# Variation in the *Melampyrum sylvaticum* group in the Carpathian and Hercynian region: two lineages with different evolutionary histories

**Variabilita *Melampyrum sylvaticum* agg. v karpatské a hercynské oblasti: dvě odlišné evoluční linie**

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We investigated variation in the *Melampyrum sylvaticum* group in the Carpathian and Hercynian regions using morphological and molecular tools. The aim of our study was to examine differences in the pattern of variation between the Eastern Carpathians and region of the Western Carpathians and the Hercynian Massif. We also tested correlations between putatively taxonomically important variation in corolla colour present in the *Melampyrum sylvaticum* group in the Eastern Carpathian region and other morphological and molecular traits. Samples were collected from populations of the *M. sylvaticum* group in the Hercynian Massif and the Eastern and Western Carpathians. Morphometric analyses of the size and shape of the corolla (based on thin plate spline with sliding semilandmarks), length of the anthers and especially molecular analyses based on sequencing the nuclear ITS and *trn*L-*trn*T regions of chloroplast DNA, confirmed that the populations occurring on the opposite sides of the Eastern-Western Carpathian biogeographic boundary are very different. It is likely that the eastern and western lineages have been isolated for a long time and the extant pattern of variation with character disagreement within the border zone, originated from hybridization and introgression. The differences in corolla colour did not coincide with the variation in morphological traits or molecular markers within the North-Eastern Carpathian region. In addition, the geographical distribution of the populations with contrasting corolla colours lacked any pattern and there are populations with both corolla colours as well as plants with transitional pale-yellow flowers. Therefore, it is suggested that *M. saxosum* and *M. herbichii*, microspecies delimited on the basis of corolla colour, are conspecific. The high level of molecular variation and its pattern indicate that the *M. sylvaticum* group may have survived in or near the Eastern Carpathians during the Weichselian Ice Age. This hypothesis is supported by several recent phytogeographical and palaeo-ecological studies, which indicate the existence of a glacial refuge in the Eastern Carpathian region. Molecular uniformity of the Western Carpathian and Hercynian populations might in contrast indicate recent (Holocene) migration from assumed perialpine refuges.

K e y w o r d s : Carpathians, geometric morphometrics, haplotype lineage, *Melampyrum*, molecular variation, phylogeography, refuge


## Introduction

The hemiparasitic genus *Melampyrum* (*Orobanchaceae*) is an important part of the European flora and is most diverse in the Balkan Peninsula followed by Caucasus and temperate Europe (Meusel et al. 1978). *Melampyrum* originated probably in the Mid Tertiary (Wolfe et al. 2005), evolved a number of species a few of which migrated outside Europe and the Caucasus and constituted taxa that do not occur in Europe (e.g., *Melampyrum roseum* and a few related species in E Asia, *M. lineare* in E North America; Soó

1926–1927, Štech 1998). Having survived the Quaternary climatic cycles, temperate species of *Melampyrum* maintained a high diversity, unlike most of the European Tertiary flora (Ložek 1973, Lang 1994). Their diversity might have even increased as a result of isolated evolution in glacial refuges.

The genus *Melampyrum* is still actively speciating, which has resulted in the evolution of several complexes of closely related microspecies that are hardly distinguishable from each other, of which the *M. nemorosum* and *M. sylvaticum* groups are good examples. The origin and distribution pattern of individual microspecies are supposed to have been predominantly affected by the migration of populations, their isolation and subsequent coming into contact in the late Pleistocene and Holocene (Wesselingh & van Groenendael 2005).

The *M. sylvaticum* group is a widespread element of the European montane and subalpine flora. Its geographical range covers mountain ranges from W Europe (the Pyrenees, Scottish Highlands; Dalrymple 2007) to the Urals and lowlands in the boreal zone (Meusel et al. 1978). Forming large populations, it is relatively common in most of its range. Nonetheless, it is sometimes considered rare or even endangered in countries near its geographical boundary, e.g. in Britain (Dalrymple 2007).

Three taxa are usually distinguished at the species level in the *M. sylvaticum* group, based on anther length, corolla size and colour. *Melampyrum sylvaticum* L. s. str. defined by short anthers and a small yellow corolla (see Soó & Webb 1972 or Těšitel & Štech 2007 for the exact range of values) is the most widespread type, believed to grow across the entire range of the group (Meusel et al. 1978). Although certain levels of variability in *M. sylvaticum* s. str. are reported from the Alps (e.g., Ronniger 1911, Soó 1926–1927) there are currently no other species level taxa in this group (Soó & Webb 1972). Long anthers and long (large) corolla characterize *M. herbichii* Woł. and *M. saxosum* Baumg., which differ from each other only in their corolla colour, being yellow and white, respectively. Beside being similar in terms of morphology, the center of the geographical distribution of the latter two species is in the Eastern Carpathians (Soó 1926–1927, Jasiewicz 1958, Paucă & Nyárady 1960, Soó & Webb 1972) and they are also often reported from the Southern Carpathians (Paucă & Nyárady 1960, Soó & Webb 1972). The taxonomic concept of the group is complicated by populations exhibiting diagnostic traits that are intermediate between those of *M. sylvaticum* s. str. and the Eastern Carpathian species. These are frequent in the Western Carpathians and eastern part of the Hercynian Massif, where there is a large zone of morphologically transitional types (Jasiewicz 1958, Štech & Drábková 2005, Těšitel & Štech 2007). Nonetheless, it is not clear whether this morphological similarity reflects genetic similarity with the Eastern Carpathian populations, which has resulted from gene flow across the boundary between the Eastern and Western Carpathians. An alternative hypothesis is that the similarity in morphological features resulted from convergent evolution under similar ecological conditions in both mountain ranges.

The main objective of this study was to evaluate the pattern of variation in the *M. sylvaticum* group across the Hercynian Massif and the Eastern and Western Carpathians using molecular markers and modern morphological methods. We asked the following questions: (i)What is the relationship between populations occurring on the opposite sides of the biogeographical boundary between the Eastern and Western Carpathians? Are they closely related or do they present two distinct lineages within the complex? (ii) Can we detect gene flow between the two Carpathian massifs? (iii) Do the Eastern Carpathian populations display any morphological or genetic variation (in non-coding loci) associated with the two corolla colour forms?

## Material and methods

### Material

The present study is based on plant material from 31 populations (596 individual specimens) of the *M. sylvaticum* group collected from the Eastern and Western Carpathian region and Hercynian Massif (Table 1, Fig. 1). Up to 31 plants were sampled from each population and used in the morphometric analysis of all the specimens collected (596 plants). The corolla of one flower per plant was put into an Eppendorf-tube filled with concentrated (96%) ethanol (denatured) and stored for digitalization. Several leaves or bracts from up to three plants per population were desiccated using silica-gel and kept at –20°C for DNA extraction (in total, 72 specimens were analyzed using molecular tools). The other parts of each plant were processed as a standard herbarium voucher and are in the herbarium of the Faculty of Science, University of South Bohemia (CBFS).

Some of the samples used in the present investigation were originally collected for a previous study of variation in the *M. sylvaticum* group (Těšitel & Štech 2007). Unfortunately, this material was destroyed during processing for the previous analysis and, therefore, not available for the current morphometric analyses. Nonetheless, those samples are a valuable source of material for the molecular part of this study (Table 1). These populations were chosen so as to represent all the mountain ranges from where the samples for our previous study originated (Těšitel & Štech 2007).

### Digitalization and morphometric analysis

Thin plate spline method with sliding semilandmarks (Bookstein 1997, Zelditsch et al. 2004) was employed to analyze corolla shape. This is an efficient way of describing the outline of an object, in particular the edges (presence of which causes difficulties when outline-based methods are used) and has been used in a number of studies on the variation in shape of biological objects (e.g., Neustupa & Hodač 2005, Macholán 2006). Preliminary trials clearly showed that this method is superior to the traditional distance-based morphometrics previously used (Štech & Drábková 2005, Těšitel & Štech 2007). The semilandmarks managed to capture e.g., variation in the corolla curvature, an important diagnostic character completely overlooked by the conventional approach based on a series of linear measurements of corolla shape (see Těšitel & Štech 2007).

The corollas kept in ethanol were flattened and scanned at 1200 dpi using CanoScan 4200 (Canon Inc., Tokyo). The images were saved as RGB colour images in JPG format (low compression). Twenty-seven landmarks were digitized on the outline of each corolla (Fig. 2a), using version 2.05 of tpsDig software (Rohlf 2006). The images were ordered randomly before performing the landmark digitization, which should minimize subjective bias caused by potential similar misplacement of some landmarks in successive images. Twenty-five landmarks were defined as semilandmarks allowing them to slide along the abscissa between their neighbours during the superimposition. Although landmarks 11, 12 and 21 seem to be well defined in two dimensional space (Fig. 2a), we decided to use them as semilandmarks. True landmarks have a slightly higher influence on the analysis than semilandmarks (Zelditsch et al. 2004), which is undesirable in the case of these points. Their position is strongly affected by bending of the lower corolla lip, which occurs when the three dimensional corolla is flattened, and the curvature of the corolla base,

Table 1. – List of the details of the samples of the *Melampyrum sylvaticum* group used in this study. Localization, type of the nuclear DNA (ITS) and chloroplast DNA (*trnL–trnT*) haplotypes are indicated. Samples marked by an asterisk are from populations included in our previous study (Těšitel & Štech 2007). Numbers of specimens analyzed by morphometric and molecular methods are indicated before and after the slash, respectively.

| No. | Country | Locality | Latitude | Longitude | Altitude (m) | Date of sampling | ITS haplotypes | cpDNA haplotypes | Corolla color | Number of specimens |
|---|---|---|---|---|---|---|---|---|---|---|
| 1* | Ukraine | Rakhiv: spruce forest on slope ca 2 km ESE of the town | 48°0236"N | 24°1513"E | 950 | 30.6.2005 | B | a | yellow | 27/3 |
| 2* | Ukraine | Yasinya: forest edge abutting the ski slopes at the tourist resort ca 8 km W of the town | 48°1425"N | 24°1424"E | 1410 | 12.7.2005 | B, B1 | a4 | yellow | 30/3 |
| 3 | Romania | Stațiunea Durău resort: side of path between Fîntînele and Dochia chalets on N slope of Mt Ceahlau, ca 3 km ESE of the mountain resort. | 46°5903"N | 25°5725"E | 1610 | 2.7.2006 | A6, A7 | a6 | white | 30/3 |
| 4 | Romania | Vatra Dornei: rocky massif Piatrele Doamnei ca 1 km SW of Mt Rărau, ca 20 km NE of the town | 47°2651"N | 25°3353"E | 1590 | 3.7.2006 | A, A5 | a | yellow | 30/3 |
| 5 | Romania | Vatra Dornei: side of path in a spruce forest on the ridge between Mt Rărau and Mt Giumalau, ca 13 km NE of the town | 47°2701"N | 25°2960"E | 1416 | 4.7.2006 | A | a2 | yellow | 20/2 |
| 6 | Romania | Vatra Dornei, Mt Giumalau: *Pinus mugo* vegetation on the E slope of the mountain ca 250 m E of the summit, ca 11 km NE of the town | 47°2613"N | 25°2904"E | 1788 | 4.7.2006 | – | – | yellow | 27/– |
| 7 | Romania | Vatra Dornei: mountain meadows of Poiană Obcina Mică ca 5 km NE of the town | 47°2246"N | 25°2239"E | 1250 | 5.7.2006 | A, A2 | a3 | mixed | 29/3 |
| 8 | Romania | Vatra Dornei: side of path in meadows on the NE boundary of the town | 47°2138"N | 25°2214"E | 946 | 5.7.2006 | A | a3 | yellow | 22/3 |
| 9 | Romania | Gura Haitii: *Pinus mugo* vegetation along a path ca 1 km S of the rocky massif Sfincile doispresece apostolii | 47°1304"N | 25°1328"E | 1589 | 5.7.2006 | A | a | white | 30/3 |
| 10 | Romania | Iacobeni: edge of spruce forest on the S slope of Mt Tarnița ca 5 km W of the village | 47°2449"N | 25°1413"E | 1421 | 6.7.2006 | A | a | yellow | 27/4 |
| 11 | Romania | Rotunda settlement: *Pinus mugo* vegetation around a path ca 1.5 km SE of the summit of Mt Omu, ca 8 km SE of the settlement | 47°2918"N | 25°0623"E | 1737 | 7.7.2006 | A | a | white | 30/3 |
| 12 | Romania | Rotunda settlement: side of the road between the Rotunda settlement and the Pasul Rotunda saddle ca 2 km SW of the settlement | 47°3327"N | 25°0114"E | 1128 | 8.7.2006 | A | a | mixed | 29/3 |
| 13 | Romania | Rotunda settlement: side of the road between the Rotunda settlement and the Pasul Rotunda saddle ca 1.5 km SW of the settlement | 47°3333"N | 25°0152"E | 1080 | 8.7.2006 | A | a3 | white | 27/2 |
| 14 | Romania | Danești (Izvoru Oltului): spruce forest and spring area on N slope of a hill ca 1 km NW of the village | 46°3446"N | 25°4647"E | 908 | 1.7.2006 | A4, A5, A7 | a1 | yellow | 29/3 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 15 | Romania | Baile Tuşnad: edge of spruce forest at the W boundary of the town ca 1 km SW of the railway station | 46°08'42"N | 25°51'09"E | 683 | 30.6.2006 | A, A1 | a, a5 | yellow | 31/2 |
| 16 | Romania | Timişu de Jos: path margin in the valley of the Şipaia creek ca 2 km SE of the railway station | 45°34'41"N | 25°38'13"E | 838 | 28.8.2006 | A, A2, A3 | a | yellow | 24/3 |
| 17* | Slovakia | Runina: alpine pastures at Sedlo pod Ďurkovcom Saddle, 3.2km NNE of the village | 49°05'08"N | 22°25'24"E | 1128 | 8.7.2005 | B | a | yellow | 29/3 |
| 18* | Czech Republic | Zalány: *Picea abies* forest on the N border of the village | 49°38'35"N | 13°51'25"E | 645 | 12.7.2005 | B | b1 | yellow | 26/3 |
| 19* | Czech Republic | Ovesná: *Picea abies* forest next to the railway station | 48°48'26"N | 13°56'21"E | 740 | 15.6.2006 | B | b | yellow | 30/2 |
| 20 | Czech Republic | Volary: edge of forest ca 1 km W of Mt Doupná hora, ca 3.5 km ESE of the town | 48°53'45"N | 13°55'28"E | 790 | 21.6.2006 | – | – | yellow | 30/– |
| 21 | Czech Republic | Javorník: edge of meadow ca 700 m S of the village | 49°07'58"N | 13°39'37"E | 900 | 21.6.2006 | – | – | yellow | 15/– |
| 22 | Czech Republic | Pec pod Sněžkou: edge of forest near the lower station of the cableway to Mt Sněžka. | 50°42'28"N | 15°44'02"E | 870 | 17.6.2006 | B | b | yellow | 24/2 |
| 23* | Czech Republic | Kvilda: group of spruce trees on a knoll in valley of Kvildský potok stream 0.5 km E of the village | 49°01'04"N | 13°35'02"E | 1045 | 11.7.2004 | B | b1 | yellow | –/2 |
| 24* | Czech Republic | Karlov, Velká kotlina Valley: montane spruce forest ca 1.5 km SE of peak of Mt Vysoká Hole | 50°03'10"N | 17°14'52"E | 1110 | 7.7.2004 | B | b | yellow | –/2 |
| 25* | Slovakia | Mt Veľký Rozsutec: spruce forest on the N slope of the mountain | 49°14'20"N | 19°06'20"E | 1385 | 22.6.2004 | B | b | yellow | –/2 |
| 26* | Slovakia | Trangoška: montane meadows on the S slope of Mt Chopok, ca 100 meters N of Kosodrevina Hotel | 48°55'57"N | 19°35'28"E | 1525 | 26.6.2004 | B | b | yellow | –/2 |
| 27* | Slovakia | Huty: montane forest near the start of the pathway leading to Mt Biela skala, ca 2 km E of the village | 49°13'24"N | 19°35'59"E | 930 | 1.7.2004 | B | b | yellow | –/2 |
| 7967 28* | Slovakia | Lysá Poľana: meadow beside the road between the village and a gamekeeper's lodge, ca 3 km S of the village | 49°14'27"N | 20°06'05"E | 1005 | 4.7.2004 | B | b | yellow | –/2 |
| 29 | Slovakia | Oravská Polhora: alpine meadows with *Pinus mugo* shrubs on Mt Babia hora, ca 0.5 km SSW of the summit, ca 8 km NE of the village | 49°34'09"N | 19°31'34"E | 1580 | 6.7.2008 | B | b | mixed | –/3 |
| 30* | Ukraine | Burkut: alpine meadows on Mt Chivchin, ca 0.5 km N of the summit, ca 8.5 km S of the village | 47°52'09"N | 24°42'38"E | 1640 | 9.7.2003 | A | b | white | –/2 |
| 31* | Ukraine | Lazeshchina: alpine pastures between Mt Hoverla and Mt Pietrosh ca 2.75 km W of the summit of Hoverla, ca 12 km S of the village | 48°09'37"N | 24°27'50"E | 1570 | 11.7.2003 | A | c | yellow | –/2 |
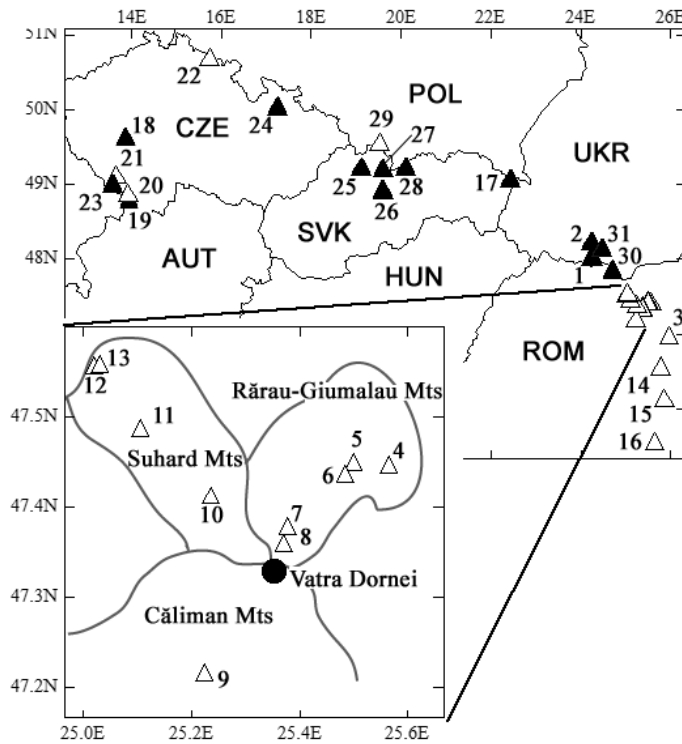
Fig. 1. – Map of the *Melampyrum sylvaticum* group localities included in this study. A magnified view of the surroundings of the town of Vatra Dornei is provided as many samples were collected in this area. Populations displayed by ▲ were sampled in our previous study (Těšitel & Štech 2007) and those depicted by △ were sampled in this study. Borders of the following Central and Eastern European countries are shown: CZE – Czech Republic, AUT – Austria, SVK – Slovakia, POL – Poland, UKR – The Ukraine, ROM – Romania, HUN – Hungary.

which is more or less stochastic and potentially connected to the phenological stage of individual flowers. Nonetheless, there was no apparent difference in the results when we performed a reference analysis in which these points were true landmarks.

Individual landmark constellations were aligned using the Procrustes superimposition (Zelditsch et al. 2004) in tpsRelw, version 1.42 (Rohlf 2005). A maximum of 10 iterations was allowed in the superimposition procedure aiming to minimize the bending energy among the shapes. Resulting scatter of superimposed landmarks can be seen in Fig. 2b. Relative warp analysis (RWA, Rohlf 1993) was subsequently performed with the parameter $\alpha$ set to 0 (resulting in shape principal component analysis) using tpsRelw, version 1.42 software (Rohlf 2005). Centroid size (i.e., sum of distances between individual landmarks and the central point defined as the hypothetical center of gravity) was extracted during the superimposition procedure and employed in subsequent analyses as a measure of size independent of shape.

Anther length was measured in individual flowers in addition to the acquisition of corolla shape and size data. The measurements were done under a dissection microscope. The metering accuracy was 0.05 mm.
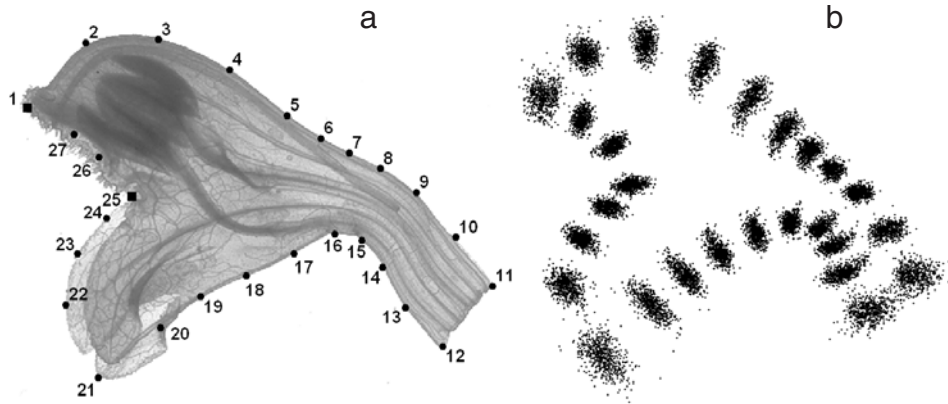
Fig. 2. – (a) Position of landmarks on flattened corolla. Landmarks are marked by boxes, semilandmarks by circles. (b) Scatter plot of superimposed specimens.

*DNA sequencing*

DNA was extracted from dried leaf tissue using a commercial Invitek Plant Extraction Kit (Invitrogen) and following the standard protocol provided by the manufacturer. Polymerase chain reaction (PCR) performed on a Biometra T3000 thermal cycler was employed to amplify the *trn*L-*trn*T region of chloroplast DNA and the ITS1, 5.8S and ITS2 region of ribosomal DNA under the following conditions. PCR was performed in a total volume of 25 μl consisting of 1X PCR Buffer, 200 μM each of dNTPs, 1.25U Taq DNA polymerase (TopBio), 1 μl DNA template solution and 7.5 pmol of each of the primers *trn*L (5'-GAGATTTTGAGTCTCGCGTGTC-3'; primer d in Taberlet et al. 1991), *trn*T2F (5'-CAAATGCGATGCTCTAACCT-3'; Cronn et al. 2002) for cpDNA amplification, or plant-specific ITS1P (5'-CTTTATCATTTAGAGGAAGGAAG-3'; Selosse et al. 2002) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'; White et al. 1990) for ITS. The amplification profile for cpDNA consisted of denaturation at 95°C (300 s), 30 cycles of denaturation at 95°C (60 s), annealing at 62°C (90 s), extension at 72°C (90 s) and final extension at 72°C (600 s). The amplification profile for ITS consisted of denaturation at 95°C (300 s), 32 cycles of denaturation at 95°C (60 s), annealing at 52°C (90 s), extension at 72°C (90 s) and final extension at 72°C (600 s). The PCR products were subsequently purified using JetQuick PCR Purification Kit (Genomed). Sequencing reaction was performed using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) in the Sequencing Centre of the University of South Bohemia.

The sequencing procedure resulted in unambiguous data for both loci. The nucleotide 583 in the ITS alignment was the only exception, oscillating erratically between C and T. It was treated as an ambiguous base Y in all specimens, which set its influence in any analysis to zero. The ITS data otherwise displayed complete concerted evolution (see e.g., Álvarez & Wendel 2003 for explanation) and were directly used in sequence grouping for haplotype definition.

*Data analysis*

We employed standard statistical techniques for detecting variability in morphological characters. The axes constructed by the relative warp analysis (RWA) are suitable for direct visualization by ordination plots as this method is identical with a principal component analysis (PCA), if an appropriate parameter setting is applied. Proportions of within-population variation were calculated using an expected mean square procedure (EMS; Quinn & Keough 2002). Indicators of populations were regarded as random-effect predictors in the calculation. Differences in the quantitative morphometric traits among populations displaying different corolla colours were analyzed only within the North-Eastern Carpathian region due to lack of morphological data for the Western Carpathian populations and the uniformity of the South-Eastern Carpathian populations. An analysis of variance (ANOVA) and a redundancy analysis (RDA) based on the relative warp scores were used to test the relationship between corolla colour and univariate morphometric characters, and corolla shape. Based on the population means or mean relative warp scores of populations (corolla shape), these tests treated populations as independent observations. Corolla colour entered this analysis as a predictor defined as a binary-coded two variable matrix (describing presence of yellow/white colour in the population); hence populations of mixed or transitional colours received 1 for both predictor variables.

We used Statistica for Windows, version 6.0 (StatSoft 2001) for basic statistical procedures, graphical visualization of data and calculation of EMS for univariate variables. Package R, version 2.3.1 (R Development Core Team 2006) was employed for ANOVA calculations. Canoco for Windows, version 4.53 (ter Braak & Šmilauer 2002) was used for the multivariate statistics and for an extraction of sum of squares from the relative warps, which served as a basis for subsequent manual calculation of EMS using a formula in Quinn & Keough (2002). A PCA based on consensual landmark configurations was computed in PAST package, version 1.67 (Hammer et al. 2001) using a singular value decomposition algorithm (which improved the PCA stability when more variables than samples were present in the analysis).

Sequences of each of the analyzed loci were aligned using Clustal W (Thomson et al. 1994) and the alignment was subsequently improved manually. Identical sequences were grouped to define haplotypes. Phylogenetic network of nuclear and chloroplast haplotypes was constructed by means of statistical parsimony (Posada & Crandall 2001) using software package TCS, version 1.21 (Clement et al. 2000). Indels were treated as independent binary characters (coded as A for absence and C for presence as TCS does not support 0/1 coding). Individual gap positions were treated as missing data.

## Results

*Continuous morphometric characters*

Within-population variation accounted for 50.3% of the variation in the shape of the corolla, 32.9% of that of the corolla centroid size and 23.8% of anther length (inferred from EMS analyses). Variation in all analyzed traits displayed continual patterns, which were more or less congruent with each other.
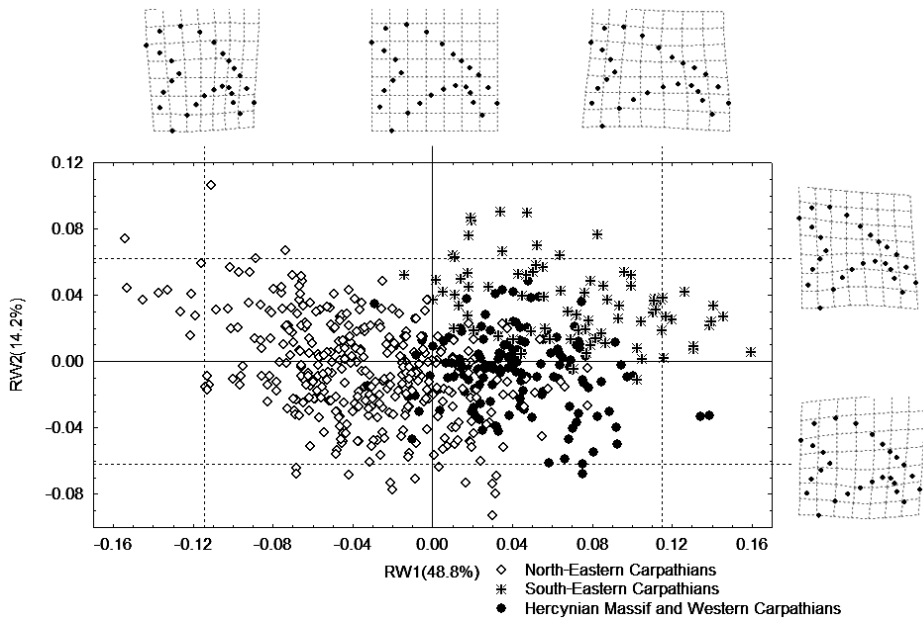
Fig. 3. – RWA ordination plot based on variation in corolla shape of individual plants. Points representing the specimens are classified according to the geographical distribution of the populations. Mean corolla shape and the shape changes associated with the first two principal warps are depicted (shapes corresponding to ± 2 SD positions are displayed on each axis).

Classification of individual specimens (Fig. 3) or populations (Fig. 4) in the relative warp ordination plots revealed that plants growing in different geographical regions tend to concentrate in certain parts of the ordination space. Most of the specimens (and all when the consensual corolla shapes within populations are considered) from the North-Eastern Carpathians (north of the southern slopes of the Câliman Mts and Ceahlau Massif) occupy the left side of the first ordination axis, and tend to have a concave shaped shorter corolla with a slightly more prominent lower lip. Differing mainly in the convex shape of their corolla, the Western Carpathian and Hercynian plants (populations) are generally located on the opposite side of this gradient. Three populations in the South-Eastern Carpathians (south of the northern limit of the Harghita Mts) differ from both of these groups, especially those in the geographically proximate North-Eastern Carpathians. Featuring very long and strongly convexly curved corollas, these plants appear similar to some extreme specimens from the Western Carpathian – Hercynian region.

The plot of variation in univariate morphometric characters showed similar opposite tendencies in samples from North-Eastern Carpathians versus Hercynian and Western Carpathian populations (Fig. 5). The former group has longer anthers and larger corollas than the latter group. The three populations in the southern part of the Eastern Carpathians differ in that their corollas are very large but anther length is variable, as one population has long and the other two rather short anthers, similar to the Hercynian specimens.
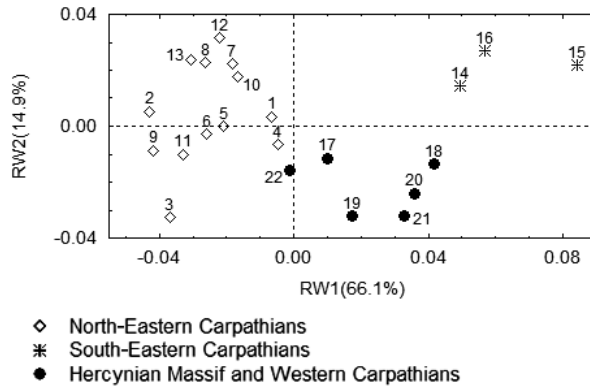
Fig. 4. – RWA ordination plot based on consensual corolla shapes in each population. Percentages of variance explained by the axes correspond only to the variation among populations. The populations are labelled with the numbers of the localities (Table 1).
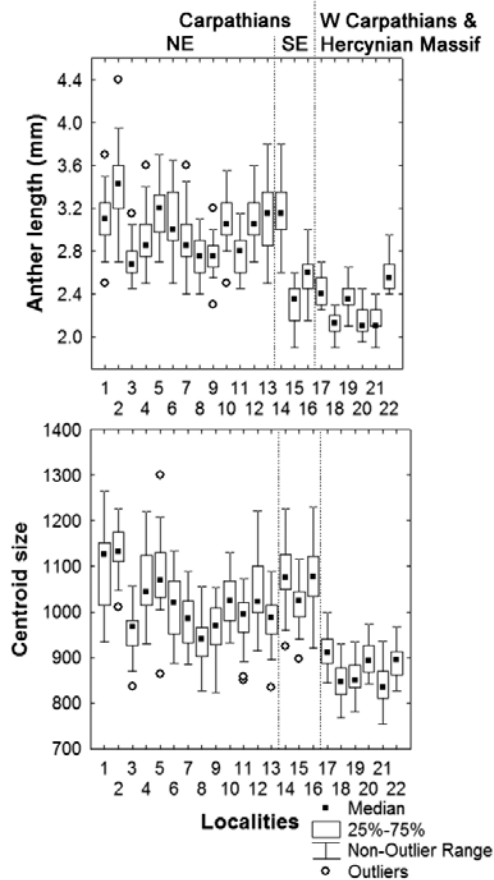


Fig. 5. – Box-and-whisker plots displaying the values of anther length (AL) and centroid size at individual localities. Population numbers correspond to those in Table 1. Their geographical location is indicated by dotted lines separating populations in the North-Eastern Carpathians (1–13), South-Eastern Carpathians (14–16) and Western Carpathians and Hercynian Massif (17–22).

Table 2. – Chloroplast DNA haplotypes defined by variable positions in the *trnL-trnT* cpDNA region. Substitutions within indels are indicated by white font on a black background. Number of plants and populations (sites) in which individual haplotypes were found are indicated.

| Haplotype | GenBank accession number | Number of plants (sites) | Variable positions (bp) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 131 | 306 | 307–315 | 347–355 | 484 | 559 | 615–622 | 637 | 678–700 | 701–723 | 798–799 |
| a | EU653274 | 27 (10) | A | C | – | – | C | G | – | A | TTATATTTCTAGAGACACTATAT | – | – |
| a1 | EU653275 | 3 (1) | A | C | – | – | C | G | – | A | TTATATTTCTAGAGA**T**ACTATAT | – | – |
| a2 | EU653276 | 2 (1) | A | C | – | – | C | A | – | A | TTATATTTCTAGAGACACTATAT | – | – |
| a3 | EU653277 | 8 (3) | A | C | – | – | C | G | – | A | TTATATTTCTAGAGACACTATAT | TTATATTTCTAGAGACACTATAT | – |
| a4 | EU653278 | 3 (1) | A | C | TATAA | – | C | G | – | C | TTATATTTCTAGAGACACTATAT | – | – |
| a5 | EU653279 | 1 (1) | A | C | – | – | C | G | – | A | TTATATTTCTAGAGACACTATAT | – | GC |
| a6 | EU653280 | 3 (1) | A | G | – | – | T | G | – | A | TTATATT**A**TAGAGACACTATAT | – | – |
| b | EU653282 | 17 (8) | A | C | – | – | C | G | AATATAGA | A | – | – | – |
| b1 | EU653283 | 5 (2) | C | C | – | – | C | G | AATATAGA | A | – | – | – |
| c | EU653281 | 2 (1) | A | C | – | AGTAATTAA | C | G | – | A | – | – | – |

Table 3. – Nuclear DNA haplotypes defined by variable positions in the internal transcribed spacer (ITS1, 5.8S, ITS2) sequences. Number of plants and populations (sites) in which individual haplotypes were found are indicated.

| Haplotype | GenBank accession number | Number of plants (sites) | Variable position (bp) | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 50 | 59 | 86-87 | 91 | 187 | 406 | 408–409 | 414 | 442 | 509 | 558–560 | 567 | 571–572 | 577–579 | 597 |
| A | EU624125 | 27 (13) | C | G | TC | A | C | T | GT | C | A | A | – | C | CG | – | C |
| A1 | EU624126 | 1 (1) | C | G | TC | C | C | T | GT | C | A | A | – | C | CG | – | C |
| A2 | EU624127 | 2 (2) | C | G | TC | A | C | T | GT | C | G | A | – | C | CG | – | C |
| A3 | EU624128 | 1 (1) | C | G | CC | C | C | T | GT | C | A | A | – | C | CG | – | C |
| A4 | EU624129 | 1 (1) | C | G | TC | C | C | T | GT | C | G | A | – | C | CG | – | C |
| A5 | EU624130 | 3 (2) | C | A | TC | C | C | T | GT | C | A | A | – | C | CG | – | C |
| A6 | EU624131 | 1 (1) | C | G | CT | C | C | T | GT | C | G | A | – | C | CG | – | C |
| A7 | EU624132 | 3 (2) | T | G | CT | C | C | T | GT | C | G | A | – | C | CG | – | C |
| B | EU624133 | 28 (13) | C | A | TC | A | A | C | GT | T | A | C | TTG | T | TC | GTA | A |
| B1 | EU624134 | 2 (1) | C | A | TC | A | A | C | AC | T | A | C | TTG | T | TC | GTA | A |
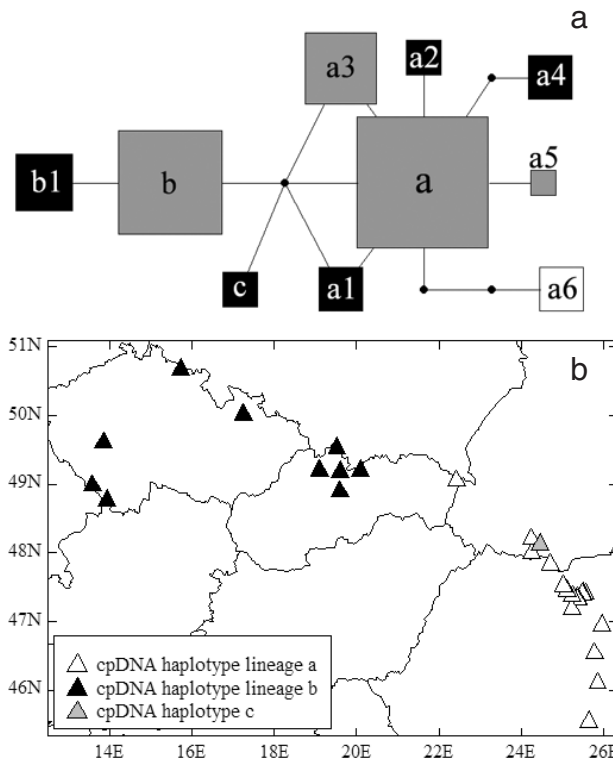
Fig. 6. – (a) The most parsimonous phylogenetic network of the cpDNA haplotypes of the *Melampyrum sylvaticum* group detected in the populations studied. Size of boxes is proportional to the number of plant specimens in which individual haplotypes were detected. Small circles symbolize missing haplotypes. Corolla colour trait is mapped onto the network: haplotypes of populations with only yellow-coloured flowers ■, haplotypes of populations with only white-coloured flowers □, haplotypes of populations with flowers of both colour types or mixed corolla colour ▩. (b) Map displaying the distribution of the cpDNA haplotype lineages of the *Melampyrum sylvaticum* group in the populations studied.

## Variation revealed by molecular markers

Both loci analyzed were variable enough to provide valuable information on the relationships among the populations. Ten haplotypes were detected in the *trn*L-*trn*T region of cpDNA (Table 2). Most of them in two haplotype lineages (**a, b**), which differed in two relatively large indel mutations (Table 2, Fig. 6a). Within each of these lineages there is a basic and widespread haplotype (haplotypes a and b) from which other generally much less frequent haplotypes were derived (these are marked by numbers) by both indel and point substitutions. Haplotype c could not be assigned to either of the large lineages and formed an independent group characterized by a unique indel combination, which positioned it between haplotype groups **a** and **b**. Lineage **a** was found in the whole Eastern Carpathian region including the Bukovské vrchy Mts (Fig. 6b), with its basic haplotype present in most of the specimens and populations (Table 1, Fig 6a). The derived haplotypes were either characteristic of small populations or only found in one population, resulting in com-
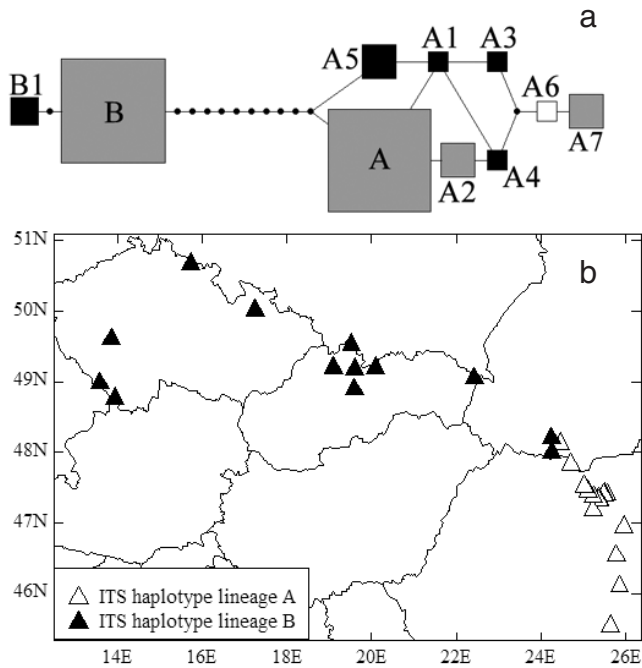
Fig. 7. – (a) The most parsimonous phylogenetic network of the ITS haplotypes of the *Melampyrum sylvaticum* group detected in the populations studied. Size of boxes is proportional to the number of plant specimens in which individual haplotypes were detected. Small circles symbolize missing haplotypes. Corolla colour trait is mapped onto the network: haplotypes of populations with only yellow-coloured flowers ■, haplotypes of populations with only white-coloured flowers □, haplotypes of populations with flowers of both colour types or mixed corolla colour ▨. (b) Map displaying distribution of the ITS haplotype lineages of the *Melampyrum sylvaticum* group in the populations studied.

paratively high genetic differentiation among populations. In contrast, within-population variation was rather low as multiple (two) haplotypes were found only in one population (Table 1). The Hercynian and Western Carpathian populations are similar in only containing haplotype b and its variant b1, distinguished by a point substitution present in two populations in the southern half of Bohemia (Table 1). Haplotype c was only found in a single population on Mt Hoverla (Table 1, Fig. 6b).

The ITS haplotypes could be assigned to the two major lineages **A** and **B**, which differ in the number of single base substitutions and two three-base indels (Table 3). The most parsimonous network describing relationships among individual haplotypes revealed a very pronounced genetic difference (corresponding to a high number of missing haplotypes) between these haplotypic groups (Fig. 7a). Occurring at more than one site, the derived haplotypes were in general not characteristic of individual populations. There was more than one haplotype in many Eastern-Carpathian populations despite the small number of specimens analyzed per population (Tables 1, 3). This was particularly pronounced in the three populations in the southern part of the mountain range where almost no two plants share the same haplotype. Therefore, the genetic pattern is characterized by low differentiation between populations and high within-population variation, at least in the Eastern Carpathians where the genetic variability is high enough for such an estima-
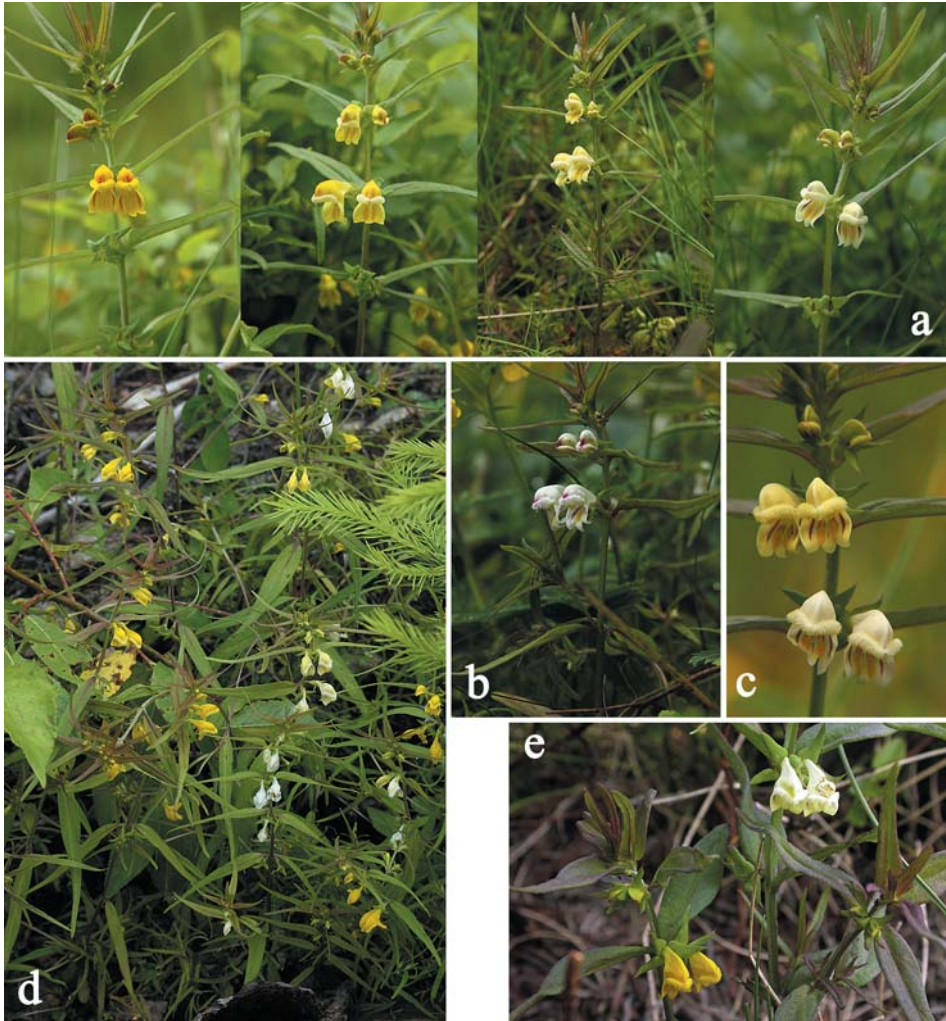
Fig. 8. – Variation in the corolla colour of populations of the *Melampyrum sylvaticum* group. (a) Variation in corolla colour of the population growing in the Poiană Obcina Mică meadows near Vatra Dornei, Romania. The plants are ordered from the yellow on the left to the lightest (almost white) specimen on the right. 5 July 2006. (b) A typical example of a white-flowered plant, Ceahlau Massif, 2 July 2006. (c) An unusual plant with flowers of different colours at different nodal positions, Poiană Obcina Mică meadows near Vatra Dornei, 5 July 2006. (d) A mixture of yellow-, white- and pale-yellow-flowered plants growing along the side of the road near Pasul Rotunda saddle, 8 July 2006. (e) Yellow- and whitish-flowered specimens from Mt Babia hora, Slovakia, 6 July 2008.

tion. The geographical range of lineage **A** includes most of the Eastern Carpathian sites except for those occurring near the Eastern-Western Carpathian boundary (Fig. 7b). In contrast to the cpDNA lineages, lineage **B** is not restricted to the Hercynian and Western Carpathian populations but also occurs in the Eastern Carpathians. The valley of the Tisa River, which crosses the Eastern Carpathians in the Ukraine, appears to be its eastern limit (Fig. 7b).

Variation in both molecular markers appears substantially higher in the Eastern Carpathians than in the western populations, which were found to be almost uniform. In spite of clear differentiation between the Eastern Carpathian and the western populations revealed by both molecular markers, the geographical borders of the distributions of the haplotype lineages do not coincide, resulting in discordance of the phylogeographical patterns. That is, there is a transitional zone on the boundary between the Eastern and Western Carpathians.

*Variation in corolla colour*

There were both yellow- and white-flowered plants in the North-Eastern and Western Carpathian populations of the *Melampyrum sylvaticum* group (Figs 8a, 8b) but only yellow-flowered plants in the populations sampled in the South-Eastern Carpathians and Hercynian Massif. White-flowered specimens were frequent in the North-Eastern Carpathian region, where they formed entire populations, but very rare in the Western Carpathians where only one population was found with yellow, intermediate pale yellow and almost white-flowered specimens on Mt Babia hora (population no. 29, Fig. 8e). The same within-population pattern in corolla colour was recorded at one North-Eastern Carpathian site near Vatra Dornei (population no. 7). In the population below the Rotunda saddle in the Suhard Mts (no. 12) there were plants with intermediate pale-yellow flowers and both extreme corolla colours (Fig. 8d). There was a continuum in corolla colour from white (or almost white in the first two cases) to yellow (Fig. 8a) in all these populations. Slight differences in colour were rarely observed even among flowers on an individual plant (Fig. 8c).

There were no significant relationships between the variation in corolla colour and anther length (logarithmic transformation; ANOVA, $F_{2,10} = 2.59$, $P = 0.226$), centroid size (square-rooted; ANOVA, $F_{2,10} = 1.73$, $P = 0.124$) or corolla shape (RDA, Monte-Carlo permutation test with 999 permutations: $F = 2.47$, $P = 0.146$). There was also no apparent agreement between corolla colour and genetic variation in either of the analyzed loci (Fig. 6a, 7a). Moreover, there were no conspicuous patterns in the geographical distributions of populations featuring different corolla colours (Fig. 9).

## Discussion

*Differentiation and gene flow on the border between the Eastern and Western Carpathians*

Our analyses revealed solid and in general concordant phylogeographical patterns in variability in all continuous morphometric characters and both molecular markers. The differentiation between the Eastern Carpathian and western populations suggested by previous studies (e.g., Jasiewicz 1958, Těšitel & Štech 2007) is clearly supported by two distinct lineages within the *Melampyrum sylvaticum* group in our data. The differences delimiting the Eastern and Western Carpathian populations were especially pronounced in the ITS sequences and in congruent patterns in variation of several morphological characters (despite continual nature of their variation and overlaps). The marked differences in the western (**B**) and eastern (**A**) ITS haplotype lineages is good evidence that the Eastern Carpathian and western types were isolated from one another for a long time in their evolutionary history.
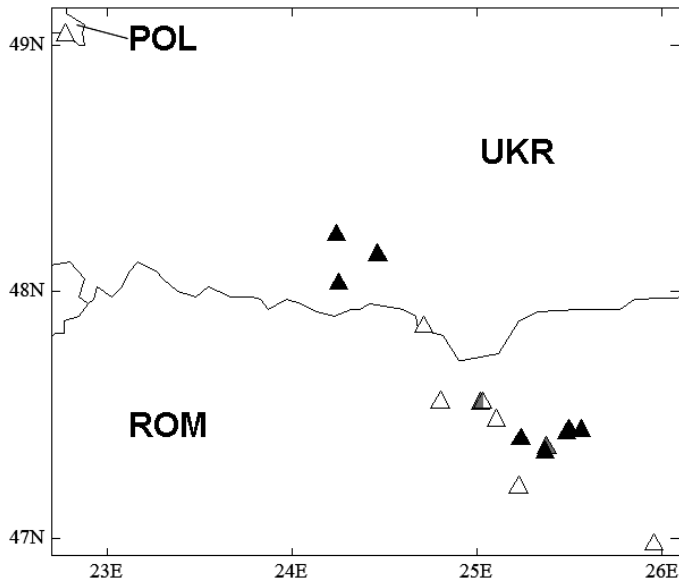
Fig. 9. – Map of the localities for the *Melampyrum sylvaticum* group in the northern part of the Eastern Carpathians for which reliable data are available. Position of an additional Polish white-flowered population is estimated on the basis of data in Zając & Zając (2001) and on the web site of the Bieszczady National Park (Anonymus 2008). The populations are classified according to corolla colour (▲ yellow corolla, △ white corolla, △ populations with mixed- and pale-yellow-flowered plants). Borders of countries are shown: UKR – The Ukraine, ROM – Romania, POL – Poland.

The origin of the transitional zone observed in the border region between the Eastern and Western Carpathians can be attributed to the meeting and subsequent hybridization of the two lineages. Although there are few samples from this area, it is likely that this zone extends from the Tisa valley (and neighbouring Pass of Yablunjitsa) to the Bukovské vrchy Mts (probably its western margin). Reaching only around 850 and 700 m a.s.l., respectively, these sites are very low and narrow parts of the Carpathian ridge, and are likely to impede the migration of alpine and upper-montane floristic elements. The pattern in variation apparently reflects these gene-flow barriers, although the *M. sylvaticum* group is generally regarded as a montane to subalpine taxon (Soó & Webb 1972, Šípošová 1997, Štech 2000) growing in mountain spruce (less frequently beech) forests and ascending to the tree-line. This description of its ecology is however based predominantly on observations in the Alps or Western Carpathians, where many populations indeed grow under spruce or beech forest canopy albeit not in heavily shaded areas (e.g., populations 19, 23 and 27 in the present dataset; Table 1). By contrast, all the populations in the North-Eastern Carpathians apparently prefer open habitats, either natural subalpine grasslands or dwarf-pine vegetation near the tree-line or man-made meadows, clearings and road sides at lower altitudes. This may account for the limited gene flow from the Eastern Carpathians westwards but not in the reverse direction. The low altitude of the Eastern Slovakian part of the main Carpathian ridge combined with the comparatively ineffective myrmecochorous seed dispersal strategy of *Melampyrum* (Winkler & Heinken 2007) may have prevented a mass migration of the central Western Carpathian populations in an east-

erly direction. The region between the Vysoké Tatry and Bukovské vrchy Mts comprises ca 100 km wide zone within which the altitude only fluctuates between 500 and 800 m a.s.l. The natural vegetation of this region is a continuous closed-canopy beech forest unfavourable for *M. sylvaticum*. On the other hand, *M. sylvaticum* might have migrated at certain periods in the Holocene when *Picea abies* forests formed a more substantial part of the local vegetation, probably between ca 8000 and 4000 BP (Latałova & van der Knaap 2006). The presence of western-type ITS haplotype in the Eastern Carpathian populations west of the Tisa valley may, therefore, be attributed to gene flow from the central part of the Western Carpathians in the past and subsequent introgression.

The pronounced genetic and morphological differences in the *M. sylvaticum* group on the East-West Carpathian boundary are similar to the recently reported patterns in genetic variation in *Hypochaeris uniflora* (Mráz et al. 2007) and *Campanula alpina* (Ronikier et al. 2008). The relatively wide transitional zone is in agreement with the continuous nature of the biogeographical boundary characterized by a gradual decrease in the diversity of Eastern Carpathian alpine floristic elements (such as *Rhododendron kotschyi*, *Alnus viridis*, *Laserpitium krapfii* subsp. *krapfii* and several diploid species of *Hieracium*; Polívka et al. 1928, Mráz & Szeląg 2004) in a westerly direction (Zemanek 1991). Many lower-montane species (i.e., those occurring mainly in beech forests), however, crossed this border and reached the Western Carpathians (e.g., *Veronica urticifolia*, *Aconitum moldavicum*, *Aposeris foetida*) and even the Hercynian Massif (e.g., *Anthriscus nitida* and *Doronicum austriacum*; Slavík 1997, Štech 2004). The latter case was recently well documented for *Rosa pendulina* using a phylogeographical study based on chloroplast DNA sequence variation (Fér et al. 2007).

*Evolution of the extant variation pattern and its palaeoecological background*

The high level of molecular variation in both chloroplast and nuclear DNA sequences in populations in the Eastern Carpathians indicates that large populations sufficient to maintain such variability have been present there for a long time. These loci are almost uniform in the Hercynian and Western Carpathian populations, which indicates markedly different evolutionary histories. Populations in the North-Eastern Carpathians probably survived the last glacial period (Weichsel, Würm) in one, or more likely, several refuges located probably either in the Eastern Carpathians or their vicinity. Locations and size of these favourable sites might have been relatively dynamic and dependent on climatic oscillations. Evolution in refuges that were isolated but connected periodically can result in the observed pattern in the genetic variation. Molecular uniformity of the populations in the Western Carpathian and Hercynian Massif indicate a recent (Holocene) migration from refuges located probably in perialpine areas.

Several recent studies have demonstrated that it is highly likely that a glacial refuge existed in the Eastern Carpathians, which supports our hypothesis of the long-term persistence of the *M. sylvaticum* group in this region. Robust evidence comes from a review of palaeobotanical finds of charcoal in Central Europe (Willis & van Andel 2004), which indicates the presence of *Picea* (one of the main *M. sylvaticum* group host species; e.g., Štech 2000) and *Alnus* in the eastern surroundings of the North-Eastern Carpathians between 35 000 and 20 000 years BP (calibrated ^{14}C chronology), i.e. during a significant part of the Last Glacial Maximum (LGM). Genetic and pollen data indicate that *P. abies*

survived in the North-Eastern Carpathian region (Tollefsrud et al. 2008). In addition, the North-Eastern Carpathian populations of *Pinus mugo,* another important species associated with the *M. sylvaticum* group, differ morphologically from other Central European and Balkan populations, which indicates their genetic isolation and that they have probably been present in the region for a long time (Boratyńska, Muchewicz & Drojma 2004). This is also supported by the results of several palynological sequences that indicate the presence of *Pinus* (probably *P. mugo*) in the late ice age (Farcas et al. 1999, Feurdean 2004), and that a refuge or several isolated refuges suitable for *Melampyrum* might have existed at favourable sites at the base of the mountains in the North-Eastern Carpathian region.

It is suggested that Siberian taiga-type boreal forest existed in the Western Carpathians during the LGM (Jankovská & Pokorný 2008) This does not accord with our hypothesis that the Hercynian Massif and Western Carpathians were recolonized by *M. sylvaticum* during the Holocene, as it suggests the species might have survived in the area during the full-glacial period. On the other hand, Tollefsrud et al. (2008) have demonstrated not only the survival of *Picea abies*, a characteristic and often dominant tree species in European boreo-montane forests, in the Western Carpathians during the Weichselian Ice Age but also very low genetic diversity in populations of this species in this region. These authors suggest a bottleneck resulting from a substantial decrease in population size during either the LGM or Younger Dryas (Tollefsrud et al. 2008), associated with a decrease in the area covered by vegetation favourable for *M. sylvaticum* and its putative local extinction. Moreover, the present distribution of *M. sylvaticum* is limited by the Uralian mountain range and does not extend substantially into Siberia (Meusel et al. 1978), where the vegetation is nowadays analogous with that reconstructed by Jankovská & Pokorný (2008). It is possible that the distribution of *Melampyrum* is limited by permafrost as it germinates in autumn and has an active overwintering stage with roots. This would account for its present distribution limit and extinction in the Western Carpathians if the reconstructed LGM forest grew on permafrost (Jankovská & Pokorný 2008). The conditions in the Eastern Carpathians were certainly more favourable as at least the southern part of the mountain range was in the permafrost free zone even during the LGM (Taberlet et al. 1998) and permafrost free sites could have occurred at more northerly situated sites (e.g., on southern slopes).

The substantial divergence between *Picea abies* genetic lineages in the northern and southern parts of the Eastern Carpathians (Tollefsrud et al. 2008) suggests a possible explanation for the morphological divergence and differences in the pattern of genetic variation found between the *M. sylvaticum* group populations occurring in these regions. Both species have similar ecological preferences and, therefore, might share the same evolutionary history characterized by the isolation in the past of the populations inhabiting the South- and North-Eastern Carpathians. The little data on *Melampyrum*, however, make this hypothesis very speculative. Nonetheless, it is an interesting idea worthy of further study.

*Taxonomic conclusions*

Our results demonstrate that the current taxonomic concept of the *Melampyrum sylvaticum* group (Jasiewicz 1958, Soó & Webb 1972) needs to be reviewed. The insignifi-

cant relationships between corolla colour and other traits, lack of a pattern in the geographical distributions of populations with different corolla colours and the presence of whitish-flowered specimens in the Western Carpathians decrease the taxonomic value of this character. Therefore, we propose that *M. saxosum* and *M. herbichii* are conspecific as their delimitation is based entirely on corolla colour. Under the terms of the priority rule, the correct name for most Eastern Carpathian plants is *Melampyrum saxosum* Baumg., as this name was published earlier (Baumgarten 1816) than *Melampyrum herbichii* Woł. (Wołoszczak 1887). Nonetheless, this nomenclatorical solution must be regarded as preliminary. The final designation of plant names must be based on type herbarium vouchers, which have not yet been studied.

We suggest that the Central European populations of the *M. sylvaticum* group be classified into two species *M. sylvaticum* s. str. and *M. saxosum* differing in the shape and size of the corolla and anther length. These species have different evolutionary histories and geographic distributions, with the approximate border zone between them on the Eastern-Western Carpathian boundary. Nevertheless, this morphological delimitation between these species applies only to the populations in the northern part of the Carpathian mountain range. The presence of morphologically specific populations in the South-Eastern Carpathians that are genetically closer to the North-Eastern Carpathian samples prevents the generalization of this delimitation between *M. sylvaticum* s. str. and *M. saxosum*, which requires further study (especially the collection of more samples from the Southern Carpathian region).

## Acknowledgements

## Souhrn

Studovali jsme variabilitu *Melampyrum sylvaticum* agg. v Hercynském Masivu a ve Východních a Západních Karpatech pomocí morfometrických a molekulárních metod. Naším cílem bylo především zjistit, jak se ve variabilitě studované skupiny odráží výrazná biogeografická hranice, oddělující Východní a Západní Karpaty a jaké jsou rozdíly ve struktuře variability na opačných stranách této hranice. Zároveň jsme hledali molekulární a další morfologické znaky, které by byly korelované s variabilitou v barvě koruny ve východokarpatských populacích. Ty se na základě barevných rozdílů obvykle oddělují do mikrospecií *M. saxosum* (s bílou barvou koruny) a *M. herbichii* (se žlutou barvou koruny).

Analýzy tvaru koruny, její velikosti a délky prašníku a zejména molekulární analýzy založené na sekvencích chloroplastové (region *trn*L-*trn*T) a jaderné ribozomální DNA (region ITS) potvrdily výrazné odlišnosti typů rostoucích na opačných stranách hranice mezi Východními a Západními Karpaty. Zjištěné rozdíly na molekulární úrovni se ukázaly natolik výrazné, že se velmi pravděpodobně jedná o dvě poměrně značně vzdálené linie v rámci komplexu. Pozorované nesoulady ve struktuře genetické variability mezi molekulárními markery přímo na hranici Východních a Západních Karpat připisujeme hybridizačnímu procesu mezi těmito liniemi a následné introgresi. Nenalezli jsme žádný znak oddělující od sebe různě barevně kvetoucí východokarpatské populace a zároveň jsme objevili bíle kvetoucí jedince i v populaci na lokalitě Babia hora v Západních Karpatech, která však byla molekulárně identická se žlutě kvetoucími rostlinami z okolních pohoří. Obě barvy květů se nevyznačují ani žádným

charakteristickým geografickým rozšířením v rámci severní části Východních Karpat, kde se společně vyskytují nejhojněji. Proto jsme navrhli považovat obě východokarpatské mikrospecie za jediný druh.

Vysoká molekulární variabilita východokarpatských populací svědčí o tom, že mohly přečkat poslední glaciál v refugiích v blízkosti svého současného areálu, což podporují i biogeografické studie druhů preferujících stejný typ vegetace (především subalpínské klečové formace). Možnou existenci glaciálního refugia v prostoru Východních Karpat podporují též paleobotanická data. Populace *M. sylvaticum* s. str. ze Západních Karpat a Hercynského masivu jsou naopak značně molekulárně uniformní, což svědčí o holocenní expanzi tohoto typu z pravděpodobně perialpských refugií.

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