methods of data interpretation and analysis are well developed (Lowe et al., 2004).

In the study we addressed the following questions

(1) What is the extent and pattern of allozyme variation in the *M. subalpinum* group? (2) Is the level of genetic variation of the *M. subalpinum* group correlated with its morphological variation across the whole distributional range? (3) Are marginal isolated populations in the

Czech Republic threatened by a loss of genetic variation? (4) Is it possible to appraise any colonization history events of the *M. subalpinum* group in Central Europe?

Methods

Sampling

Sampled populations of the *M. subalpinum* group were chosen to cover the whole distributional range and all morphotypes. Sampling was thorough especially in the Vienna Forest (esp. broader surroundings of Baden) with morphologically diverse populations (Fig. 1, Table 1). Outside of the Alps and Vienna Forest, all regions in which *M. subalpinum* occurred are represented by at least one population. Because of the assumed former hybridization of *M. subalpinum* with *M. nemorosum* in the area of Vienna Forest, 4 local populations of *M. nemorosum* were also included. From each population 10 randomly selected plants were collected and in total 31 populations were sampled. A sample from each plant contained approximately 8 fresh leaves or bracts. Upon collection leaves were wrapped in wet tissues and stored in plastic bags on ice until extraction. Voucher specimens from all sampled populations are deposited in CBFS herbarium in České Budějovice (Czech Republic).

Extraction

Extraction was carried out within 24 h of collection. Approximately 80 mg of leaf tissue was ground in ice-cold Tris–HCl extraction buffer with the addition of a small amount of DOWEX 1 × 8–100 (Cl) and quartz sand. The extraction buffer contained 0.1 M TRIS–HCl pH 8.3, 1% (w/v) l-glutathione reduced, 10 mM $MgCl_2 \cdot 6H_2O$, 5% (w/v) sucrose and 0.1% (v/v) 2-mercaptoethanol. Crude homogenates were centrifuged for 10 min at 15,000 rpm. Clear supernatant was stored in deep freeze at −75°C.

Electrophoresis

The extracts (30 µl per sample) were subjected to electrophoresis in vertical polyacrylamide gel slabs (Hoefer SE 600 vertical unit) using separating gel (8.16%) with 1.82 M Tris–HCl buffer, pH 8.9, and stacking gel (4%) with 0.069 M Tris–H_3PO_4 buffer, pH 6.9. Electrode buffer was 0.02 M Tris, 0.24 M glycine, pH 8.3. Ice-refrigerated electrophoresis was carried out by applying a pulsed current at 80 mA for ca. one hour (until the front of samples left the stacking gel) and subsequently at 100 mA for 3 h and 15 min. The following 18 enzymes were tested with a focus on well stained and clearly interpretable zymograms exhibiting some variability: SKDH (1.1.1.25), ADH (1.1.1.1), SOD (1.15.1.1), AAT (2.6.1.1), ENP (3.4.23.6), PRX (1.11.1.7), MDH (1.1.1.37), IDH (1.1.1.42), GDH (1.4.1.2), ACP (3.1.3.2), HEX (2.7.1.1), 6-PGDH (1.1.1.44), PGM (2.7.5.1), PGI (5.3.1.9), DIA (1.6.-.-), LAP (3.4.11.-), EST (3.1.1.-), G-6-PDH (1.1.1.49). From these enzymes 4 were chosen for further analysis (shikimic acid dehydrogenase SKDH, alcohol dehydrogenase ADH, superoxide dismutase SOD and endopeptidase ENP). The other tested enzymes were either invariable or did not provide zymograms of the required quality.

Staining

The staining procedures followed Vallejos (1983) to visualize ADH and Wendel and Weeden (1989) for ENP, SOD and SKDH with the following modifications. The SOD staining ingredients were 50 ml of 0.05 M Tris–HCl (pH 8.2), 5 mg of NBT, 4.5 mg of EDTA and 1.5 mg of riboflavin. The gel was incubated in the dark at 37°C for 20 min and then placed under

lamp light until bands appeared on the dark background. The SKDH ingredients were 30 ml of 0.1 M Tris–HCl (pH 8.4), 30 mg of shikimic acid, 5 mg of NADP, 6 mg of MTT and 1 mg of PMS. The gel was incubated in the dark at 32°C until bands appeared. Ingredients for ENP for solution A were 50 ml 0.2 M Tris–maleic acid (pH 5.5) and 50 ml for rinsing of the gel, 20 mg of Fast Black K salt and 50 mg of $MgCl_2 \cdot 6H_2O$. Ingredients for solution B were 2 ml *N,N*-dimethylformamide and 25 mg BANA. Solution A was poured into solution B in the dark. The gel was rinsed in chilled Tris–maleic acid and then incubated in a mixture of solutions A and B at 37°C until bands appeared. ADH staining ingredients for solution A were 40 ml 0.1 M Tris–HCl (pH 7.5), 15 mg NAD, 10 mg MTT and 1 mg PMS. Solution B was 10 ml of chilled ethanol. Solution A was poured over the gel and left to incubate at 32°C. After 3 min solution B was added. If the gel was not sufficiently stained, more ethanol was added after 1 h of incubation. All gels were rinsed in distilled water and wrapped in two cellophane sheets and dried.

Data analysis

Zymograms were scored according to Soltis and Soltis (1989). One variable and easily interpretable locus was chosen in each enzyme system. Within this locus in monomeric SKDH and ENP zymograms alleles were numbered with increasing migration distance from the origin. In dimeric SOD and ADH zymograms heterozygotes possess 3 bands. From these only outer bands were scored as alleles whereas the middle (heterodimeric) band was not considered as an allele. Occasionally occurring secondary bands with notably lower intensity were not scored as alleles. Allele frequencies, percentage of polymorphic loci (P), mean number of alleles (A), mean effective number of alleles (A_e), observed (H_o) and expected (H_e) heterozygosity and fixation index (F_{st}) were calculated using POPGENE version 1.31 (Yeh et al., 1999). Coefficients of inbreeding (F_{is}) were calculated using FSTAT version 2.9.3.2 (Goudet, 1995); their significance (assuming the null hypothesis of $F_{is}= 0$) was tested by a permutation test with 1000 replicates. A dendrogram based on Nei's genetic distances between populations (Nei, 1972) was generated using POPGENE. Nei´s genetic distances were used as a metric in a Principal Coordinate Analysis, which was calculated using Canoco version 5.01 (ter Braak and Šmilauer, 2012).

To determine if genetic distances were correlated with geographic distances, a Mantel test (Mantel, 1967) was performed for different subsets of the studied populations (all populations of *M. subalpinum* agg.; Czech, Slovak, and Austrian narrow-leaved; all Austrian; Austrian narrow-leaved; Austrian wide-leaved; only Czech and Slovak populations). Correlation coefficients between matrices of Nei´s genetic distances of populations and their geographical distances were calculated using zt software (Bonnet and van de Peer, 2002); significance of the correlation coefficients was tested by a permutation test with 10,000 replicates.

Reproduction experiment

A pollination test was carried out on the Czech DoubB population. Very young unopened flowers on several plants were used for the test. Flowers were alternately either castrated and pollinated a few days later with pollen from another plant (allogamy), not castrated and left to self-pollinate (autogamy) or only castrated (control). After the treatment, the experimental plants were covered with monofilament sacks to prevent access by pollinators. The flowers were visually checked for the formation of capsules and seeds (well formed × aborted/defective × absent).

Results

Allozymes

For SOD two loci were detected, but only the faster locus provided clear and variable pattern (dimeric, 2 alleles). For ADH two loci were detected as well and only the faster locus was variable and clear enough for scoring (dimeric, 7 alleles). SKDH yielded one monomeric locus with 7 alleles and ENP had one monomeric locus with 4 alleles.

The total number of alleles per population ranged from 6 to 7 in Czech and Slovak populations, 8 to 12 in narrow-leaved Austrian populations and 9 to 15 in wide-leaved populations from the Vienna Forest (Fig. 1). The highest number of alleles (15) was detected in the EinoS population which is morphologically close to *M. nemorosum*. The number of alleles in *M. nemorosum* populations ranged from 8 to 11.

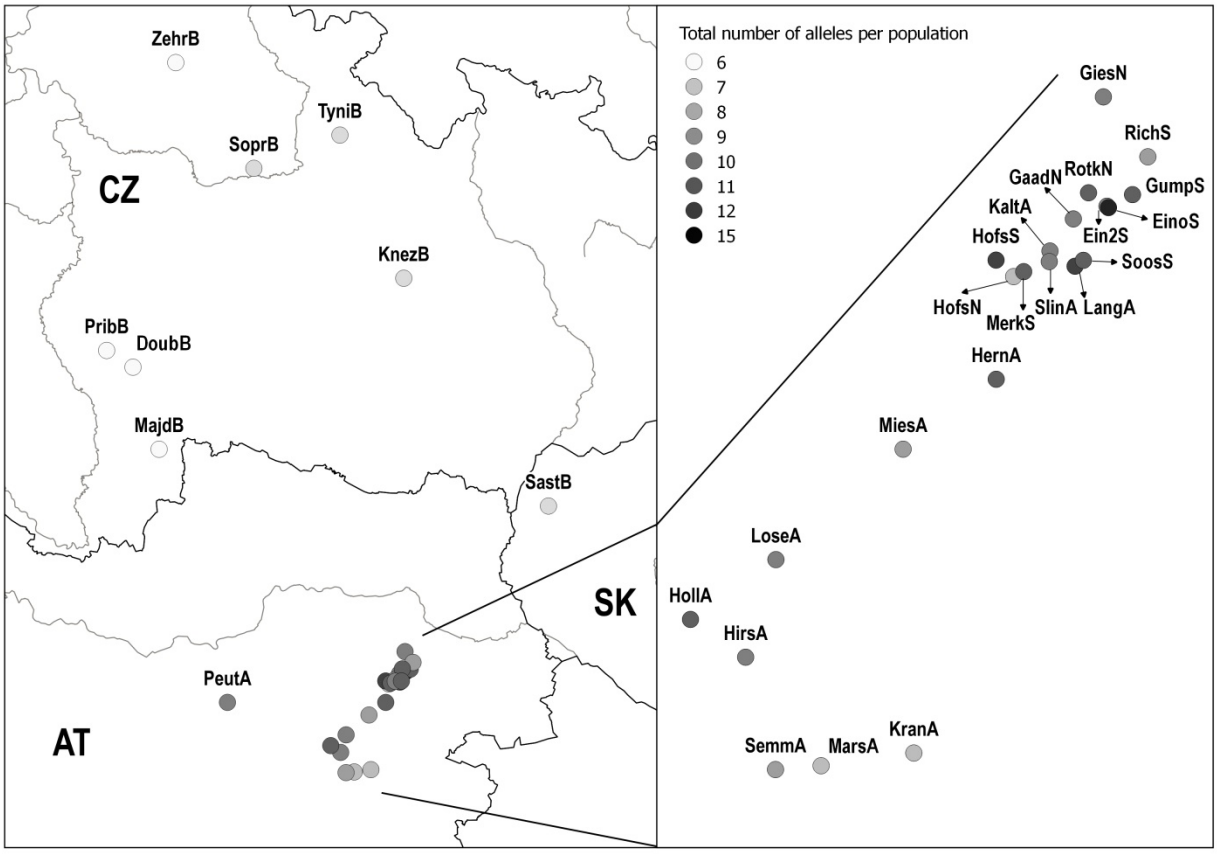

Fig.1. Sampled populations of the *M. subalpinum* group with the total number of alleles per population represented by different shades of grey.

The rarest alleles were ADH 7 with a frequency of 0.002 (LoseA population), SKDH 7 with a frequency of 0.005 (HirsA population), ADH 1 with a frequency of 0.006 (EinoS and HollA populations) and ADH 4 with a frequency of 0.032 (EinoS and SastB populations). The most frequent alleles were ENP 2 with an overall frequency of 0.640 and SOD 2 and ADH 6 with a frequency of 0.613. These alleles were present in most or all of the populations including some *M. nemorosum* populations. Apart from rare alleles that occurred only in one or two populations, we found a unique allele (ADH 5) that occurred only in *M. nemorosum* populations and one wide-leaved *M. subalpinum* population (EinoS) that is morphologically close to *M. nemorosum*. The SKDH 6 allele was found only in 3 wide-leaved *M. subalpinum*

Table 1: Sampled populations with description of locality, altitude, geographical coordinates, date of sampling and approximate number of plants per population.

Population code	Locality	Altitude (m a. s. l.)	Coordinates (WGS84)	Date of collection	Size of the population (order)
Czech and Slovak populations					
DoubB	Doubí u Tábora: forest near the road 230 m SSW of the railway station	410	49°19'8.8"N 14°43'1.7"E	26.8.2008	10^2 - 10^3
KnezB	Kněževes: forest along the path ca 0.7 km NW of the village	550	49°35'30.4"N 16°24'59.8"E	7.8.2006	10^3
MajdB	Majdaléna: forest edge along the path 2.5 km NNW of the village	445	48°59'4.8"N 14°50'39.7"E	30.8.2005	10^2
PribB	Příběnice: forest around of the castle ruin of Příběnice	420	49°23'32.8"N 14°33'44.8"E	5.9.2006	10 - 10^2
SastB	Šaštín: pine forest near the road to Borský Mikuláš 1.4 km ESE of the village	185	48°37'52.3"N 17°10'12.8"E	29.8.2006	10^2 - 10^3
SoprB	Sopreč: forest near the crossroad 1.8 km SSE of the village	230	50°4'42.9"N 15°32'46.3"E	5.9.2006	10^3
TyniB	Křivice: forest 1.2 km WNW of the village	280	50°11'1.7"N 16°5'48.4"E	5.9.2006	10^3
ZehrB	Žehrov: forest near the road 0.9 km SE of the village centre	280	50°31'22.8"N 15°6'23.5"E	5.9.2006	10^2
Austrian narrow-leaved populations					
HernA	Hernstein: forest margin along the road to Neusiedel 1.6 km NNW of the village	390	47°54'26.6"N 16°5'41.2"E	24.6.2008	10^3
HirsA	Hirschwang an der Rax: forest above the Höllental road 1.9 km NNW of the village	525	47°43'20.9"N 15°48'15.9"E	21.8.2008	10^2
HollA	Höllental: forest margin and shrubs near the mouth of the G. Kesselgraben valley	580	47°45'9.6"N 15°44'55.5"E	23.6.2008	10^3
KaltA	Baden, Kaltenberger Forst: pine forest along the path on the northeast slope of the Soosser Lindkogel 5 km W of the town	590	47°59'46.2"N 16°9'48.0"E	21.8.2008	10^2
KranA	Kranichberg: hazel shrubs SE of the castle of Kranichberg	660	47°38'38.6"N 15°58'31.4"E	23.6.2008	10^3
LangA	Sooß: forest along the path on the ridge southward of the Langer Graben valley	430	47°59'1.5"N 16°11'19.7"E	21.8.2008	10^2
LoseA	Losenheim: forest along the path 200 m N of the castle ruin of Losenheim	760	47°47'26.8"N 15°50'40.6"E	30.8.2005	10^3
MarsA	Maria Schutz: spruce forest margin along the road 0.5 km ESE of the Mariaschutz church	730	47°38'25.0"N 15°52'32.9"E	23.6.2008	10^3
MiesA	Miesenbach: forest margin along the road 2.5 km N of the village	440	47°51'45.7"N 15°59'22.0"E	21.8.2008	10^2 - 10^3
PeutA	Peutenburg: pine forest above the path 0.3 km WNW of the Peutenburg railway station	430	47°57'20.5"N 15°9'15.1"E	29.8.2006	10^2
SemmA	Semmering: forest along the road 250 m N of the Hotel Panhans	1030	47°38'24.4"N 15°49'37.3"E	29.8.2006	10^4
SlinA	Sooß: forest along the path 0.3 km S of the Soosser Lindkogel hilltop	640	47°59'19.2"N 16°9'42.0"E	21.8.2008	10^2

Population code	Locality	Altitude (m a. s. l.)	Coordinates (WGS84)	Date of collection	Size of the population (order)
Austrian wide-leaved populations					
Ein2S	Einöde: forest and shrubs along the road to Gaaden 750 m NW of the village centre	320	48°01'29.7"N 16°13'41.8"E	21.8.2008	10^3
EinoS	Einöde: forest and shrubs along the road to Gaaden600 m NW of the village centre	320	48°01'25.6"N 16°13'47.0"E	30.8.2005	10^3
GumpS	Gumpoldskirchen: open forest on the rock outcrops ca 500 m WNW of the village	420	48°2'45.4"N 16°15'40.9"E	29.8.2006	10^2
HofsS	Rohrbach, Hofstätten: shrubs (Corylus, Carpinus) along the road to the hermitage Hofstätten	420	47°59'34.4"N 16°6'17.5"E	30.8.2005	10^3
MerkS	Merkenstein: forest above the castle ruin of Merkenstein	450	47°58'58.6"N 16°8'0.3"E	29.8.2006	10^2 - 10^3
RichS	Gumpoldskirchen, Richardhof: shrubs and forest edge along the road 300 m NNE of the Hotel Richardhof	360	48°3'28.2"N 16°16'34.9"E	21.8.2008	10^3
SoosS	Sooß: shrubs along the road 1.4 km W of the centre of the village centre	310	47°59'15.0"N 16°11'53.7"E	21.8.2008	10^3
***M. nemorosum* populations**					
GaadN	Rosental: shrubs along the road ca 1.7 km SSE of the village	260	48°1'4.0"N 16°11'28.2"E	24.6.2008	10^3
GiesN	Gießhübl, Tirolerhof - Siedlung: forest on the western edge of the satllement	420	48°6'12.6"N 16°14'2.6"E	30.8.2005	10^4
HofsN	Bad Vöslau, Großau: forest along the road ca 4.3 km WNW of the village Großau	380	47°58'48.0"N 16°7'20.0"E	30.8.2005	10^2
RotkN	Rotes Kreuz: forest margin ca 2 km SSE of the village Gaaden near Rotes Kreuz crossroad	400	48°2'8.3"N 16°12'34.8"E	29.8.2006	10^3

populations morphologically close to *M. nemorosum*, but not in any *M. nemorosum* population. Allele ADH 2 occurred only in Austrian populations, both wide- and narrow-leaved, from the Vienna Forest region and in one *M. nemorosum* population, and was absent in narrow-leaved populations from the Alps and Czech and Slovak populations. The ADH 3 allele was very common in both Austrian *M. subalpinum* morphotypes, however it also occurred in one *M. nemorosum* population and in one isolated Czech *M. subalpinum* ZehrB population. The ENP 4 and SKDH 5 alleles occurred in wide- and narrow-leaved populations, both Austrian and Czech, but did not occur in *M. nemorosum*. The SKDH 2 and 3 alleles did not occur in the Czech populations, but were present in *M. nemorosum* and wide- and narrow-leaved Austrian populations. The SKDH 4 allele occurred in all *M. nemorosum* populations and 1 wide-leaved population (MerkS) and 1 narrow-leaved Austrian population (LoseA); hence, although it is not unique for any group, it is clearly much more common in *M. nemorosum* populations than in the *M. subalpinum* agg. The allelic frequencies for each locus and each population are given in Table 2.

The effective number of alleles (A_e) per population ranged from 1.112 to 2.940 (Table 3). Among different groups (Table 4), the wide-leaved *M. subalpinum* group had the highest value (A_e= 2.160) and the Czech narrow-leaved group had the lowest value (A_e= 1.340).

Observed heterozygosity (H_o) ranged from 0.050 to 0.593 (mean 0.340), and mean expected heterozygosity (H_e) ranged from 0.092 to 0.572 (mean 0.373). The Czech and Slovak populations had the lowest H_e suggesting the lowest genetic variation. The inbreeding coefficient (F_{is}) of Czech and Slovak populations ranged from high (0.481) to very low (−0.416), which was also the lowest from all studied populations. The highest value of F_{is} was 0.570 for the Austrian LoseA population. Population characteristics are given in Table 3.

The total fixation index (F_{st}) for all populations was 0.378 which indicates a very high genetic differentiation between populations. In the case of groups of populations, the highest F_{st} was found for the Czech-Slovak group (0.514) and the lowest, but still a considerably high F_{st} was found for the *M. subalpinum* group (0.224). A summary of population characteristics for the groups is given in Table 4.

The UPGMA dendrogram (Fig. 2) and the PCoA based on Nei's genetic distances showed similar population relationships. The most isolated population in UPGMA is the Austrian narrow-leaved PeutA population. The remainder of the populations clustered into two major groups. The first group contains all *M. nemorosum* populations and three *M. subalpinum* populations (EinoS, Ein2S, HofsS), which are morphologically closest to *M. nemorosum*. The structure of the second major group is less clear. The remaining Austrian wide-leaved populations are dispersed between narrow-leaved populations. Most of the Czech populations are grouped together, yet the KnezB population, the Slovak SastB population, and especially ZehrB are distant from the rest of the Czech populations. These populations have a rather isolated position as seen in the PCoA analysis (outlying *M. nemorosum* populations were excluded for better resolution). *M. subalpinum* populations morphologically close to *M. nemorosum* (EinoS, Ein2S, HofsS) lie remote from the main patch of the Austrian populations with HofsS being the closest (Fig. 3).

The results of the Mantel test show a significant positive correlation ($r = 0.698$, $p < 0.001$) between Nei´s genetic distances and the geographic distances of the Austrian narrow-leaved populations. Correlations in the most remaining subsets of populations (all populations of *M. subalpinum* agg., Czech, Slovak, and Austrian narrow-leaved, all of the Austrian populations, and Czech and Slovak populations) are positive as well, but markedly lower and they are not, or are only weakly, significant. Weakly significant but negative correlation was revealed in Austrian wide-leaved population (Table 5).

Reproduction experiment

From 35 flowers without stamens pollinated by pollen from different plants (allogamy), 31 flowers developed into a ripe capsule, 1 developed into an aborted capsule and 3 flowers did not producea capsule. From 49 non-pollinated flowers with preserved stamens (autogamy), 42 did not develop into a capsule and 7 flowers self-pollinated and developed capsules. From 58 non-pollinated control flowers with removed stamens 56 did not develop, 1 developed into the aborted capsule and 1 developed into a ripe capsule.

Table 2: Allelic frequencies at four polymorphic loci for each population. Alleles are numbered for each enzyme system/locus.

Population	Locus/allele																			
	SOD		SKDH							ENP				ADH						
	1	2	1	2	3	4	5	6	7	1	2	3	4	1	2	3	4	5	6	7
HofsN	0.850	0.150	0.000	0.050	0.400	0.550	0.000	0.000	0.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.444	0.556	0.000
GiesN	1.000	0.000	0.050	0.050	0.450	0.450	0.000	0.000	0.000	0.500	0.300	0.200	0.000	0.000	0.000	0.000	0.000	0.350	0.650	0.000
GaadN	0.950	0.050	0.150	0.000	0.200	0.650	0.000	0.000	0.000	0.150	0.650	0.200	0.000	0.000	0.000	0.000	0.000	0.750	0.250	0.000
RotkN	0.950	0.050	0.100	0.000	0.100	0.800	0.000	0.000	0.000	0.000	0.950	0.050	0.000	0.000	0.050	0.200	0.000	0.250	0.500	0.000
EinoS	0.900	0.100	0.150	0.150	0.000	0.300	0.050	0.350	0.000	0.000	0.650	0.000	0.350	0.050	0.550	0.150	0.100	0.100	0.050	0.000
Ein2S	0.850	0.150	0.300	0.000	0.000	0.250	0.000	0.450	0.000	0.000	0.500	0.200	0.300	0.000	0.389	0.000	0.000	0.000	0.611	0.000
HofsS	0.550	0.450	0.150	0.150	0.000	0.000	0.250	0.450	0.000	0.000	0.900	0.050	0.050	0.000	0.389	0.167	0.000	0.000	0.444	0.000
GumpS	0.250	0.750	0.800	0.000	0.100	0.000	0.100	0.000	0.000	0.056	0.611	0.333	0.000	0.000	0.050	0.600	0.000	0.000	0.350	0.000
MerkS	0.650	0.350	0.150	0.000	0.350	0.100	0.400	0.000	0.000	0.000	0.750	0.050	0.200	0.000	0.000	0.550	0.000	0.000	0.450	0.000
RichS	0.200	0.800	0.000	0.000	0.650	0.000	0.350	0.000	0.000	0.000	0.800	0.000	0.200	0.000	0.050	0.400	0.000	0.000	0.550	0.000
SoosS	0.250	0.750	0.250	0.100	0.200	0.000	0.450	0.000	0.000	0.000	0.550	0.250	0.200	0.000	0.111	0.000	0.000	0.000	0.889	0.000
LoseA	0.050	0.950	0.850	0.000	0.000	0.150	0.000	0.000	0.000	0.000	0.450	0.400	0.150	0.000	0.000	0.400	0.000	0.000	0.550	0.050
PeutA	0.050	0.950	0.050	0.950	0.000	0.000	0.000	0.000	0.000	0.050	0.050	0.400	0.500	0.000	0.000	0.750	0.000	0.000	0.250	0.000
SemmA	0.000	1.000	0.600	0.100	0.300	0.000	0.000	0.000	0.000	0.000	0.550	0.300	0.150	0.000	0.000	0.250	0.000	0.000	0.750	0.000
HernA	0.400	0.600	0.450	0.100	0.000	0.000	0.450	0.000	0.000	0.000	0.600	0.350	0.050	0.000	0.050	0.250	0.000	0.000	0.700	0.000
HollA	0.050	0.950	0.750	0.000	0.050	0.000	0.200	0.000	0.000	0.000	0.700	0.200	0.100	0.150	0.000	0.150	0.000	0.000	0.700	0.000
HirsA	0.200	0.800	0.600	0.250	0.000	0.000	0.000	0.000	0.150	0.000	0.600	0.150	0.250	0.000	0.000	0.350	0.000	0.000	0.650	0.000
KaltA	0.200	0.800	0.000	0.000	0.800	0.000	0.200	0.000	0.000	0.000	0.550	0.350	0.100	0.000	0.050	0.300	0.000	0.000	0.650	0.000
KranA	0.200	0.800	0.900	0.100	0.000	0.000	0.000	0.000	0.000	0.000	0.600	0.000	0.400	0.000	0.000	0.100	0.000	0.000	0.900	0.000
LangA	0.300	0.700	0.400	0.250	0.300	0.000	0.050	0.000	0.000	0.000	0.650	0.300	0.050	0.000	0.286	0.143	0.000	0.000	0.571	0.000
MarsA	0.400	0.600	0.250	0.750	0.000	0.000	0.000	0.000	0.000	0.000	0.500	0.000	0.500	0.000	0.000	0.111	0.000	0.000	0.889	0.000
MiesA	0.150	0.850	0.950	0.050	0.000	0.000	0.000	0.000	0.000	0.000	0.800	0.150	0.050	0.000	0.000	0.450	0.000	0.000	0.550	0.000
SlinA	0.400	0.600	0.450	0.000	0.250	0.000	0.300	0.000	0.000	0.000	0.450	0.550	0.000	0.000	0.150	0.600	0.000	0.000	0.250	0.000
MajdB	0.500	0.500	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.700	0.300	0.000	0.000	0.000	0.000	0.000	0.000	1.000	0.000
SastB	0.300	0.700	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.950	0.000	0.050	0.000	0.000	0.000	0.900	0.000	0.100	0.000
PribB	0.150	0.850	0.950	0.000	0.000	0.000	0.050	0.000	0.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000	0.000
SoprB	0.300	0.700	0.900	0.000	0.000	0.000	0.100	0.000	0.000	0.000	0.950	0.050	0.000	0.000	0.000	0.000	0.000	0.000	1.000	0.000
ZehrB	0.000	1.000	0.000	0.000	0.000	0.000	1.000	0.000	0.000	0.000	0.700	0.000	0.300	0.000	0.000	0.800	0.000	0.000	0.200	0.000
TyniB	0.200	0.800	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.667	0.111	0.222	0.000	0.000	0.000	0.000	0.000	1.000	0.000
KnezB	0.750	0.250	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.150	0.300	0.550	0.000	0.000	0.000	0.000	0.000	1.000	0.000
DoubB	0.000	1.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.400	0.550	0.000	0.050	0.000	0.000	0.000	0.000	0.000	1.000	0.000
Mean	0.387	0.613	0.490	0.098	0.134	0.105	0.127	0.040	0.005	0.037	0.640	0.169	0.154	0.006	0.069	0.217	0.032	0.061	0.613	0.002

Table 3: Summary of genetic population characteristics for all studied populations based on allozymes. P (%) = percent of polymorphic locus; A = average number of alleles per locus; A_e = effective allele number; H_o = observed heterozygosity; H_e = expected heterozygosity; F_{is} = inbreeding coefficient (values above 0.25 in bold; values marked with * are significant at 5% level).

Population	P (%)	A	A_e	H_o	H_e	F_{is}	No. of alleles
HofsN	75	2.000	1.710	0.342	0.339	-0.010	8
GiesN	75	2.500	1.640	0.525	0.438	-0.212	10
GaadN	100	2.500	1.720	0.350	0.395	0.119	10
RotkN	100	2.750	1.550	0.250	0.309	0.200	11
EinoS	100	3.750	2.940	0.533	0.506	-0.034	15
Ein2S	100	2.500	2.174	0.569	0.526	-0.089	10
HofsS	100	3.000	2.220	0.547	0.526	-0.044	12
GumpS	100	2.750	1.690	0.431	0.459	0.067	11
MerkS	100	2.750	2.161	0.400	0.534	**0.262***	11
RichS	100	2.250	1.160	0.350	0.429	0.192	9
SoosS	100	2.750	2.123	0.350	0.488	**0.294***	11
LoseA	100	2.500	1.880	0.175	0.395	**0.570***	10
PeutA	100	2.500	1.490	0.275	0.303	0.096	10
SemmA	75	2.250	1.590	0.350	0.395	0.119	9
HernA	100	2.750	2.049	0.375	0.533	**0.308***	11
HollA	100	2.750	1.970	0.325	0.372	0.133	11
HirsA	100	2.500	1.950	0.425	0.496	0.150	10
KaltA	100	2.500	1.540	0.475	0.445	-0.072	10
KranA	100	2.000	1.820	0.350	0.305	-0.156	8
LangA	100	3.000	2.350	0.593	0.572	-0.038	12
MarsA	100	2.000	1.230	0.364	0.411	0.120	8
MiesA	100	2.250	1.280	0.23	0.311	**0.286**	9
SlinA	100	2.500	2.180	0.500	0.572	0.133	10
MajdB	50	1.500	1.431	0.300	0.242	-0.256	6
SastB	75	1.750	1.220	0.125	0.183	**0.328**	7
PribB	50	1.500	1.112	0.050	0.092	**0.471**	6
SoprB	75	1.750	1.220	0.175	0.183	0.045	7
ZehrB	50	1.500	1.870	0.200	0.195	-0.029	6
TyniB	50	1.750	1.150	0.183	0.215	0.155	7
KnezB	50	1.750	1.240	0.350	0.253	-0.416	7
DoubB	25	1.500	1.760	0.075	0.141	**0.481**	6

Table 4: Summary of genetic characteristics for groups of populations. P (%) = percent of polymorphic locus; A = average number of alleles per locus; A_e = affective allele number; H_o = observed heterozygosity; H_e = expected heterozygosity; F_{st} = fixation index.

Group	P(%)	A	A_e	H_o	H_e	F_{st}
CZ + SK	100	2.750	1.340	0.182	0.369	0.514
AU narrow	100	4.250	2.120	0.368	0.523	0.226
AU wide	100	4.500	2.160	0.457	0.615	0.224
M. nemorosum	100	3.250	1.740	0.368	0.419	0.146

Table 5: Results of the Mantel test. r = correlation coefficient, p = significance by probability test with 10,000 replicates (value below 0.01 in bold).

Group	r	p
All populations of *M. subalpinum* agg.	0.090	0.224
CZ +SK + AU narrow	0.158	0.113
AU narrow + AU wide	0.396	0.015
AU narrow	**0.698**	**<0.001**
AU wide	-0.350	0.027
CZ + SK	0.213	0.205

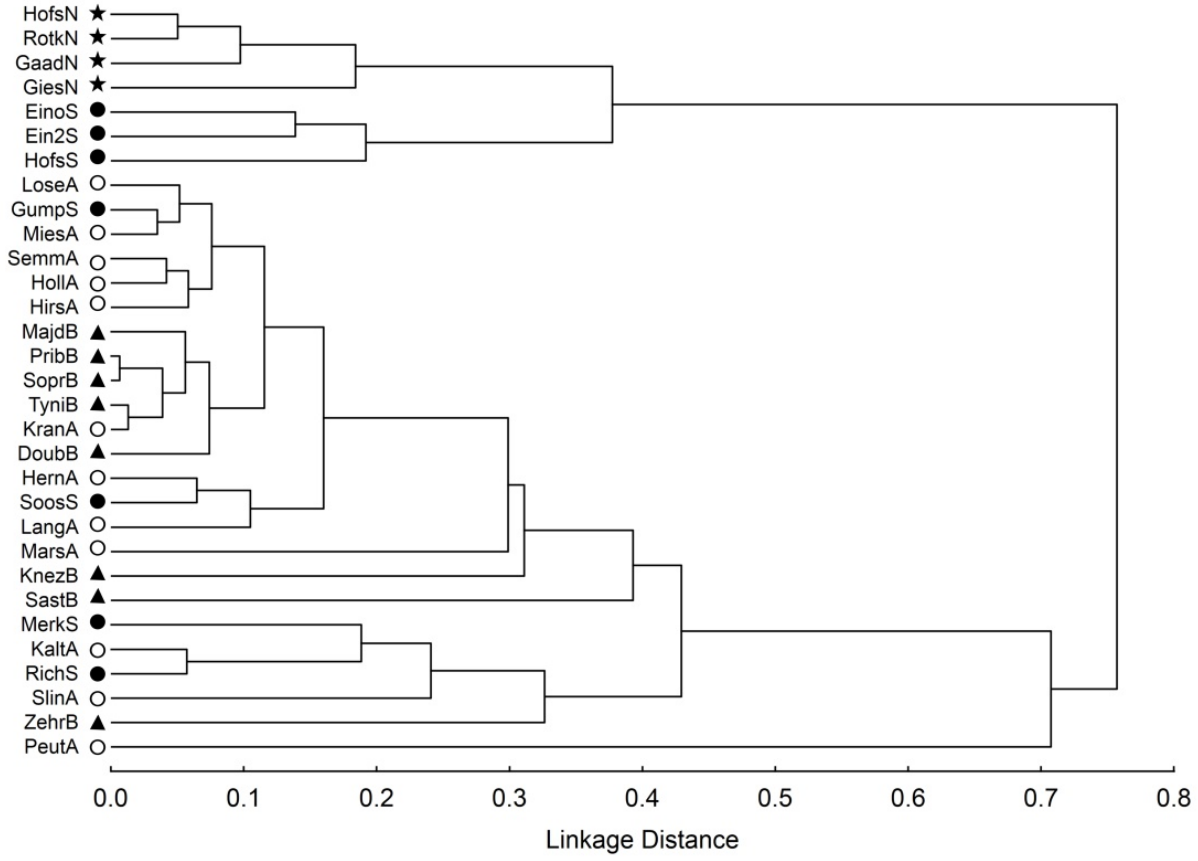

Figure 2: The UPGMA dendrogram based on Nei's genetic distances (Nei, 1972). Symbols explanation: * *M. nemorosum*; ● wide-leaved *M. subalpinum*; ○ Austrian narrow-leaved *M. subalpinum*; ▲ Czech or Slovak *M. subalpinum.*

Discussion

The central-marginal concept claims that within-population genetic diversity declines and among-population differentiation increases from the centre of the species' geographical range to the periphery (Eckert et al., 2008). Although there are some studies questioning the central-marginal concept in the genetic variation of populations (e.g., Mandák et al., 2005; Wróblewska, 2008), our results are consistent with this theory.

The highest genetic variation of *M. subalpinum* agg. among Austrian wide-leaved populations (H_e= 0.615) and the highest effective number of alleles per locus (A_e= 2.160) is in accordance with the assumptions based on morphology about the diversity centre of the group in the

Vienna Forest (Štech, 2006). The adjacent narrow-leaved Austrian populations occurring from the Vienna Forest towards the Lower Austria/Styria Alps display a lower level of variation (H_e= 0.523, A_e= 2.120) and the marginal Czech and Slovak narrow-leaved populations have the lowest variation (H_e= 0.369, A_e= 1.340) and lowest number of alleles per population as well as a lower percentage of polymorphic loci. The highest genetic variation of the wide-leaved populations may also be partly influenced by an assumed hybridization with *M. nemorosum* populations (Štech, 2006).

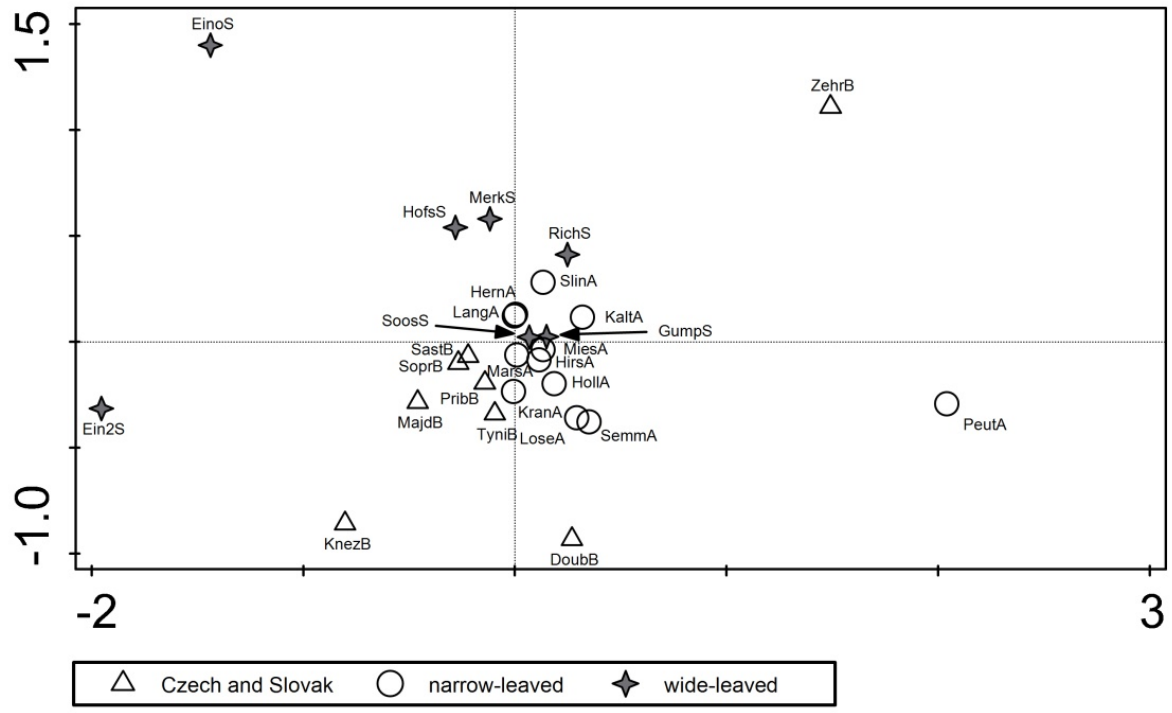

Figure 3: Principal Coordinates Analysis for all studied populations of *M. subalpinum*. For better resolution *M. nemorosum* populations were removed.

This assumption is partly supported by the Mantel test results which showed the highest significant correlation of genetic and geographic distance among Austrian narrow-leaved populations. For wide-leaved populations the correlation was weaker (yet still significant) and negative. The lower correlation is probably caused by the low number of wide-leaved populations which are also restricted to a relatively small area. Another reason might be the possible hybridogenous origin of wide-leaved populations. Hybridogenous populations were observed to possess no significant geographic component in genetic variation, whereas obvious correlations between genetic and geographic distances were detected in non-hybrid populations of the genus *Lotus* (Kramina, 2013).

The highest average fixation index (F_{st}= 0.514) for the Czech and Slovak populations supports the marginal status of this group and clearly shows that the disjunction of these populations is not a recent event. It is possible that the ancestral taxon was spread in the early Holocene across large areas which included today's localities and started to withdraw when the tree canopy became more dense, and later due to substantial changes to suitable habitats made by man. Recent localities are often found in sparse woods on well-illuminated sites such as slopes and terrace edges of river floodplains, forest margins, or the surroundings of forest roads and clearings (Chlumský and Štech, 2011). Another possibility is that *M. subalpinum* was never

widespread in today's marginal areas, but was restricted to small regions with appropriate environmental conditions, and recent localities are the remains of scattered historical distribution.

Nevertheless, genetic drift (Amos and Hardwood, 1998) as a result of either persistent isolation, bottleneck, or founder effect has had enough time to substantially enhance the genetic differentiation between populations.

The average inbreeding coefficient (F_{is}) was close to 0 in all groups suggesting that there is not a high risk of inbreeding depression. However, a closer look at single populations shows a more complicated pattern. There were 8 populations that had a rather high value for the inbreeding coefficient (F_{is}> 0.25, Table 3; note that in four of them it is not significantly different from 0 in a permutation test, which is, however, caused by their low overall variation or even invariability of some loci, which are then omitted from the computation). One of the reasons could be the isolation of populations and therefore higher inbreeding due to crossing with close relatives. The pollination test also showed the partial ability to self-fertilise, which may as well add to the amount of inbreeding. Adaptations for self-fertility are known to increase the probability of establishment following dispersal (Larson and Barrett, 1998) and self-fertility may also be selected in peripheral populations (Lipow and Wyatt, 2000). However, allogamy is obviously still the preferred reproduction mode in *M. subalpinum* agg.

A common feature of populations with high inbreeding coefficient is that they occupy secondary stands which were presumably colonised by rapid radiation from a small number of plants originating from vanished primary stands. The density and regular distribution of these populations is often very high. It has been observed that land use changes may lead to a decreased genetic diversity within populations shortly after the colonization of secondary stands (Vellend, 2004; Jacquemyn et al., 2004, 2009).

On the contrary, the populations with the lowest inbreeding coefficients are often spatially structured and comprised of a system of patches. Although their sites are usually secondary, they often occur close to their putative primary habitats such as the edges of terraces above watercourses. Disturbance dynamics realized by the river ensured proper light conditions for the survival of the species during the Holocene. The continual occurrence of appropriate conditions can reduce a bottleneck effect and such populations may act as allelic refugia and present higher genetic variability (Comps et al., 2001). In case of sufficient genetic diversity in the population and gradual expansion of population size there is still sufficient opportunity for non-relative allogamy and F_{is} thus may reach even moderate negative values.

In concordance with Honnay and Jacquemyn (2007) there was no significant relationship between population size and the inbreeding coefficient.

Despite the low number of studied loci (but high total number of alleles), the dendrogram (Fig. 2) and principal coordinates analysis (Fig. 3) based on Nei's genetic distances were easily interpretable. The dendrogram separated a cluster containing *M. nemorosum* populations together with some wide-leaved Austrian populations that are morphologically closest to *M. nemorosum*. This pattern supports a hypothesis supposing old hybridization between *M. subalpinum* and *M. nemorosum* assumed on the basis of morphological characteristics (Štech, 2006).

The EinoS population had also the allele ADH 5 which was otherwise specific for *M. nemorosum* populations. Another interesting allele, SKDH 4, common for *M. nemorosum* populations, was present in 3 wide-leaved populations. However, it is also present in the population LoseA, which morphologically belongs to the narrow-leaved populations and occurs in a region without the presence of any *M. nemorosum* population. It is hard to say if SKDH 4 is

an ancestral allele or evidence for an old hybridization event with an inconspicuous morphological manifestation.

As expected, *M. grandiflorum* MarsA population did not differ in any way from the rest of the narrow-leaved Austrian populations. The allelic pool of Czech and Slovak populations is obviously pauperized compared to the Austrian populations and there are no unique alleles present for this area. An interesting fact is that the ADH 4 allele is shared by the Slovak SastB population and Austrian wide-leaved EinoS population, which are morphologically different, but geographically relatively close.

The ZehrB population was, due to its geographic isolation and rather late year of discovery, considered to be introduced (Holub, 1996). However, within the Czech populations unique ADH 3 allele discovered in the ZehrB population (common for Austrian *M. subalpinum* agg. populations and one Austrian *M. nemorosum* population) suggests that the ZehrB population might be considered relic.

Conclusions

Genetic variation estimated by isozyme analyses is congruent with the known pattern of morphological variation of *M. subalpinum* agg.

The allelic richness is higher in the Austrian populations than in the marginal Czech and Slovak populations.

Some wide-leaved populations from the Vienna Forest have a rather high number of alleles. This allelic abundance may be caused by allelic enrichment due to an old hybridization with *M. nemorosum*. The Czech and Slovak populations traditionally designated as *M. bohemicum* are, according to their allozymes, genetically derived from Austrian narrow-leaved populations.

The high differentiation among populations suggests that the current gene flow between populations is not common and pop-ulations do not interbreed often. The highest between-population differentiation (F_{st}) in the group of Czech and Slovak populations suggests that they have been isolated long enough for the genetic drift to divide these populations.

The high inbreeding coefficient (F_{is}) in some populations together with the pollination experiment indicates that there might be selfing within the populations.

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