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Variation in the *Melampyrum sylvaticum* group



Master thesis

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Annotation

This study deals with morphological variation in the *Melampyrum sylvaticum* group (Orobanchaceae). Populations of the Hercynian Massif, Western and Eastern Carpathians were investigated using both conventional and geometric morphometric methods. Individual morphological characters were evaluated in detail. Preliminary taxonomic concept of the group is presented. Biogeographical context of detected morphological variation is discussed.

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Introduction

This thesis presents results of my research of variation in the *Melampyrum sylvaticum* group, one of so-called critical taxa displaying complex variation which is difficult to understand. The genus *Melampyrum* (Orobanchaceae) comprises annual hemiparasitic species occurring predominantly in Europe. Complexes of closely related and hardly distinguishable species present quite typical pattern of variability in the genus. Moreover, individual species frequently display complicated infraspecific variation and considerable phenotypic plasticity. Such a complex background of the morphological variation in species and their complexes could not have resulted in anything but serious confusions in species delimitations and taxonomic concepts. Individual researchers have constituted a chaotic system of low rank taxa which are often just extreme deviances on morphological gradients of phenotypic plasticity or low-scale genetic variation.

The state of our knowledge does not naturally allow for understanding the character of variation in the species complexes or individual species. Identification of factors affecting the variation pattern or formulation of biogeographical hypotheses is hardly possible. Extensive studies based on comprehensive sampling across the whole geographical ranges of studied groups or at least their substantial parts can only provide a sufficient insight into variation of individual taxa. Such knowledge is a necessary prerequisite for further formulation of phylogenetic and phylogeographical hypotheses and their consequent testing.

I made effort to follow the rules mentioned above during my work and tried to perform as comprehensive sampling as possible across relatively large geographical regions. The general aim of my study was to capture and describe variation patterns in the *M. sylvaticum* group in the Carpathian and Hercynian regions including the Eastern Carpathians, presumed diversity center of the group.

My research was projected to answer following questions:

1. Which morphological traits reflect large-variation among populations and are thus potentially usable for identification of individual microspecies?
2. Do patterns in the traits selected as taxonomically important correspond to the current taxonomic concept of the group?
3. What is the relationship between the variation patterns and geographical distribution of the populations?

Addressing these questions provides a solid base for constitution of phylogeographical hypotheses about origin of individual types (species) and migratory pathways of the populations in the past.

The first part of my work was focused on variation in *M. sylvaticum* group in the Western Carpathians and Hercynian Massif across anticipated transitional zone between two microspecies. The field sampling of plant material for this part of the study was comprehensive enough to allow for a detailed evaluation of numerous morphological characters. This was probably the most important outcome of the first phase of the investigation.

The following research step is more or less complementary to the first phase. I tried to understand and describe variation in the *M. sylvaticum* group directly in its center – in the Eastern Carpathians. The preceding experience and reference to the results of the Western Carpathians substantially contributed to the success of this part of my study.

Part I.

Morphological variation in the *Melampyrum sylvaticum* group within the transitional zone between *M. sylvaticum* s. str. and *M. herbichii*

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Morphological variation in the *Melampyrum sylvaticum* group within the transitional zone between *M. sylvaticum* s. str. and *M. herbichii*

Morfologická variabilita *Melampyrum sylvaticum* agg. v přechodné zóně mezi *Melampyrum sylvaticum* s. str. a *Melampyrum herbichii*

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The *Melampyrum sylvaticum* group is a complex of three closely related species. This group is most variable in the Carpathian region. Interactions among different levels (within-population to interspecific) of genetic variation and influence of the environment are considered to be the main sources of the complicated morphological variation in this region. Morphological variation in the *M. sylvaticum* group was studied in mountain ranges of the Hercynian Massif and in the Western and Ukrainian Carpathians. Several populations were sampled at different altitudes within each mountain range. Hierarchical partitioning of morphological variation at different levels (within populations, among populations within a mountain range and among mountain ranges) was calculated. Correlations among groups of morphological characters and altitude were calculated. The largest proportion of variation on a large geographic scale (i.e. among mountain ranges) was detected in anther length and several corolla characters (length of the lower corolla lip, height of upper corolla lip), whereas these traits were homogeneous at a local scale (within populations and among populations in one mountain range). An opposite pattern (i.e. high proportion of variation at the low levels, which blurred possible large scale differences) was found in bract traits and several calyx characters. Moreover, a strong correlation between bract length and altitude was observed. The observed changes in the proportions of morphological variation and response to altitude suggest a close connection between bract characters and environmental factors (or lower levels of genetic variation). On the other hand, some of the flower characters seem to be genetically determined and thus might reflect evolutionary processes (early diversification, potential hybridization, introgression) on which the taxonomic treatment of the group should be based. The most distinct differences were detected between samples from the Ukraine and south-western part of Bohemia. Populations from the the Sudeten Mts and the Western Carpathians were variable and morphologically intermediate, forming a continuum between the two extremes.

Key words: *Melampyrum herbichii*, *Melampyrum sylvaticum*, morphometrics, Sudeten Mts, taxonomy, variance components, Western Carpathians

Introduction

The genus *Melampyrum* L. belongs to the family *Orobanchaceae* (Olmstead et al. 2001), which consists of species mostly having a (hemi)parasitic life cycle. Diversification of individual microspecies within relatively distinct species complexes in most of the genera is supposed to have taken place in the late Pleistocene. Quaternary climatic cycles (glacial/interglacial periods) and consequent migration are thought to have played an important role in the diversification and distribution pattern of individual microspecies (Wesselingh & van Groenendael 2005). Their recent origin by various evolutionary pro-

cesses (hybridization, introgression) may complicate the identification and delimitation of particular taxa (Wesselingh & van Groenendael 2005).

Three probably closely related species are distinguished in the *M. sylvaticum* group. These species have usually been delimited from each other on the basis of flower traits, especially length of the anthers, colour and size of the corolla (Baumgarten 1816, Wołoszczak 1888, Jasiewicz 1958). Typical specimens can be classified appropriately using these characters. *Melampyrum sylvaticum* L. is characterized by a yellow and relatively small corolla, (5–) 7–9 (–11) mm long and the shortest anthers, (1.4–) 1.6–2.1 (–2.3) mm long, within the group (Jasiewicz 1958). This species is the most widespread of the three microspecies. Its geographical range corresponds to the range of the whole group, i.e. it covers the European boreal zone and mountains in the European temperate zone from the Pyrenees to the Urals (Meusel et al. 1978).

Melampyrum herbichii Woł. is similar to the previous species, particularly in corolla colour. The length of the anthers and corolla, which reach (2.0–) 2.4–3.6 (–4.2) mm and (7.0–) 9.0–12.5 (–14.0) mm respectively, are supposed to be the main diagnostic characters differentiating this species from *M. sylvaticum* s. str. (Jasiewicz 1958) although significant overlaps are obvious in both characters. This species was first described from three localities in the Ukrainian Carpathians by Wołoszczak (1888). The description of the species is based on samples from three localities including the Hoverla massif in the Chernogora region. Its geographical range was believed to be restricted to the Eastern and Southern Carpathian region (Soó & Webb 1972). However, populations classified as *M. herbichii* are reported from the regions of the Western Carpathians and the Sudeten Mts (e.g. Jasiewicz 1958, Šípošová 1997, Štech 2000) together with specimens featuring diagnostic characters with values transitional between *M. herbichii* and *M. sylvaticum* (Štech 1998, Štech & Drábková 2005). In addition, significant variation in bract shape has been detected in samples from this region (Štech 1998). Bract morphology was investigated in the most recent studies (Štech 1998, Štech & Drábková 2005) suggesting that bract proportions can be used to discriminate between species in the transitional (potential hybrid) zone. Beside the morphological differentiation, a significant difference in habitat preferences between *M. herbichii* and *M. sylvaticum* is described by Šípošová (1997). According to this taxonomical treatment, the habitat preferences of *M. sylvaticum* are supposed to be relatively wide, as it grows in mountain spruce forests up to the dwarf-pine communities at the tree-line. By contrast, *M. herbichii* appears to be restricted to montane meadows around the tree-line (Šípošová 1997). However, Jasiewicz (1958) also reports *M. herbichii* from forests at lower altitudes.

The third species, *M. saxosum* Baumg., is characterized by a white corolla. Quantitative morphological characters seem to overlap completely those of *M. herbichii*; thus the corolla colour is the only diagnostic trait separating these two species (Jasiewicz 1958). *Melampyrum saxosum* occurs in the Eastern (and probably Southern) Carpathians (Soó & Webb 1972), any overlap with the other species reported elsewhere can be considered as a misidentification (Štech 2000).

Although several authors have attempted to elucidate and interpret morphological variation in the *M. sylvaticum* group (the exact delimitation between *M. herbichii* and *M. sylvaticum*, and relationships among transitional populations are usually considered to be the most important questions), their effort have never been completely successful, because a few factors confuse the large-scale morphological variation gradient. A high

phenotypic plasticity is expected to have a great impact on the overall morphology of plants; however its impact is unlikely to be the same for all morphological traits. So-called seasonal variation, a specific type of low-scale genetic variation affecting plant architecture, characteristic of many hemiparasitic *Orobanchaceae* (Wettstein 1895, S6o 1926–1927, Zopfi 1993a, 1993b, 1995, 1997, 1998a, 1998b), is another phenomenon with considerable influence on morphology. Seasonal variation produces polytopic locally adapted ecotypes, which differ primarily in stem internode number (Zopfi 1993b); however, some flower and bract traits might be directly correlated with this character. Phenotypic plasticity together with seasonal variation and their interactions produce local gradients in morphological variation, which can interfere with large geographical gradients in variation resulting in the geographical distribution of individual morphotypes showing a very complex pattern (Štech & Drábková 2005, Těšitel 2005). Thus, neglecting the low-scale variability of phenotypic factors (e.g. building a taxonomical treatment of the group on the basis of sampling a single population within individual mountain ranges) may lead to biased conclusions.

There exist many environmental gradients that may affect the morphological features of specimens of the *M. sylvaticum* group (e.g. light conditions, climate, host plant species and competition with other species). But the major factors are directly connected to altitude and seasonal variation (Štech 1998, Těšitel 2005). The altitudinal gradient is suitable for direct analyses and easy to interpret, which is why this variable was chosen as a reference for gradients in low-scale morphological variation in this study.

The aim of the current study is to elucidate patterns in morphological variability in the *M. sylvaticum* group in the Western Carpathian and Sudeten regions, where transitional morphological types between *M. sylvaticum* and *M. herbichii* prevail (typical specimens of individual species were also included for reference). Detailed analysis of variation in morphological traits is a crucial part of this assessment. It should help to exclude the characters, which were considered discriminatory by previous authors but display significant low-scale variation. Consequent analysis of geographical distribution of morphotypes can be used as a basis for formulating phylogeographic hypotheses and a taxonomic treatment of the group. Population sampling differed slightly and was more complex than in other recent studies (Štech 1998, Štech & Drábková 2005). The objective of this was to obtain a more robust data set suitable for such a rigorous assessment.

Material and methods

Material

Morphometrical data were collected from 24 populations (658 plants) of the *M. sylvaticum* group within the transitional zone between *M. sylvaticum* and *M. herbichii* (Fig. 1 and Appendix 1). The localities were selected to cover the whole transitional zone including typical populations of the microspecies. Several populations within a homogeneous geographically-defined mountain range were sampled in order to evaluate the distribution of variation among these geographical units (further referred to also as regions) and among populations within them. Populations from the Šumava Mts and the Brdy massif were combined into one region named “South and Central Bohemia” due to the low number of localities studied (two and one, respectively) and overall morphological similarity among the

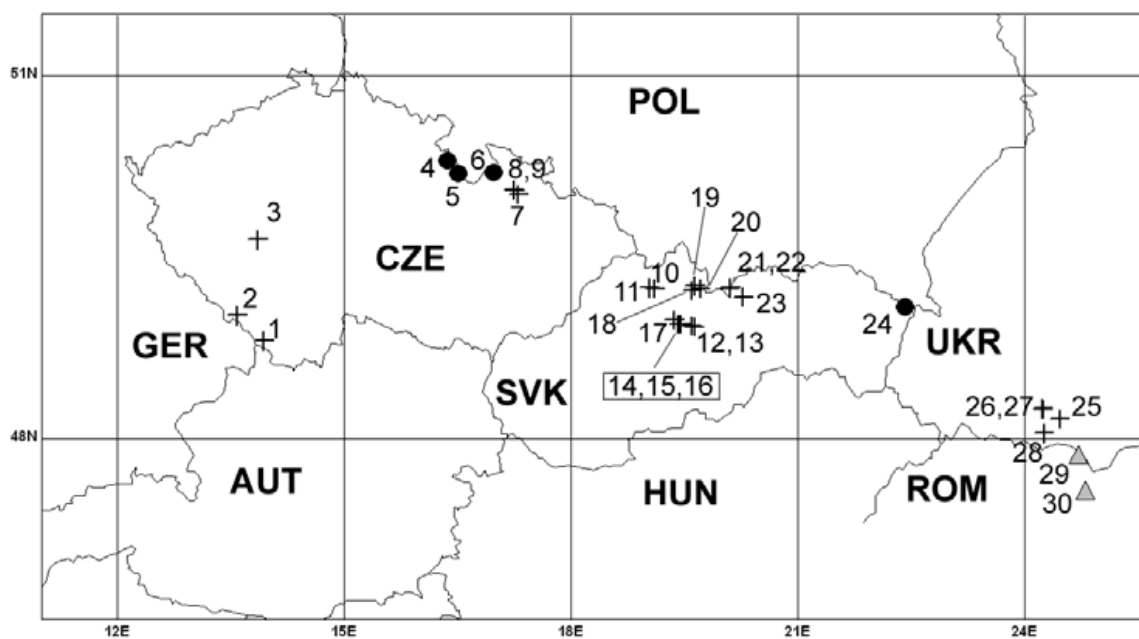


Fig. 1. – Map of the localities of the *Melampyrum sylvaticum* group included in this study. Borders of Central European countries are also displayed. CZE – Czech Republic, SVK – Slovakia, UKR – The Ukraine, ROM – Romania, HUN – Hungary, AUT – Austria, GER – Germany, POL – Poland. + populations included in morphometric analyses, ● additional populations referred to in Discussion ▲ populations of *M. saxosum*.

plants growing in these districts, which were always classified as typical *M. sylvaticum* (Štech 2000). Considering the other end of the geographical gradient, all the population samples collected in the Ukrainian Carpathians are a priori classified as *M. herbichii*, as this species was described from this region (Wołozszczak 1888). Beside the specimens included in the analyses, several additional populations were included in a survey of the geographical distribution of variation. These are the specimens collected in the Orlické hory Mts and the Rychlebské hory Mts, for which data on bract shape are lacking, and populations of *M. saxosum*, which were used as a reference. The sampling was conducted over a short period of time at the start of the flowering season in order to minimize the influence of phenological divergence on the morphology of the flowers (1st to 3rd, exceptionally 4th lowermost flower of all the plants were taken and analyzed). The only exception was the sample of plants from the Bukovské vrchy Mts, which were collected at a later ontogenetical stage and 5th or 6th lowermost flower had to be taken.

A population sample of 21–30 (– 38) plants were collected at every locality (see Appendix 1 for the exact number at each locality). The calyx and corolla of one flower of each plant were put into an Eppendorf-tube filled with ethanol and stored until measured. The first and the fifth bracts were stuck on a sheet of paper using a transparent tape. The other parts of the whole plants were processed as standard herbarium specimens and are kept in the herbarium of the Faculty of Biological Sciences, University of South Bohemia (CBFS).

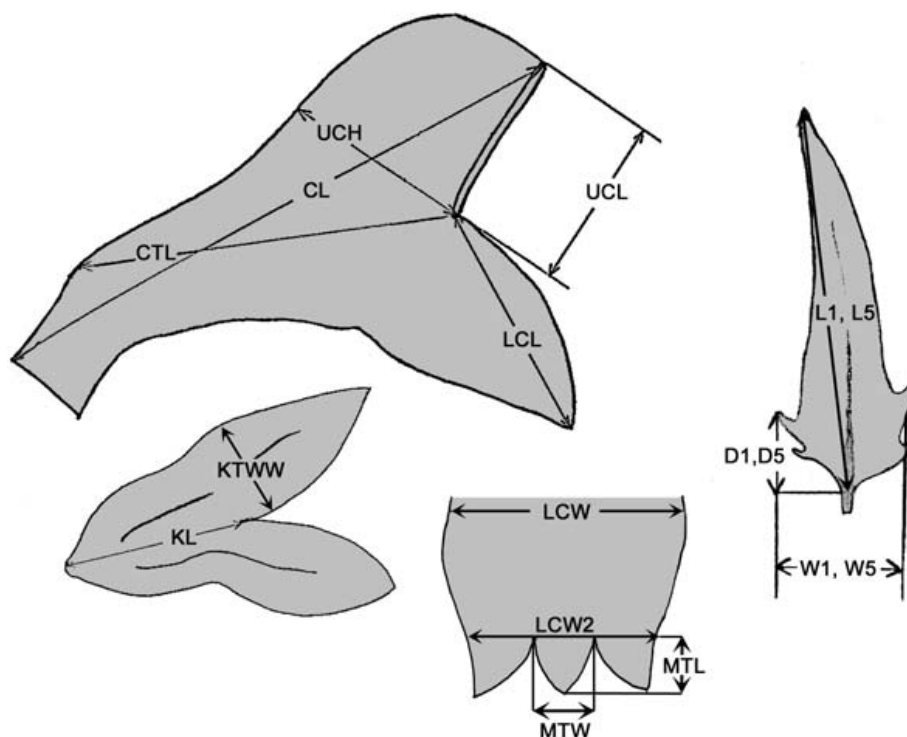


Fig. 2. – Morphological characters of the corolla, calyx, lower lip of corolla and bracts. See the “Morphological characters” paragraph in the methods section for definitions.

Morphological characters

Morphological characters were obtained by a series of measurements conducted on each plant. Twelve traits were measured on flowers (anther length – AL, corolla length – CL, length of the corolla tube – CTL, length of the lower corolla lip – LCL, length of the upper corolla lip – UCL, height of the upper corolla lip – UCH, width of the lower corolla lip – LCW, width of the lower corolla lip at the base of corolla teeth – LCW2, width of the middle tooth of lower corolla lip – MTW, length of the middle tooth of lower corolla lip – MTL, calyx tube length – KL, width of the upper calyx tooth at its widest point – KTW) and three traits (length – L, width – W, distance of the widest part from its base – D) on the 1st and 5th bract (see Fig. 2). Some of the traits were not available in a few cases due to damaged material. This happened rather frequently for the fifth bract which was often not developed, particularly when the plants were sampled at an early ontogenetic stage (i.e. immediately after flowering).

Statistical analyses

Multivariate statistical techniques were employed to investigate the morphological variation in all characters together. An unconstrained ordination method, principal component analysis (PCA, Lepš & Šmilauer 2003), based on the matrix of correlations among the trait values (and thus centered and standardized data), was used to display general patterns in

the variation. The patterns were correlated with the altitude of the sites, the most obvious environmental gradient in the data, using a constrained ordination method, redundancy analysis (RDA, Lepš & Šmilauer 2003). Furthermore, Pearson correlation coefficients were calculated to quantify the relationships detected.

Proportional variation was estimated for single traits at different geographical levels by extracting the variance components from a linear mixed effect model (Quinn & Keough 2002). The regions and populations were random-effect terms in this model. Expected mean squares estimation (EMS) and restricted maximum likelihood estimation (REML; Quinn & Keough 2002, Pinheiro et al. 2005) were employed for processing multivariate and univariate data, respectively.

Multivariate statistical analyses were performed using Canoco for Windows, version 4.52 (terBraak & Šmilauer 2002). Variance components were computed with the R package, version 2.2 (R Core Development Team 2005), package nlme version 3.1-65 (Pinheiro et al. 2005). Statistica for Windows, version 6 (StatSoft 2001) was used for correlation analyses and other basic statistics.

Results

Overall variation in morphological characters

Within-population morphological variation of all characters accounted for 48.6% of the total variation. Regarding particular characters, considerable differences can be identified in their patterns of variation (Fig. 3, Table 1). A highly significant proportion of the variation among populations was detected in all traits (see fourth and fifth column in Table 1). However, the structure of this variation differed noticeably among individual traits. The length of anthers (AL) had the largest proportion of variation connected to the among-region level but the lowest proportion of within-regional variability and within-population variation. Some corolla traits (LCL, UCH, LCW, CL, and CTL) appeared to have a similar pattern of variation, but the proportion of within-population and within-regional variation was substantially higher. In contrast, there was no bract trait with a significant variance component based on the differences among regions. Variation in these characters was concentrated at the within-regional and within-population levels.

Two independent groups of traits can be distinguished in PCA plots based either on the variation among individual plants, or on the means of character values within particular populations (Figs 4 and 5, respectively). These show a rather strong correlation with the first or the second principal axes. The group correlated with the first axis is formed entirely by flower traits whereas the second group consists of bract characters. Populations from different regions appear not to be clearly separable from each other using these morphological traits, but there is only a very small overlap in the morphological features of the populations from the regions at the opposite sides of the gradient of the first PCA axis (Figs 5, 6).

Morphological variation in relation to altitude

Morphological variability induced by differences in environmental conditions was investigated by evaluating the relationships between morphometric trait values and altitude. Results of the RDA (Fig. 7) indicate that variation in most flower traits (except for LCW,

Table 1. – Likelihood-ratio test results for the variance components of individual morphological traits (see Fig. 3). Significant results ($P < 0.05$) are displayed in bold.

Morphological character	Variation among regions		Variation among populations	
	Likelihood-ratio	P	Likelihood-ratio	P
Anther length (AL)	37.13	< 0.0001	796.73	< 0.0001
Length of the lower corolla lip (LCL)	19.43	< 0.0001	583.01	< 0.0001
Height of the upper corolla lip (UCH)	13.96	0.0002	415.90	< 0.0001
Width of the lower corolla lip (LCW)	11.31	0.0008	448.99	< 0.0001
Corolla length (CL)	12.57	0.0004	490.30	< 0.0001
Width of the lower corolla lip at the base of corolla teeth (LCW2)	11.77	0.0006	410.41	< 0.0001
Length of the corolla tube (CTL)	10.44	0.0012	356.58	< 0.0001
Length of the upper corolla lip (UCL)	6.37	0.0116	558.78	< 0.0001
Width of the middle tooth of lower corolla lip (MTW)	5.28	0.0216	210.69	< 0.0001
Calyx tube length (KL)	5.76	0.0164	248.54	< 0.0001
Distance of the widest part of the 5th bract from its base (D5)	1.64	0.2007	250.44	< 0.0001
Length of the middle tooth of lower corolla lip (MTL)	2.37	0.1239	203.96	< 0.0001
Width of the upper calyx tooth at its widest point (KTWW)	0.65	0.4213	340.91	< 0.0001
Length of the 1st bract (L1)	0.45	0.5040	351.84	< 0.0001
Distance of the widest part of the 1st bract from its base (D1)	0.13	0.7139	294.17	< 0.0001
Length of the 5th bract (L5)	0.00	0.9440	322.26	< 0.0001
Width of the 5th bract (W5)	0.00	0.9995	283.73	< 0.0001
Width of the 1st bract (W1)	0.00	0.9995	300.59	< 0.0001

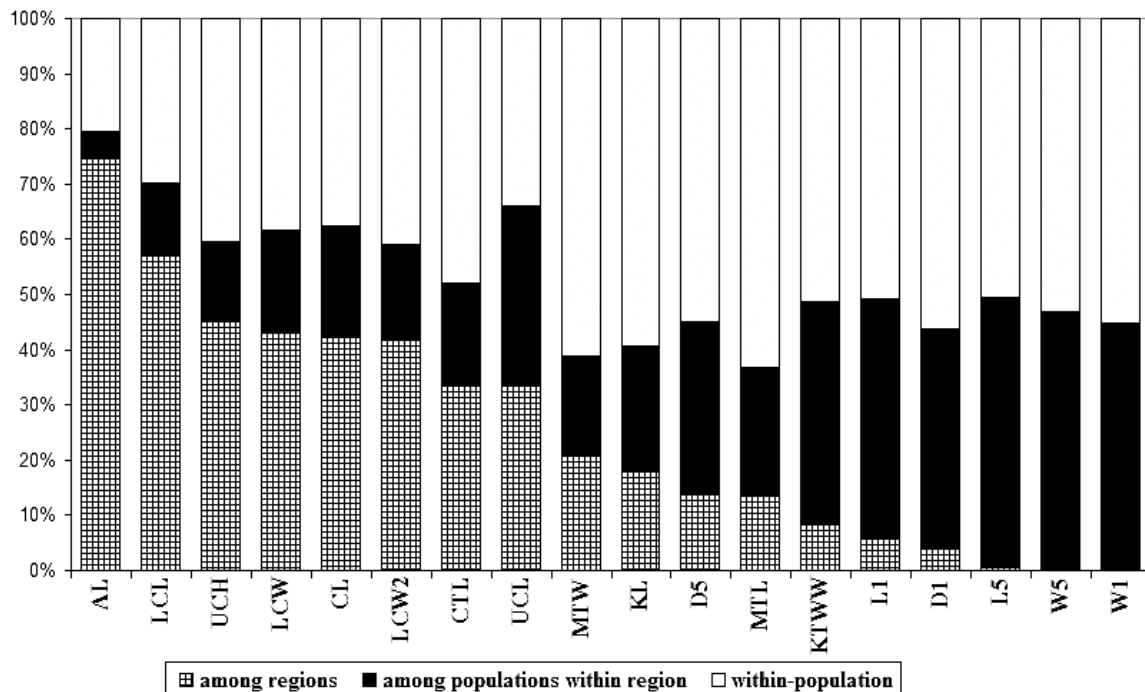


Fig. 3. – Variance components of individual characters corresponding to the hierarchical geographical levels. Variance component estimates are based on a random effect extraction from a linear mixed effect model using restricted maximum likelihood estimation (REML). See Table 1 for significance tests of the variance components. See Fig. 2 and the “Morphological characters” paragraph in the methods section for definitions of the traits.

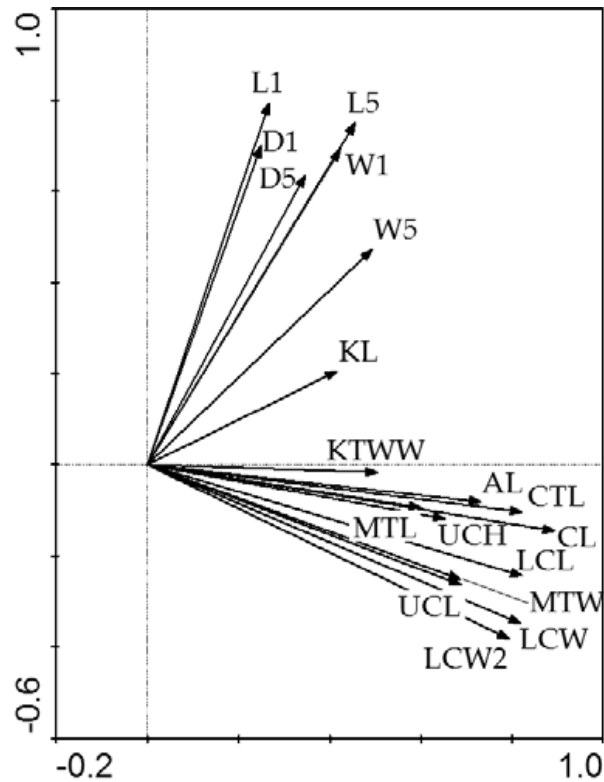


Fig. 4. – PCA plot based on individual plants. Directions of changes in morphological characters are displayed in relation to the first two principal component axes. The first and second ordination axes explain 39% and 18.6% of the variation, respectively.

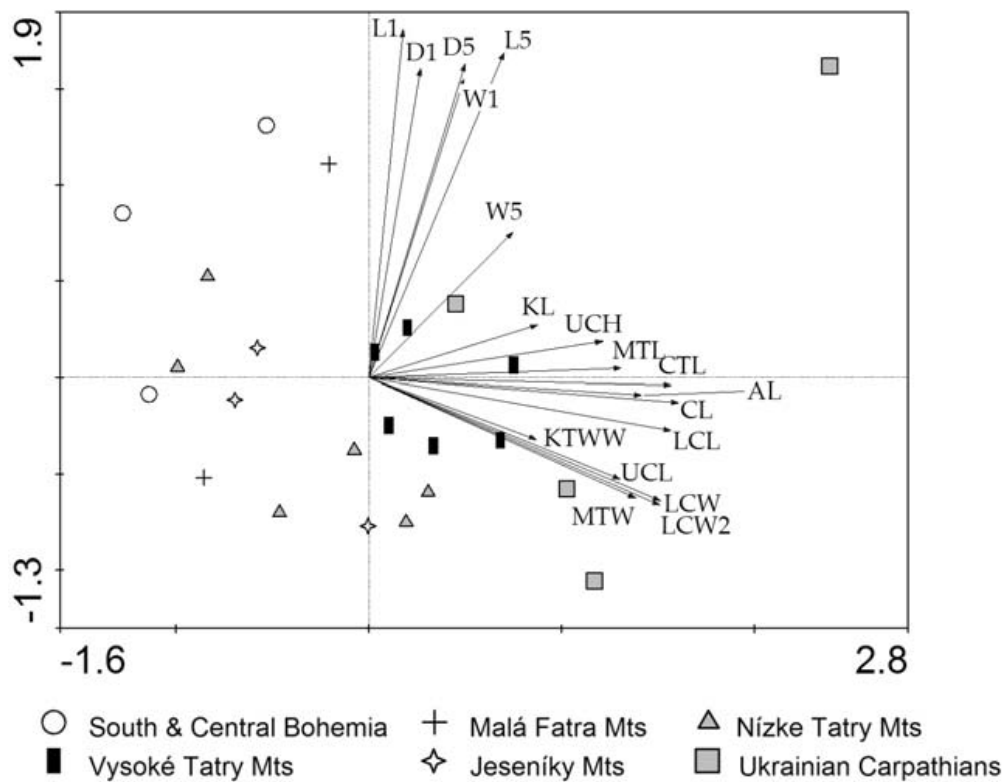


Fig. 5. – PCA plot based on means of character values within populations. Directions of changes in the morphological characters are demonstrated. Regional geographical distribution of the populations is depicted using different symbols for the ordination scores of individual populations. First two ordination axes are displayed. First axis explains 46.7% and the second 21.0% of the total variation.

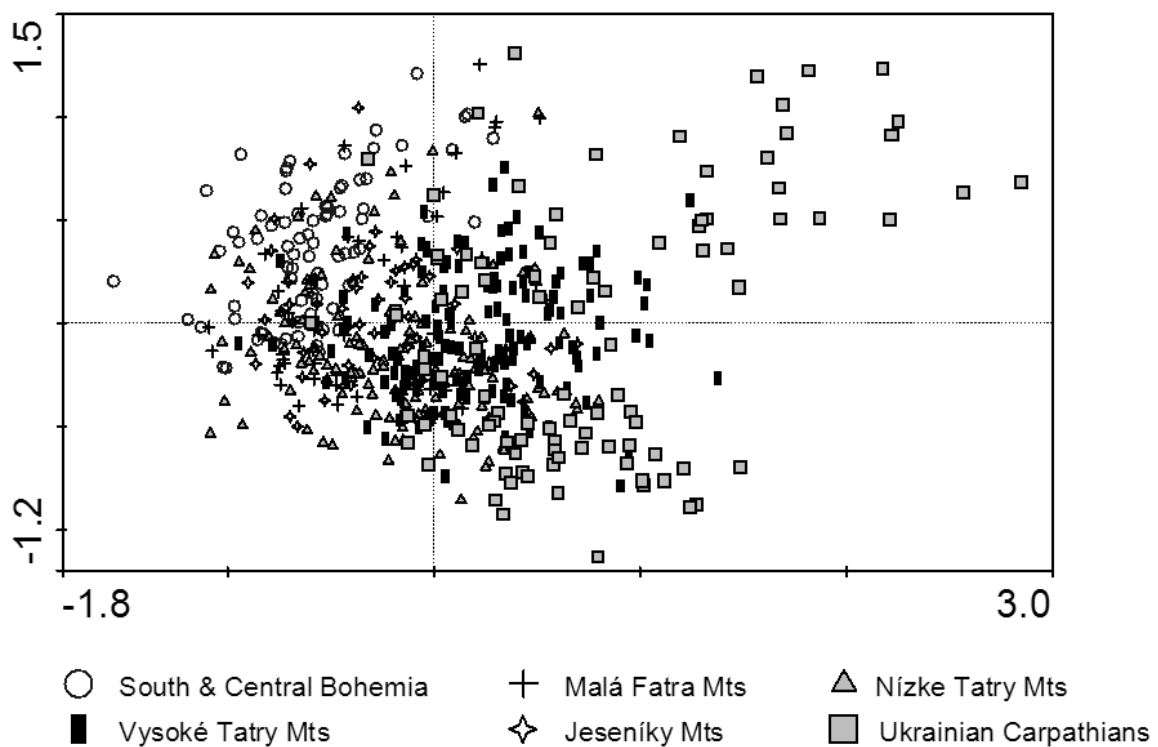


Fig. 6. – PCA plot of individual plants. Ordination scores of the plants are displayed. Regional geographical distribution of the specimens is displayed using different symbols for the ordination scores of individual plants. The first and second ordination axes explain 39% and 18.6% of the variation, respectively.

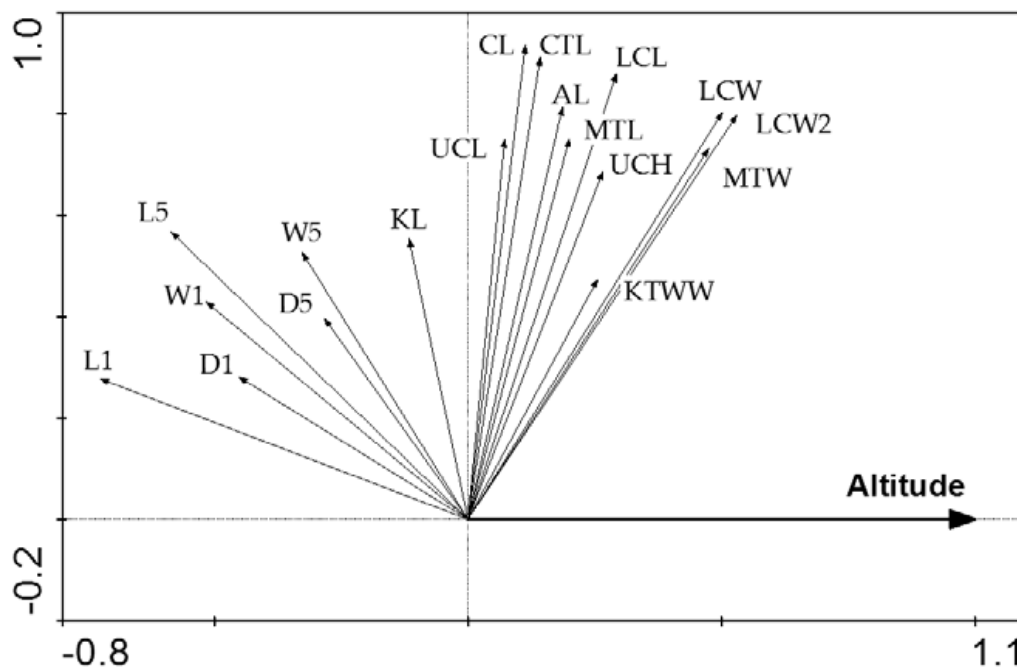


Fig. 7. – RDA plot describing directions of changes in morphological characters in relation to altitude. RDA is based on mean trait values for individual populations thus only among-population variation is considered. The constrained (horizontal) axis explains 14.6% of the variability. Monte-Carlo permutation test of significance of the canonical axis: $F = 3.76$, $P = 0.012$ (with 999 permutations).

LCW2 and MTW, for which there is a weak correlation) are independent of altitude, whereas bract traits (especially L1 and L5) seem to be correlated with this environmental variable. A more detailed survey of these relationships is provided by a correlation analysis (Table 2) confirming the pattern visible on the RDA plot (Fig. 7). A significant correlation was detected for most of the bract traits (except for W5 and D5), whereas LCW, LCW2 and MTW were the only flower characters for which there were significant correlations.

Table 2. – Pearson correlation coefficients (r) and significance of the correlation (p) between the means of morphological trait values within the populations of the *Melampyrum sylvaticum* group and altitude. Significant ($P < 0.05$) relationships are displayed in bold.

Morphological character	Correlation with altitude	
	r	P
Corolla length (CL)	0.11	0.5975
Length of the corolla tube (CTL)	0.14	0.5066
Length of the upper corolla lip (UCL)	0.07	0.7378
Height of the upper corolla lip (UCH)	0.27	0.2081
Length of the lower corolla lip (LCL)	0.29	0.1647
Width of the lower corolla lip (LCW)	0.50	0.0125
Width of the lower corolla lip at the base of corolla teeth (LCW2)	0.53	0.0077
Width of the middle tooth of lower corolla lip (MTW)	0.47	0.0191
Length of the middle tooth of lower corolla lip (MTL)	0.20	0.3480
Anther length (AL)	0.19	0.3821
Calyx tube length (KL)	–0.12	0.5887
Width of the upper calyx tooth at its widest point (KTWW)	0.26	0.2274
Width of the 1st bract (W1)	–0.52	0.0098
Distance of the widest part of the 1st bract from its base (D1)	–0.45	0.0265
Length of the 1st bract (L1)	–0.73	0.0001
Width of the 5th bract (W5)	–0.33	0.1182
Distance of the widest part of the 5th bract from its base (D5)	–0.28	0.1795
Length of the 5th bract (L5)	–0.59	0.0026

Discussion

Evaluation of morphological characters

The distribution of variation in particular morphological characters (Fig. 3, Table 1) provides an important baseline for further considerations. Assuming that a great genetic divergence manifests itself at the higher geographical levels, the traits that vary most at the among-mountain-range level are the only potentially appropriate diagnostic characters for delimiting *M. sylvaticum* and *M. herbichii*. Variation in the length of anthers (AL) and some of the corolla traits (LCL, UCH, LCW, CL and LCW2) appear to show such a pattern, whereas an opposite constellation was detected in most bract traits. The PCA plots (Figs 4, 5, 6) imply that the first principal axes on these diagrams correspond to higher-scale variation, whereas the second axes can be interpreted as lower-scale morphological variation gradients (resulting either from low-scale genetic variability or phenotypic plasticity).

In accordance with the analyses of overall variation, the correlation analyses comparing values of the morphological traits with altitude (Table 2, Fig. 7) suggest that the proportions of bracts (especially the length) and the width proportions of the lower corolla lip (LCW, LCW2, MTW) are mostly related to either phenotype plasticity or small-scale genetic variation. In contrast, the stability of anther length (AL) and lower corolla lip length (LCL) in relation to altitude confirms the results of previous analyses and suggests that these traits reflect high-scale genetic divergence more closely than any of the other characters.

This pattern of variation supports the delimitation of *M. sylvaticum* and *M. herbichii* on the basis of anther length, as found in previous studies (Jasiewicz 1958, Soó & Webb 1972). However, corolla length, another trait frequently used for determination (e.g. Jasiewicz 1958, Šípošová 1997), is obviously less robust in differentiating among populations from different regions than some other corolla characters. This might be caused by differences in the curvature of the corolla base (see Fig. 2) which adds some error variance in its values. Using another trait, the length of the lower corolla lip (LCL), instead of simple corolla length for classification seems to be more appropriate as this character appears to reflect geographical distribution pattern more precisely than any other corolla trait.

These conclusions do not agree with those of Štech (1998) and Štech & Drábková (2005). In these studies, within-population variation seemed to blur substantially the differences between individual species (even considering the traits considered diagnostic such as AL or CL), which led to a search for other traits, which can be used to delimit *M. sylvaticum* and *M. herbichii*. This disagreement can be explained by a difference in the sampling technique. As stated in the methods section, plant specimens for the current study were collected over a short period of time at the beginning of the reproductive season. Thus, the 1st – 3rd (exceptionally 4th) lowermost flower from the main inflorescence was collected for measurement. In the previous studies (Štech 1998, Štech & Drábková 2005), the sampling continued over the whole flowering season. Flowers for measurement were chosen randomly within the plant (i. e. flowers from higher nodes on the main inflorescence and from branch inflorescences were also processed). Hence, this flower trait values may have included additional variance associated with differences in the morphological features of flowers from different positions on a plant. This explanation is furthermore supported by the genetic variation detected by RAPDs (Štech & Drábková 2005). The proportion of within-population variation detected in the whole set of morphological characters in the current study coincides closely with that obtained by a RAPD marker analysis (48.6% versus 47.6% for RAPD data) but is considerably larger (68.5%) for the set of morphological characters analyzed by Štech & Drábková (2005).

Variation in the proportions of bracts needs detailed evaluation. The length of bracts (L1 and L5) does not seem to differ between the two species. However, this trait was found to have a great discriminatory power between the two species in the most recent study dealing with variation in the *M. sylvaticum* group (Štech & Drábková 2005). Such a discrepancy might be caused by slight differences in the environmental conditions experienced by the populations analyzed by Štech & Drábková (2005) and those analyzed in the current study. The populations chosen as training data-sets for classification by discriminant analysis by Štech & Drábková (2005) differed not only in their geographical distribution but also in the environmental conditions at the sites from which the samples were collected. The samples of *M. herbichii* from the Ukraine and the Bukovské vrchy

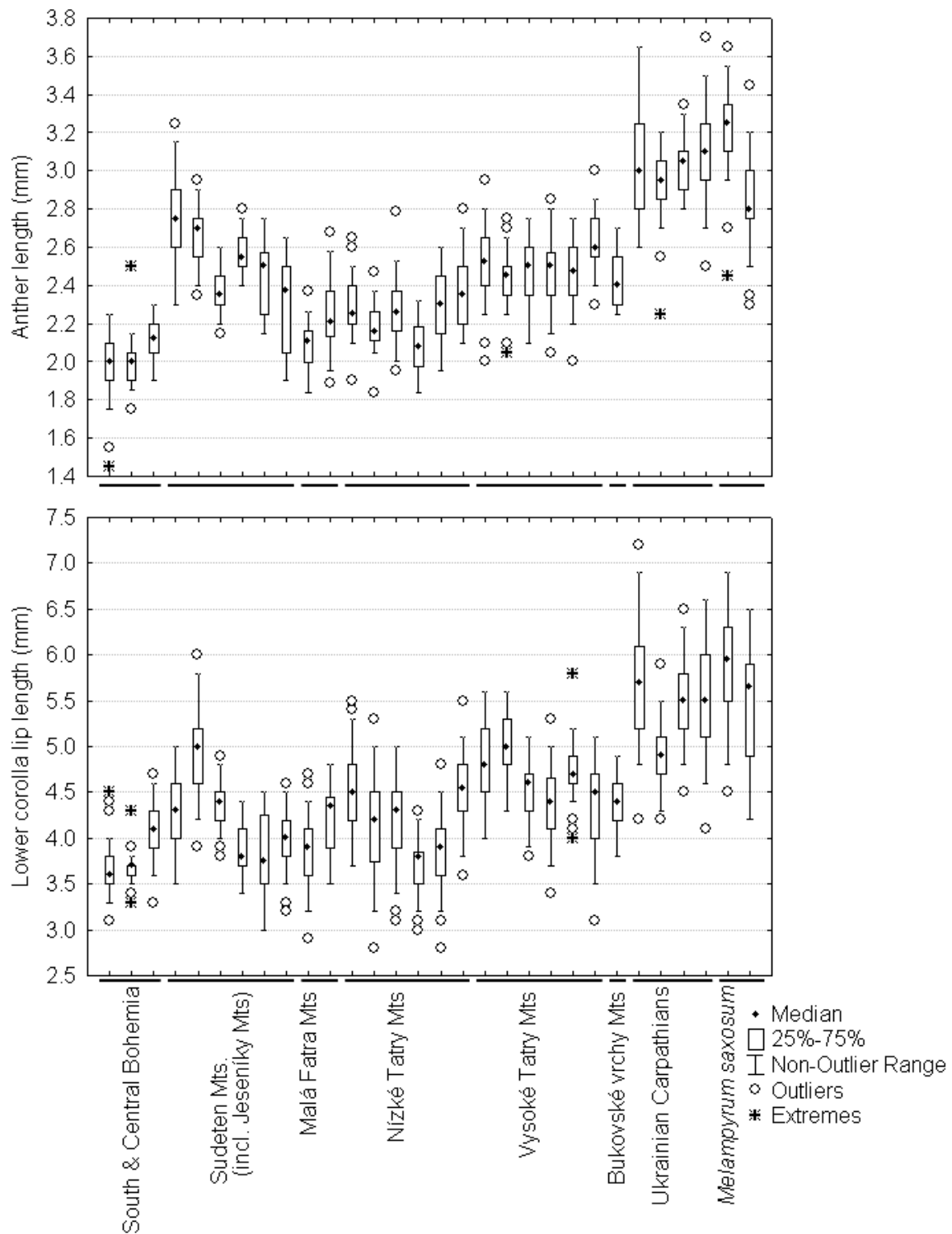


Fig. 8. – Box-and whisker plots displaying the values of anther length (AL) and lower corolla lip length (LCL) at individual localities in different regions. The populations are displayed along the horizontal axes in the order (from left to right) of the descriptions of localities in Appendix 1.

Mts included in that study were collected from alpine meadows at altitudes, mainly between 1150 and 1580 m a.s.l., whereas specimens of *M. sylvaticum* from the western group of localities (the Alps and Bohemian Forest) originated from both meadows and forests at altitudes between 500 and 1100 m a.s.l. (see Appendix in Štech & Drábková 2005). Two populations from the Alps, at 1600 and 1900 m a.s.l., were the only exceptions, but these included a very small number of plants (4 and 6, respectively) and had little influence on the coefficients of the discriminatory function. This difference in environmental conditions between the training data-sets must have biased the discriminatory function so that their consequent classification was not only based on the morphological divergence between the typical samples of the two species but also on the basis of morphological differences induced by the environment (altitude and habitat type). The a priori classification of plants from the Bukovské vrchy Mts as *M. herbichii* poses another problem because there is no evidence that these populations are closely genetically related to the Ukrainian populations of *M. herbichii*, they even seem to be morphologically more proximate to plants from the Vysoké Tatry Mts than to plants from the Ukraine (Fig. 8). It is obvious that other bract characters (W1, W5, D1, D5) are also strongly affected by various environmental conditions (e.g. the bract width is influenced by the presence of bract teeth, which was observed to be more frequent at sites exposed to direct sunlight; Štech 2000); however, it is still likely that they also reflect some large-scale genetic differences. Specimens of *M. sylvaticum* tend to have narrower bracts and the distance of the widest point of the bract from its base is usually greater than in *M. herbichii*, moreover significant differences are expected regarding frequency and shape of bract teeth (Štech 1998, 2000).

Obviously, conclusions on the pattern of variation in particular traits cannot be made based only on the data presented in this study. In particular, it is not possible to decide whether the low-scale variation detected is due to phenotypic plasticity or low-level genetic divergence (local adaptations), because these factors are correlated with each other. However, future transplantation experiments are expected to differentiate between phenotypic plasticity and genetically determined morphological variation and thus provide useful information.

Geographical distribution of variation

Regarding variation in the morphological characters (Figs 4, 5, 6), it is clear, that differences among populations form a continuum within which no obvious split can be identified. The Ukrainian populations appear the most diverse compared to the other regions. The population from Rakhiv (no. 28) is the most distinct not only from the other Ukrainian populations but from the complete set of populations studied. This could suggest some kind of differentiation from the other populations of the same region; however the morphological differences are based mainly on bract traits and some flower characters which are strongly intercorrelated, which increases the weight of a single independent measure. This biased the PCAs and made the differentiation more apparent than it is. Considering the traits reflecting large-scale variation (AL, LCL), the differentiation between the Rakhiv population and other Ukrainian populations is negligible (Fig. 8).

The largest differences in anther and lower corolla lip length can be found between populations from the Ukraine and the south-western part of Bohemia (Fig. 8). The Ukrainian plants, described as *M. herbichii*, had significantly higher values for both of these characters.

The overlap between the two morphological extremes was negligible. The other populations display intermediate values and form a continuum between them. Differentiation among these regions was substantially smaller, although some slight differences can be detected and interpreted. Plants from localities in the Vysoké Tatry Mts, Bukovské vrchy Mts, Orlické hory Mts, Rychlebské hory Mts and Jeseníky Mts appear to have longer anthers and lower corolla lips. In some of these populations, values of these traits reach those of Ukrainian plants. Thus, populations in these regions are slightly less distinct from *M. herbichii* than other populations. This supports the conclusions of previous studies (Jasiewicz 1958, Šípošová 1997, Štech 1998, 2000, Štech & Drábková 2005), which report plants similar to *M. herbichii* in the Western Carpathians and the Sudeten Mts (Jasiewicz 1958, Šípošová 1997). Lower values for both anther length and lower corolla lip length were detected in the population from the Bukovské hory Mts than reported by Štech & Drábková 2005. This can be explained by the sampling of plants at later ontogenetic stages (see methods section) and consequent analyzing of flowers from higher nodes (which are sometimes reported to differ slightly from the basal ones; Těšitel 2005).

The background of this pattern in morphological variation remains to be resolved. There are two hypotheses. The morphologically transitional populations may have arisen through hybridization between different groups of plants migrating from their glacial refuges in the early Holocene. The alternative explanation is based on the evolution of different morphotypes within isolated mountain ranges after the immigration of a common variable ancestor. Application of molecular tools is the only way to decide which of these hypotheses is correct because only a phylogeographic, genetically-based analysis can reconstruct the migratory pathways of particular groups of populations in the Holocene and explain their origin.

The stability of diagnostic traits within different mountain ranges and the lack of a correlation between their values and altitude suggest that there are hardly any differences in habitat preferences between *M. sylvaticum* and *M. herbichii*. Both species were found to grow in both montane forests and meadows at the tree-line, even though typical samples of *M. herbichii* growing in the Transcarpathian Region in the Ukraine seem to be rare at low altitudes. Considering transitional morphotypes from the Western Carpathians and Sudeten Mts, no ecological differentiation can be found between populations proximate to *M. herbichii* and populations similar to *M. sylvaticum*. Thus, the ecological differences between the two species reported in the past (Šípošová 1997) should be reconsidered. In contrast, the pattern of morphological variation and its connection to their ecology described by Jasiewicz (1958) appears to correspond well with the results of our study.

It is not possible to evaluate the morphological variation in *M. saxosum* on the basis of two population samples. The overlap in quantitative morphological characters with *M. herbichii* reported by Jasiewicz (1958) was recorded in these specimens. Difference in anther length between the two populations may suggest the possibility of a significant pattern of variation in *M. saxosum*.

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Souhrn

Melampyrum sylvaticum agg. patří mezi velmi variabilní a taxonomicky problematické skupiny flóry Střední Evropy. Komplikovaná morfologická variabilita je způsobena interakcemi mezi různými úrovněmi genetické variability a vlivy prostředí. Cílem této studie bylo oddělit jednotlivé úrovně variability a nalézt tak morfologické znaky, které vykazují malou variabilitu v lokálních měřítkách (na úrovni vnitropopulační či mezi populacemi v rámci jednoho pohoří), minimální závislost na podmínkách prostředí v různých biotopech a zároveň dostatečnou variabilitu na vyšší geografické škále. Geografická škála tak byla použita jako reference vyjadřující přibližně i genetickou rozdílnost mezi vzorky. Za účelem zjištění, jaké rozdíly v morfologii odpovídají jednotlivým geografickým úrovním (mezi homogenními, geograficky definovanými pohořími, mezi populacemi v rámci pohoří, uvnitř populací), byl proveden hierarchický rozklad variability jednotlivých znaků. Závislost morfologie rostlin na vlastnostech biotopu v němž rostou byla testována pomocí korelací mezi hodnotami znaků a gradientem nadmořské výšky, s nímž jsou těsně korelovány i další potenciální rozdíly mezi podmínkami prostředí v jednotlivých biotopech.

Délka prašníku a spodního korunního pysku se ukázaly jako znaky nejlépe odrážející variabilitu ve velkých měřítkách, což částečně odpovídá výsledkům některých starších studií (jako diagnostický znak se však tradičně používá celková délka koruny, její hodnota však vykazovala poměrně velkou vnitropopulační variabilitu). Hodnoty těchto znaků byly navíc dostatečně odlišné v různých regionech, což umožnilo provést zhodnocení geografického rozložení variability v populacích a vytvořit základ pro taxonomické hodnocení skupiny. Naopak se ukázalo, že převážná část variability ve tvaru listenů je zapříčiněna buď fenotypovou plasticitou anebo velmi lokálními genetickými rozdíly. Zároveň byla zjištěna i silná korelace znaků na listenech s gradientem nadmořské výšky.

Značné rozdíly v délkách prašníků a spodních korunních pysků byly zjištěny mezi populacemi *M. herbichii* z Ukrajiny a *M. sylvaticum* s. str. ze Šumavy a Brd. Populace ze Západních Karpat a Sudet dosahují v těchto znacích intermediálních hodnot, které v konkrétních případech poněkud blíží buď ukrajinským populacím (rostliny z Vysokých Tater a Sudet) anebo šumavským a brdským populacím (rostliny z Malé Fatry a Nízkých Tater). Mezi těmito skupinami však neexistuje žádná ostrá hranice, která by přerušila kontinuum v morfologické variabilitě. Otázka druhové klasifikace těchto rostlin tak zůstává otevřená, přestože v některých starších studiích jsou tyto rostliny přiřazovány k druhu *M. herbichii*.

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Appendix 1. – List of localities of the *Melampyrum sylvaticum* group studied. Those marked with asterisk are only discussed, not included in the analysis.

Czech Republic. Southern & Central Bohemia (Šumava Mts and Brdy Massif): 1 – Šumava Mts, Ovesná: spruce forest next to the railway station; 48°48'26"N, 13°56'21"E, 740 m a.s.l.; 10. 7. 2004; 30 specimens analyzed. 2 – Šumava Mts, 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 m a.s.l.; 11. 7. 2004; 29 specimens analyzed. 3 – Brdy Massif, Zalány: spruce forest at the N border of the village; 49°38'35"N, 13°51'25"E, 645 m a.s.l.; 12. 7. 2005; 26 specimens analyzed. **Orlické hory Mts** (Sudeten Mts): *4 – Deštné v Orlických horách: forest border between ski slopes ca 0.5 km S of the village; 50°17'49"N, 16°21'59"E, 730 m a.s.l.; 7. 6. 2003; 37 specimens analyzed. *5 – Rokytnice v Orlických horách: forest border ca 100 m N of the Hanička settlement ca 5 km NE of the town; 50°11'22"N, 16°30'38"E, 750 m a.s.l.; 7. 6. 2003; 38 specimens analyzed. **Rychlebské hory Mts** (Sudeten Mts): *6 – Velké Vrbno: forest edge ca 0.5 km W of the village; 50°11'56"N, 16°59'09"E, 840 m a.s.l.; 6. 6. 2003; 37 specimens analyzed. **Jeseníky Mts** (Sudeten Mts): 7 – Karlov: small forest on the left bank of the Moravice river in S part of the village; 50°01'12"N, 17°18'12"E, 670 m a.s.l.; 7. 7. 2004; 21 specimens analyzed. 8 – 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 m a.s.l.; 7. 7. 2004; 28 specimens analyzed. 9 – Karlov, Mt Vysoká hole: montane meadows on the E slope of the mountain; 50°03'31"N, 17°14'30"E, 1290 m a.s.l.; 7. 7. 2004; 30 specimens analyzed.

Slovakia. Malá Fatra Mts: 10 – Mt Velký Rozsutec: spruce forest on the N slope of the mountain; 49°14'20"N, 19°06'20"E, 1385 m a.s.l.; 22. 6. 2004; 29 specimens analyzed. 11 – Terchová: NW slope of the

Sokolie massif; 49°14'42"N, 19°02'11"E, 695 m a.s.l.; 23. 6. 2004; 28 specimens analyzed. **Nízke Tatry Mts:** 12 – Trangoška: margin of pathway in spruce forest between Trangoška settlement and Štefánikova chata chalet; 48°55'34"N, 19°37'58"E, 1375 m a.s.l.; 25. 6. 2004; 30 specimens analyzed. 13 – Trangoška: montane meadows on the S slope of Mt Chopok, ca 100 m N of the Kosodrevina Hotel; 48°55'57"N, 19°35'28"E, 1525 m a.s.l.; 26. 6. 2004; 28 specimens analyzed. 14 – Magurka: montane meadows near the summit of Mt Mestská hora E of the village; 48°56'51"N, 19°27'06"E; 1505 m a.s.l.; 28. 6. 2004; 29 specimens analyzed. 15 – Magurka: clearing in spruce forest on the W slope of Mt Mestská hora on E of the village; 48°56'46"N, 19°26'23"E, 1175 m a.s.l.; 28. 6. 2004; 24 specimens analyzed. 16 – Magurka: margins of a road next to Kapustisko settlement 1 km E of the village; 48°56'50"N, 19°25'09"E, 960 m a.s.l.; 29. 6. 2004; 25 specimens analyzed. 17 – Lužná: montane shrubs on the summit of Mt Salatín above the village; 48°58'53"N, 19°21'47"E, 1615 m a.s.l.; 30. 6. 2004; 30 specimens analyzed. **Vysoké Tatry Mts:** 18 – Huty: montane forest near a starting point of a pathway leading to Mt Biela skala, ca 2 km E of the village; 49°13'24"N, 19°35'59"E, 930 m a.s.l.; 1. 7. 2004; 30 specimens analyzed. 19 – Zuberec: montane forest near Zverovka chalet ca 4,5 km E of the village; 49°14'33"N, 19°42'36"E, 985 m a.s.l.; 3. 7. 2004; 25 specimens analyzed. 20 – Zuberec: montane forest around road leading to Zverovka chalet, ca 1 km E of the village; 49°15'37"N, 19°38'10"E, 820 m a.s.l.; 3. 7. 2004; 29 specimens analyzed. 21 – Lysá Pořana: montane forest at a tourist shelter ca 3 km S of the village; 49°14'53"N, 20°06'07"E, 990 m a.s.l.; 4. 7. 2004; 25 specimens analyzed. 22 – Lysá Pořana: meadow at the road leading from the village to a gamekeeper's lodge, ca 3.5 km S of the village; 49°14'27"N, 20°06'05"E, 1005 a.s.l.; 6. 7. 2004; 29 specimens analyzed. 23 – Tatranská Lomnica: montane spruce forest on the N border of the town; 49°10'11"N, 20°16'31"E, 910 m a.s.l.; 6. 7. 2004; 26 specimens analyzed. **Bukovské Vrchy Mts:** *24 – Runina: alpine pastures at Sedlo pod Ďurkovcom Saddle, 3.2 km NNE of the village; 49°05'08"N, 22°25'24"E, 1128 m a.s.l.; 8. 7. 2005; 29 specimens analyzed.

U k r a i n e . **Ukrainian Carpathian Mts:** 25 – Chernogora Mts, Lazeshchina: alpine pastures between Mt Hoverla and Mt Pietrosh ca 2.75 km W of the Hoverla summit, ca 12 km S of the village; 48°09'37"N, 24°27'50"E, 1570 m a.s.l.; 11. 7. 2003; 21 specimens analyzed. 26 – Svydovets Mts, Yasinya: alpine pastures at NE slopes of the Mt Blyznitsa ca 1.75 km N of the Blyznitsa summit, 48°14'25"N, 24°14'24"E, 1410 m a.s.l.; 12. 7. 2005; 30 specimens analyzed. 27 – Svydovets Mts, Yasinya: forest edge by ski slopes at tourist base ca 8 km W of the town; 48°14'50"N, 24°14'11"E, 1375 m a.s.l.; 12. 7. 2003; 29 specimens analyzed. 28 – Rakhiv: montane forest on slope ca 2 km ESE of the town; 48°02'36"N, 24°15'13"E; 950 m a.s.l.; 30. 6. 2005; 27 specimens analyzed.

Localities of *Melampyrum saxosum*:

*29 – Ukrainian Carpathians, Chivchin Mts, Burkut: alpine meadows at 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 m a.s.l.; 9. 7. 2003; 26 specimens analyzed. *30 – Romania, Munții Rodnei Mts: Stațiunea Borșa: N slope below mountain edge ca 6 km S of the village; 47°34'15"N, 24°48'00"E, 1848 m a.s.l.; 12. 8. 2004; 26 specimens analyzed.

Part II.

Morphological variation in the *Melampyrum sylvaticum* group in the Eastern Carpathians

a manuscript by Jakub Těšitel, based on cooperation with Tamara Malinová and Milan Štech

Introduction

The hemiparasitic genus *Melampyrum* (*Orobanchaceae*) displaying the highest diversity in temperate Europe and the Balkan Peninsula is an important part of the European flora. Having originated probably in the Early Tertiary (Wolfe et al. 2005), *Melampyrum* formed a number of species of which only a few migrated outside Europe (or near surrounding) and after subsequent speciation constituted taxa that do not occur in Europe (e.g. *Melampyrum roseum* and a few related species in East Asia, *M. lineare* in Eastern North America; Soó 1926-1927, Štech 1998). *Melampyrum* managed to overcome Quaternary climatic cycles and maintained high species diversity, unlike the major part of the European Tertiary flora (Ložek 1973, Lang 1994). The diversity of the genus might have even increased as a result of isolated evolution of particular populations in glacial refuges.

The speciation process is still active in the representatives of *Melampyrum*, which has resulted in the existence of several complexes comprising closely related microspecies that are hardly distinguishable from each other. The *Melampyrum nemorosum* and *M. sylvaticum* groups are good examples of this variation pattern. It is supposed that the origin and distribution pattern of individual microspecies were predominantly affected by migration of populations, their isolation and subsequent reconstitution of contact among them in the late Pleistocene and the Holocene (Wesselingh & van Groenendael 2005). An interplay among the evolutionary mechanisms has created a complex variation pattern in affected taxa, which is difficult to understand and interpret.

The *Melampyrum sylvaticum* group is a widespread element of the European subalpine and alpine flora. Its geographical range covers mountain ranges from Western Europe (the Pyrenees, Scottish Highlands; Dalrymple 2007) to the Urals and it descends to lowland in the boreal zone (Meusel et al. 1978). The taxon is relatively common in the major part of its range forming large populations. Despite its relative abundance, it can be sometimes considered rare or even endangered in countries near its geographical limit, e.g. in Britain (Dalrymple 2007).

Three taxa have usually been distinguished at the species level in the *M. sylvaticum* group on the basis of anther length, corolla size and color. *M. sylvaticum* s. str. defined by short anthers and a small golden-yellow corolla (see Soó & Webb 1972, 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). Certain levels of variability in *M. sylvaticum* s. str. were reported from the Alps (e.g. Soó 1927-1827), however it was always considered infraspecific and no higher-rank taxa (species) were ever described on its basis. Long anthers and long (big) corolla characterize *M. hebichii* and *M. saxosum* which differ only in corolla color being golden-yellow and white respectively. Beside being similar in terms of morphology, the latter two species display also a single geographical distribution center in the Eastern Carpathians (Soó 1926-1927, Jasiewicz 1958, Paucă & Nyárady 1960, Soó & Webb 1972) but both species have been often reported also from the Southern Carpathians (Paucă & Nyárady 1960, Soó & Webb 1972). A transitional zone exists between *M. sylvaticum* s. str. and *M. herbichii* in the Western Carpathians and the eastern part of the Hercynian Massif, which we have documented in our earlier studies (Štech & Drábková 2005, Těšitel & Štech 2007).

The amount of data on distribution and variation in the *M. sylvaticum* group in the Eastern Carpathians appears sufficient at the first glance as three detailed studies exist covering the topic (Soó 1926-1927, Paucă & Nyárady 1960, Jasiewicz 1958). Nevertheless the section describing *M. sylvaticum* group by Paucă & Nyárady (1960) in the Flora of Romania is more or less based on the monograph of the genus *Melampyrum* by Soó (1926-1927) and both these works contain a chaotic system of infraspecific taxa and the information is often confusing (e. g. occasional reports of two species from one locality). The monograph by Jasiewicz (1958) provides a more reliable information source which is however restricted to the northern part of the Eastern Carpathians. Major or at least considerable part of the plant material used in all previous studies originated from herbaria. Nevertheless, the corolla color is usually not well preserved in herbarium specimens as it tends to fade and the preservation of the quantitative flower traits is often no better. Hence, any conclusions based on this kind of material could have been significantly biased and should be regarded with a special attention.

Being aware of the limitations of the previous studies, we projected our research to answer following questions i) What is the relationship between *M. herbichii* and *M. saxosum*, are they really distinguishable as two distinct species? What is the pattern of their geographical distribution in the Eastern Carpathians? Are there any transitional populations on presumed contact between these two types? ii) What are the plants growing in the southern part of the Eastern Carpathians (i. e. area not covered by Jasiewicz 1958)? Are those populations more proximate to the other Eastern Carpathian types, or is there a similarity with Western European *M. sylvaticum* s. str? Addressing these questions will provide a solid basis for understanding taxonomical and biogeographic relations among populations of *M. sylvaticum* group in the Eastern Carpathian center of the group diversity and its surrounding.

Our experience with the previous study investigating variation in the *M. sylvaticum* group across the transitional zone between *M. sylvaticum* s. str. and the Eastern Carpathian species has revealed that an effective method is essential to capture the morphological variation of the studied populations. Despite being efficient in recording and analysis of some of the morphological traits (e. g. anther length), conventional distance-based morphometrics predominantly produced a set of highly intercorrelated variables which were rather ineffective in description of corolla shape and size (these two traits were in fact combined together without a possibility to separate them from each other). We have overcome the shortcomings of previously used methods by employment of geometric morphometric approach. We have chosen sliding semilandmark based thin plate spline analysis (Bookstein 1997, Zelditsch et al. 2004) for analysis of corolla shape. This method is efficient in outline description of studied objects, handles well also with edges (presence of which causes difficulties when outline-based methods are used) and was successfully employed in a number of studies dealing with shape variation of biological objects (e. g. Neustupa & Hodač 2005, Macholán 2006).

Material and methods

Material

Plant material was collected from 16 populations (442 plants) of the *M. sylvaticum* group in

the whole Eastern Carpathian region and additional 6 populations (154 plants) from the Western Carpathians and Hercynian Massif were included as a reference (Fig. 1 and Appendix 1). Up to 31 plants were sampled within each population. The corolla of one flower per plant was put into an Eppendorf-tube filled with ethanol and stored for digitization. The other parts of each plant were processed as a standard herbarium specimen and are kept in the herbarium of the Faculty of Biological Sciences, University of South Bohemia (CBFS).

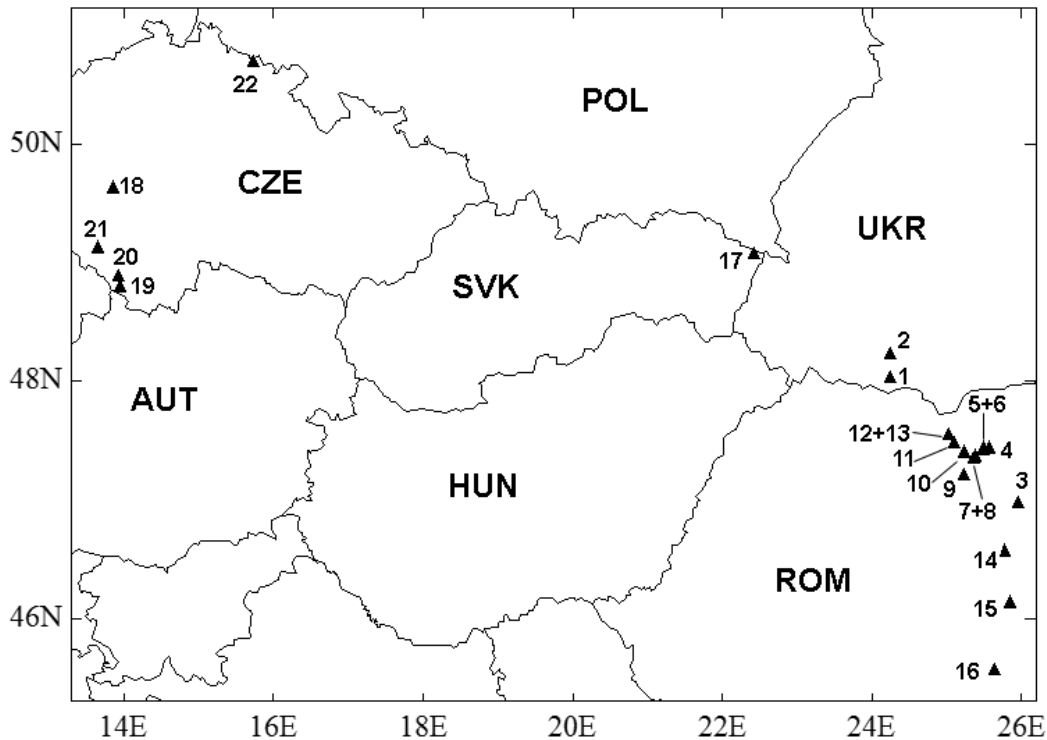


Fig. 1. – Map of the localities of the *Melampyrum sylvaticum* group from which morphometric data were collected, locality numbers corresponding to Appendix 1 are supplied. Borders of the Central European countries are also displayed. CZE – Czech Republic, SVK – Slovakia, UKR – The Ukraine, ROM – Romania, HUN – Hungary, AUT – Austria, GER – Germany, POL – Poland.

Sampling was conducted over a short time period at the start of the flowering season in order to minimize the influence of phenological divergence on the morphology of the flowers (1st to 3rd, exceptionally 4th lowermost flower were taken and analyzed). The only exception was the sample of the Bukovské vrchy Mts, which was collected at a later ontogenetic stage and 5th or 6th lowermost flower had to be taken.

The field sampling was designed to cover the whole region of the Eastern Carpathians. We tried to include more than one population sample from a mountain range to avoid potential bias caused by low-scale morphological variation (especially phenotypic plasticity) but it was not always possible to follow this rule. Unfortunately, the sampling was rather difficult in the woody southern part of the Eastern Carpathians, where it was hard to find any localities due to their rareness in the area and general floristic data deficiency. Hence, only three populations from that region are included in this study which cannot be considered sufficient for a detailed taxonomic assessment.

Specimens originating from several localities in the Eastern Carpathians (Rodna Mts, Chivchin Mts) were included in our previous study of variation in the *M. sylvaticum* group (Těšitel & Štech 2007). Unfortunately, this material is not more available for the current study as the morphometric measurements we performed were destructive. Nevertheless, these samples are still valuable for the biogeographical comparisons and are used in the discussion.

Digitalization and Morphometric Analysis

The corollas kept in ethanol were flattened and scanned at 1200 dpi using CanoScan 4200 (Canon Inc., Tokyo). Obtained images were saved as RGB color images in JPG format (low compression). 27 landmarks were digitized on the outline of each corolla (Fig. 2) using tpsDig software, ver. 2.05 (Rohlf 2006). The images were ordered randomly before performing the digitization, which should minimize subjective bias caused by potential misplacement of some landmarks in successive images. 25 landmarks were defined as semilandmarks allowing them to slide along the abscissa between their neighbors during the superimposition. Although the landmarks 11, 12 and 21 seem to be well defined in the two dimensional space, we decided to use them as semilandmarks. True landmarks have slightly higher power in the analysis compared to semilandmarks (Zelditsch et al. 2004), which we do not consider desirable for these points as their position is strongly affected by bending of the lower corolla lip when the three dimensional is being flattened and the curvature of the corolla base, which is more or

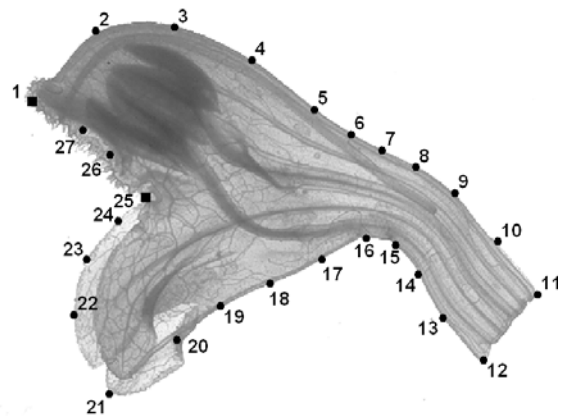


Fig. 2. – Position of landmarks on flattened corolla. Landmarks are symbolized by boxes, semilandmarks by circles.

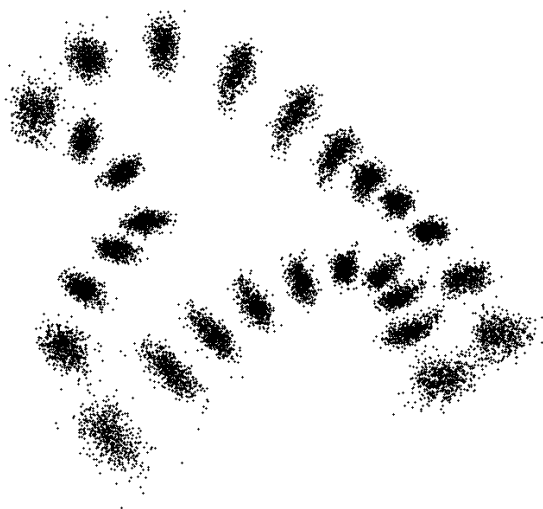


Fig. 3. – Scatter plot of superimposed specimens.

less stochastic and potentially connected to the phenological stage of individual flowers. All in all, there was almost no difference in the results when we performed a reference analysis considering these points as 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, the option of recursive procedure was disabled. Resulting scatter of

superimposed landmark can be seen in Fig. 3. Relative warp analysis (RWA, Rohlf 1993) was subsequently performed with the parameter α set to 0 (resulting in shape principal component analysis) using tpsRelw, ver. 1.42 software (Rohlf 2005). Centroid size (i.e. sum of distances between individual landmarks and 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. A dissection binocular microscope was employed for this purpose. The metering accuracy was 0.05 mm.

Statistical analyses

Standard statistical techniques were employed for detecting variability in morphological characters. The axes constructed by the RWA are suitable for direct visualization by ordination plots as this method is analogous to a PCA, if appropriate parameter setting is applied. Anther length and centroid size were the only univariate morphometric variables. This allowed for a direct visualization of their variability using a simple scatter plot.

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.

Combination of shape variables with centroid size and anther length required a rather complicated procedure. A standardization was necessary for anther length and centroid size values. This procedure is nevertheless inappropriate for relative warp scores of specimens as their scales correspond to the eigenvalues and a standardization preceding a PCA removes the scales. We solved this problem by conducting a standardized (correlation matrix based) PCA of anther length centroid size. Then we used the principal components as new variables. Subsequently, we standardized them and RW axes to unit variance and finally rescaled the standardized variables by corresponding eigenvalues so that the weights of univariate and shape variables in further analysis were the same. Then a PCA based on covariance matrix (and thus non-standardized variables) could have been calculated.

We employed a cross-validated linear discriminant analysis to assess the capability of morphometric dataset to distinguish plants that were sampled in different geographical regions and that were found to differ in their morphological traits according to the RWA and PCA. The same method was used for an analysis of delimitation between the Eastern Carpathian specimens differing in their corolla color.

We used the software package Statistica for Windows, version 6.0 (StatSoft 2001) for basic statistical procedures, graphical visualization of data, PCAs based on individual samples and calculation of EMS for univariate variables. Canoco for Windows, version 4.54 (terBraak & Šmilauer 2002) was used 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 by Quinn & Keough (2002). PCAs based on consensual landmark configurations and population means of univariate variables were computed in PAST package, version 1.67 (Hammer, Harper & Ryan 2001) using a singular value decomposition algorithm (which improved the PCA stability

when more variables than samples were present in the analysis). Cross-validated linear discriminant analysis was performed in R, version 2.3 (R Development Core Team 2006), package MASS (Venables & Ripley 2002).

Results

Variation in continuous morphometric characters

Within-population variation accounted for 50.3% in shape of the corolla, 32.9% in corolla centroid size and 23.8% in anther length (inferred from expected mean square analyses). We detected a considerable pattern in the variation of all these morphometric characters related to the geographical distribution of the populations under investigation. Three groups of populations can be distinguished on the relative warp plot based on consensual landmark configuration

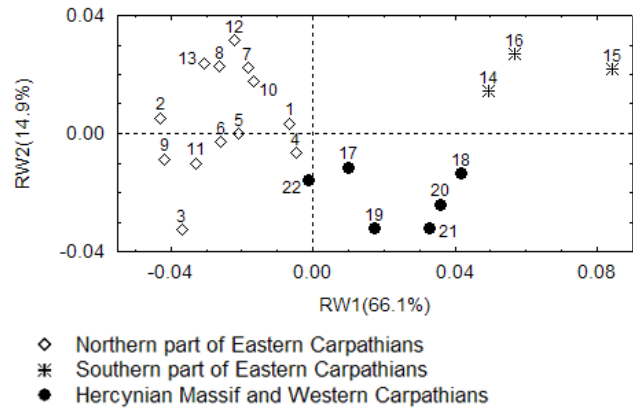


Fig. 4a. – RWA ordination plot based on consensual corolla shapes within each population. Percentages of variance explained by the axes correspond to among-population variation only. The populations are labeled by corresponding numbers of localities.

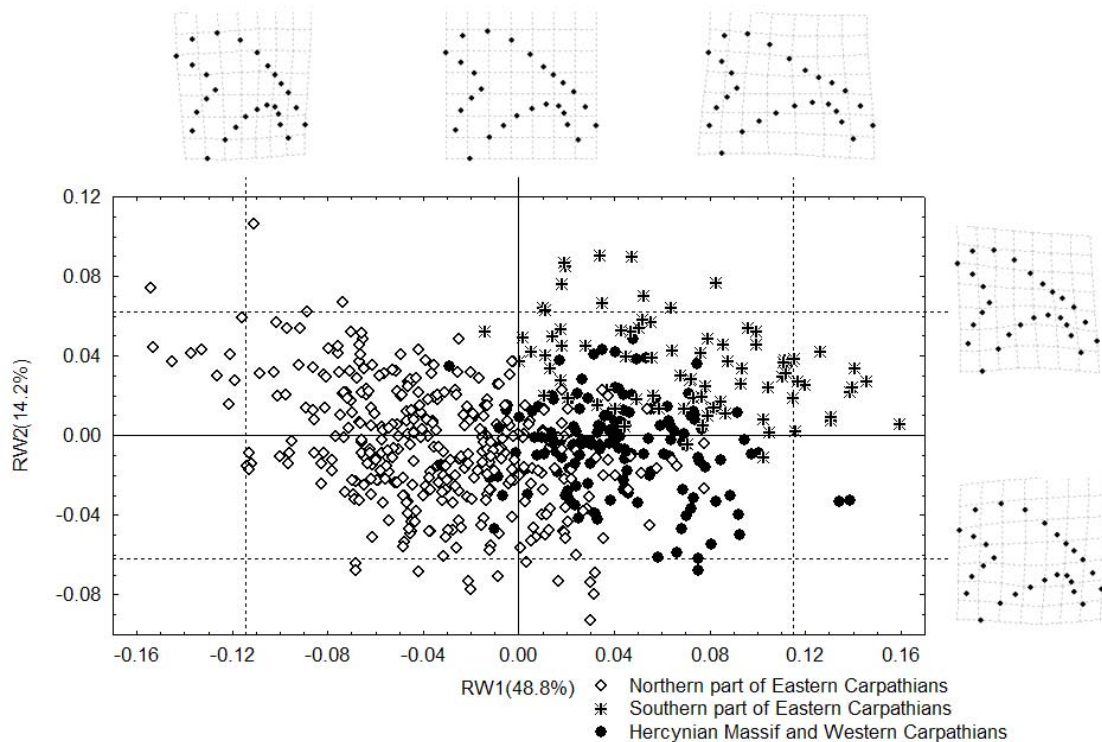


Fig. 4b. – RWA ordination plot based on variation in corolla shape in individual plants. Points representing the specimens are classified according to the geographical distribution of the populations. Mean corolla shape as well as direction of shape changes associated with first two principal warps are depicted (shapes corresponding to ± 2 SD positions at each axis are displayed).

within populations and the scatter plot of mean centroid size and anther length within populations (Figs. 4a, 5a). These groups comprise i) populations from the northern part of the

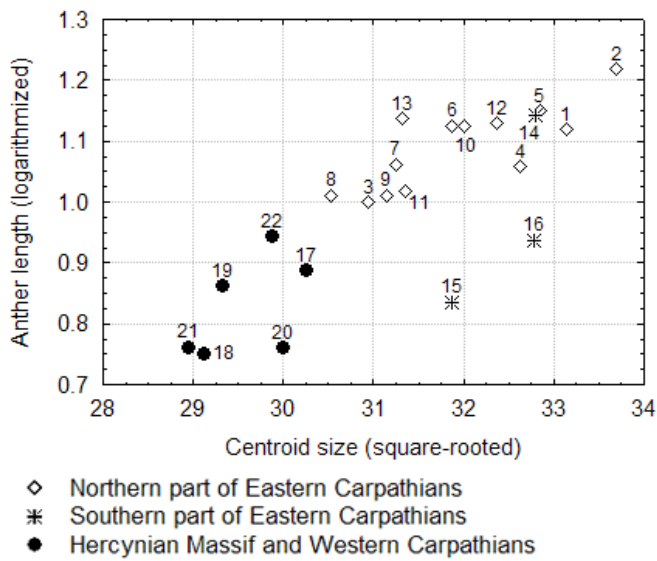


Fig. 5a. – Scatter plot of mean centroid size and anther length values in individual populations. The populations are labeled by corresponding locality numbers. The natural logarithm was applied on anther length values.

regions becomes more pronounced if a dataset combining both shape variables and univariate morphometrical characters is used in the analysis (Fig. 6a, 6b). A cross-validated discriminant analysis based on the first eleven relative warps (accounting for more than 96% of variation in corolla shape), anther length and centroid size yielded 98.84% of correct assignments of individual plants into the groups based on the geographical distribution (Table 1).

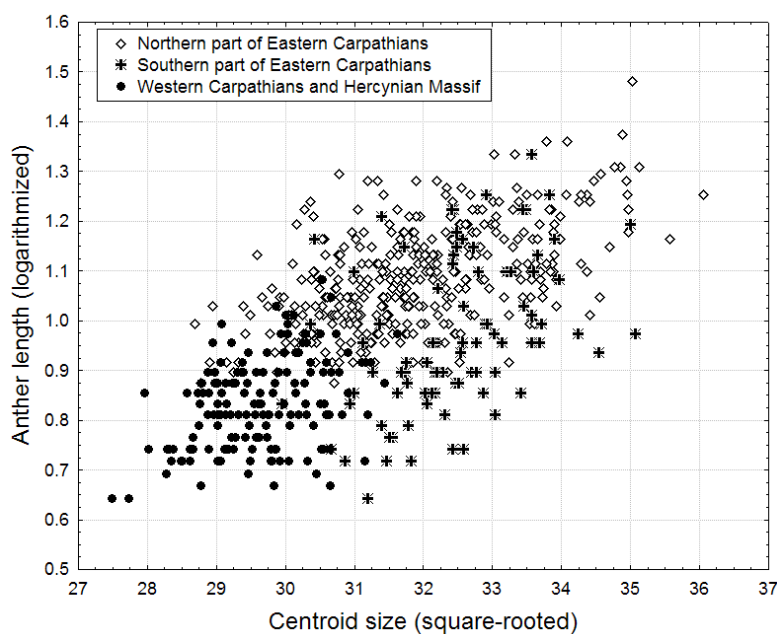


Fig. 5b. – Scatter plot of centroid size and anther length values in individual studied plants. The specimens are classified according to the geographical distribution of the populations. The natural logarithm was applied on anther length values

Eastern Carpathians ii) populations from the southern part of the Eastern Carpathians and iii) populations from the Hercynian Massif together with the population from Bukovské Vrchy Mts. (the most eastern part of the Western Carpathians). This pattern can be also observed in the plots of individual specimens (Figs 4b, 5b) although the groups are not distinct there and substantial overlaps are present. The variation appears more continuous as the differences are blurred by within-population variability. Nevertheless, the morphological distinction among plants from different geographical

The populations of the northern part of the Eastern Carpathians seem to form two slightly distinct groups on the PCA plot based on mean anther length, corolla shape and size within populations (Fig. 6a). Populations 1, 2, 4 and 5 appear to differ slightly from the rest. This difference does not have an obvious geographical basis as these populations are located in remote parts of the Eastern Carpathians.

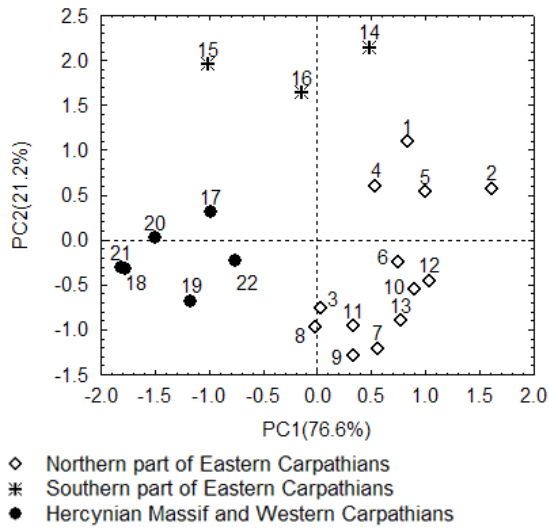


Fig 6a. – PCA ordination plot based on combined dataset of relative warps describing consensual corolla shape in individual populations and population means of anther length and centroid size of the corolla. The populations are labeled by corresponding locality numbers.

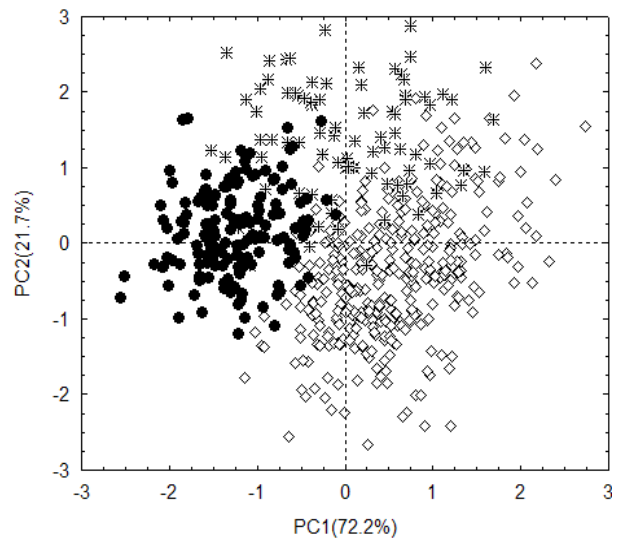


Fig. 6b. – PCA ordination plot based on combined dataset of relative warps describing corolla shape and univariate variables – anther length and centroid size of the corolla. The specimens are classified according to the geographical distribution of the populations.

Population	Region	% correct	Correct	Incorrect	Misclassified as
1	NE	96.30%	27	1	SE
2	NE	100.00%	30	0	
3	NE	100.00%	30	0	
4	NE	96.67%	29	1	HW
5	NE	100.00%	20	0	
6	NE	100.00%	27	0	
7	NE	100.00%	29	0	
8	NE	100.00%	22	0	
9	NE	100.00%	30	0	
10	NE	100.00%	27	0	
11	NE	100.00%	30	0	
12	NE	100.00%	29	0	
13	NE	100.00%	27	0	
14	SE	96.55%	28	1	HW
15	SE	100.00%	31	0	
16	SE	95.83%	23	1	HW
17	HW	100.00%	29	0	
18	HW	100.00%	26	0	
19	HW	100.00%	30	0	
20	HW	100.00%	30	0	
21	HW	100.00%	15	0	
22	HW	87.50%	21	3	NE
total:		98.84%	590	7	

Table 1. – Results of a cross-validated linear discriminant analysis separating individual plants according to geographical distribution of the studied populations on the basis of first eleven relative warps, anther length (logarithmized) and centroid size (square-rooted). Percentage and counts of correct assignments together with incorrect assignment counts and type of misclassification are presented. Following abbreviations of geographical regions are used: NE – northern part of the Eastern Carpathians, SE – southern part of the Eastern Carpathians, HW – the Hercynian Massif and Western Carpathians.

Variation in corolla color

We found both yellow- and white-flowering individuals of *Melampyrum sylvaticum* group (Figs 7a, 7b) in the Eastern Carpathian region. This trait was usually uniform in individual populations but there were noticeable exceptions. We discovered plants displaying pale yellow color of their corollas (Figs 7a, 7d) at three localities. These plants were growing together with yellow-flowering individuals in two of the cases (one of these populations is included only as an observation in this study, no morphometric data were collected). We also found a population that comprised plants displaying both corolla colors as well as intermediate pale yellow flowering individuals (Fig. 7c). The pale yellow flowering plants were variable in the color tone of their corolla, which formed a continuum from almost white to golden-yellow (Fig. 7a). In rare cases, slight differences in color tone could have been observed even between flowers of an individual plant (Fig 7d).

We performed a PCA based on combined data-set of all morphometric variables to see whether there is a relationship between corolla color on one side and anther length, corolla shape and size on the other side. The two groups of populations from the northern part of the Eastern Carpathians detected by the PCA performed on population means of morphometrical variables appear to display also a slight difference in the corolla color (Fig. 8). Both groups contain yellow flowering populations; nonetheless the occurrence of white flowering, mixed and pale flowering plants seems to be restricted on one group only. PCA based on individual plants of populations of the northern part of the Eastern Carpathian region revealed substantially higher variation in morphology of yellow flowering plants compared to white flowering and transitional populations (Fig. 9). A cross-validated discriminant analysis was generally unsuccessful in discrimination between samples from yellow and white flowering populations (Table 2).



Fig. 7a. – Variation in corolla color population at Poiană Obcina Mică meadows near Vatra Dornei. The plants are ordered from the golden-yellow flowering specimen (= typical “*Melampyrum herbichii*”) on the left to the lightest (almost white) flowering specimen on the right. 5th July 2006.



Fig. 7b. – Typical specimen of „*Melampyrum saxosum*“, Ceahlau Massif, 2nd July 2006.



Fig. 7c. – A mixture of yellow-, white- and pale-yellow- flowering plants at the road margin near Pasul Rotunda saddle, 8th July 2006.



Fig. 7d. – An unusual plant displaying difference in color between flowers at different nodal positions. Poiană Obcina Mică meadows near Vatra Dornei, 5th July 2006.

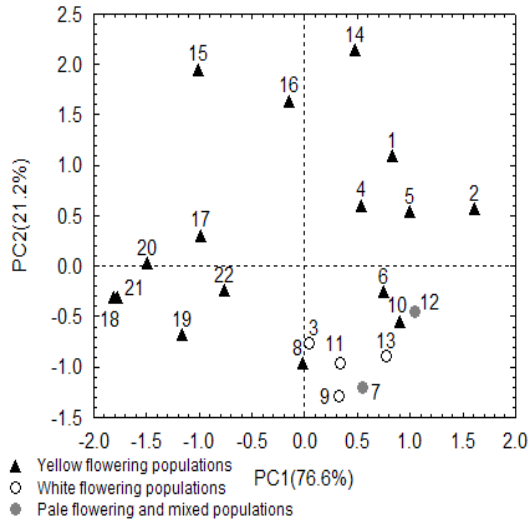


Fig. 8 – PCA ordination plot based on combined dataset of relative warps describing mean corolla shape in individual populations and population means of anther length and centroid size of the corolla. The populations are classified according to corolla color of plants and labeled by corresponding locality numbers.

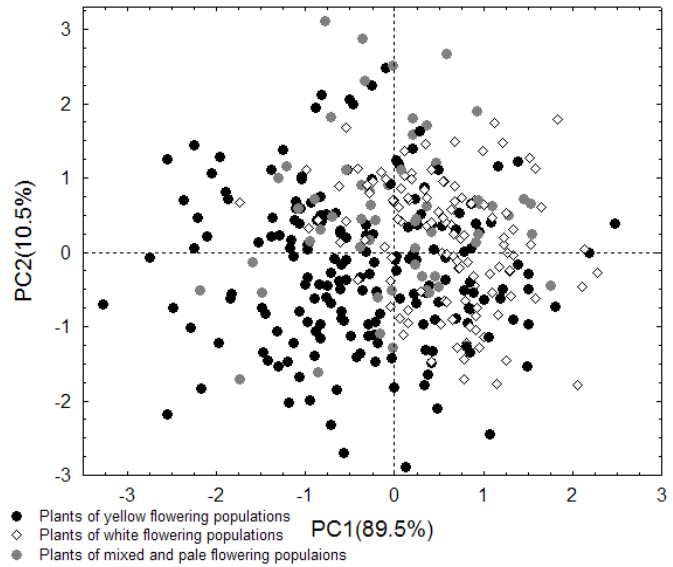


Fig. 9 – PCA ordination plot based on combined dataset of relative warps describing corolla shape, anther length and centroid size of the corolla. Plants of the northern part of the Eastern Carpathian region are included in the analysis. Individual specimens are classified according to the corolla color found in the populations.

Table 2. – Results of a cross-validated linear discriminant analysis separating individual plants from populations in the northern part of the Eastern Carpathians according to the corolla color on the basis of first eleven relative warps, anther length (logarithmized) and centroid size (square-rooted). Populations comprising pale flowering plants and mixture of white and yellow flowering individuals were excluded from the analysis. Percentage and counts of correct and incorrect assignments are given.

Population	Flower color	%correct	Correct	Incorrect
1	Yellow	100.00%	27	0
2	Yellow	96.67%	29	1
3	White	93.33%	28	2
4	Yellow	76.67%	23	7
5	Yellow	90.00%	18	2
6	Yellow	70.37%	19	8
8	Yellow	68.18%	15	7
9	White	63.33%	19	11
10	Yellow	81.48%	22	5
11	White	83.33%	25	5
13	White	22.22%	6	21
total		77.00%	231	69

Discussion

Evaluation of characters

Employed morphometric methods proved to be very efficient in capturing the pattern in variation among studied populations of *Melampyrum sylvaticum* group. The RWA separating corolla size and shape indeed brought additional information which would be lost if conventional morphometric methods were used. The within population variation in the corolla shape appears relatively high (the within-population variance component is ca twice as high compared to the univariate characters). However, the proportion of within population variation is 34.4% in the first relative warp when analyzed separately, which corresponds well to the ratio of variation in corolla size (32.9%). Anther length displays substantially lower within population variation than the corolla characters. The distribution of morphological trait variation within and among the populations is generally identical to that we reported for Western Carpathian populations (Těšitel & Štech 2007).

The relationship between corolla color and quantitative morphometric characters is weak in general (Fig. 9). The existence of populations with plants of intermediate corolla

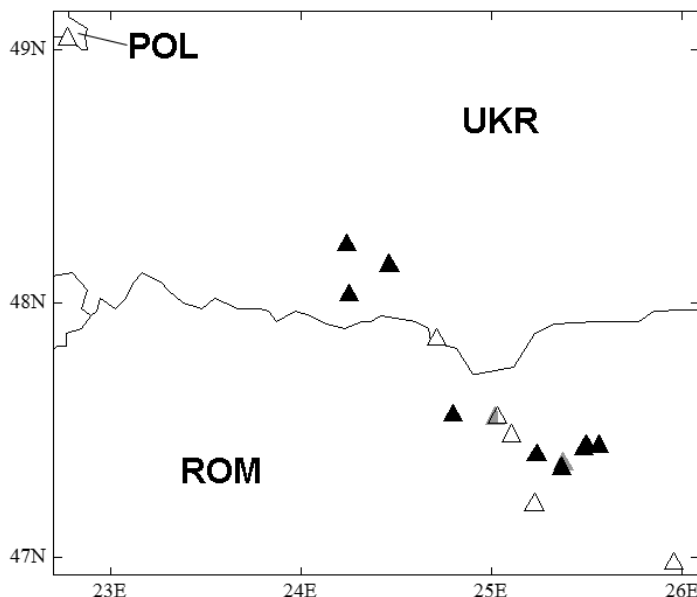


Fig. 10. – Map of the localities of the North-Eastern Carpathian morphotype of *Melampyrum sylvaticum* group (i. e. *M. herbichii* and *M. saxosum*) for which doubtless data are available. Localities used in the current work and in Těšitel & Štech (2007) are used. The position of an additional Polish white-flowering population is estimated on the basis of Zajac & Zajac (2001) and the official web site of the Bieszczady National Park (<http://www.bdpn.pl>).

The populations are classified according to corolla color of specimens (▲ – yellow corolla, △ – white corolla, ▲ – populations with mixed and pale flowering plants).

Borders of countries are displayed. UKR – The Ukraine, ROM – Romania. POL – Poland.

color and lack of any conspicuous geographical pattern (Fig 10) in this character furthermore put the corolla color role in species delimitation in question.

The observed pattern in this trait can be explained by two main hypotheses: i) white corolla color has originated repeatedly in isolated populations of *M. sylvaticum* group in the north Eastern Carpathians. ii) all north Eastern Carpathian populations were originally white flowering. Yellow corolla color is a product of (relatively recent) gene flow from yellow flowering populations which grow in proximate regions (on the west and on the south). This latter hypothesis has already been mentioned by Jasiewicz

(1958) and although hybridization has not been reported in *Melampyrum*, recent research on *M. subalpinum*, *M. bohemicum* and *M. angustissimum* has shown that the this process is likely

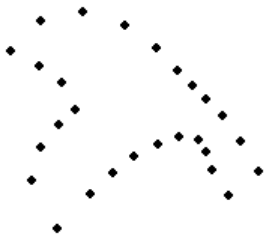
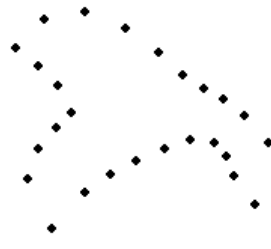

to affect these closely related *Melampyrum* species (Štech 2006). Unfortunately, given data do not allow for any decision, which of the hypotheses is more likely. The first theory is not supported by any straightforward mechanism which would have caused repeated shifts in corolla color and cannot explain, why the color shifts are not observed also in other regions. The second theory does not provide any explanation for lack of obvious geographical pattern in corolla color. Slight differentiation among the Eastern Carpathian populations (Fig. 8) does not provide any answer here. It may appear interesting at the first glance that all white-flowering plants are gathered in one cluster in the morphometric analysis but we can not really exclude the role of chance in this slight delimitation. In any case, all of the population samples from the north Eastern Carpathians are more proximate to each other (regardless corolla color) than to the populations from the other two regions.

Delimitation of morphological types

The morphometric analyses have detected existence of three morphological types to which studied populations of *Melampyrum sylvaticum* group can be assigned. However, these groups do not exactly correspond to the current definition of species in the *M. sylvaticum* group. Lack of any real morphological trait delimiting *M. saxosum* and *M. herbichii* (except the corolla color) suggests classifying these two traditional microspecies as one entity. Even if the yellow corolla color was a result of agene flow, the major part of the genotype of yellow flowering plants comes very likely from the north Eastern Carpathian type and the gene flow has character of rather an introgression than a hybridization. The southern group of the Eastern Carpathian populations is on the other hand markedly distinct from the northern one. Hence, a boundary should be placed between these two morphological types even though no previous author distinguished the southern type. If we follow these suggested modifications, the preliminary taxonomic concept of the *M. sylvaticum* group will be as following:

The morphological type integrating *M. herbichii* and *M. saxosum* is characterized by long anthers and big but rather short corolla which is curved concavely (Table 3). Corolla color is varying from white to golden yellow but is mainly uniform within populations. This type seems to prefer particularly sites near tree-line but often descends to lower altitude where it occurs on margins of paths, roads and secondary meadows. Its geographical distribution appears limited to the north Eastern Carpathians. It reaches its northern and eastern limit probably on the southern boundary of Bieszczady Zachodne Mts (SE Poland; Zając & Zając 2001). We found the southernmost populations in the Calimani Mts and the Ceahlau Massif, but naturally this type can spread even more southwards to the outer mountain ranges of the southern part of the Eastern Carpathians (e. g. Ciucului and Nemirei Mts). Both *M. herbichii* and *M. saxosum* were reported also from the Southern Carpathians in most previous studies (Paučá & Nyárady 1960, Soó & Webb 1972 – *M. herbichii* only, Meusel et al. 1978) but not by Soó (1926-1927). Unfortunately, we do not have any population samples from that region at our disposal at the present time. We cannot thus confirm nor reject the possibility that *M. herbichii/saxosum* type really occurs in that area and its geographical range is split into two parts.

Table 3 – Proposed delimitation of morphological types in the *M. sylvaticum* group. The ranges indicated in bold are based on mean \pm standard deviation, numbers in brackets denote 2.5%- and 97.5%-quantiles respectively.

Morphological type	<i>M. herbichii/saxosum</i>	<i>M. sylvaticum</i> s. str. and related types transitional to <i>M. herbichii/saxosum</i>)	Morphological type of the southern part of the Eastern Carpathians
Mean shape of the corolla			
Anther length (mm)	(2.5–) 2.7–3.3 (–3.7)	(1.95–) 2.1–2.5 (–2.75)	(2.05–) 2.3–3.1 (–3.5)
Corolla centroid size (square-rooted)	(29.4–) 30.6–33.3 (34.9)	(28.0–) 28.9–30.4 (–31.2)	(30.4–) 31.4–33.5 (–34.5)
Corolla color	golden-yellow, white, pale yellow	golden-yellow	golden-yellow
Geographical distribution (direct observations only)	North-Eastern Carpathians	Hercynian Massif and Western Carpathians	South-Eastern Carpathians

The traditional definition of the type corresponding to *M. sylvaticum* s. str. (e. g. Jasiewicz 1958, Soó & Webb 1972) remained more or less unaltered. We only suggest a slightly pronounced convex corolla curvature as one more trait differentiating this species from the north Eastern Carpathian type (Table 3). The eastern limit of this species lies probably in Bukovské Vrchy Mts; nonetheless, populations growing in the northern Hercynian Massif (Sudeten Mts) and Western Carpathians generally belong to a transitional type displaying substantial similarities with the *M. herbichii/saxosum* type (Těšitel & Štech 2007). We can assume that the populations of the Alps and Western Europe belong also to *M. sylvaticum* s. str as no substantial differences seem to differentiate the plants from the Alps and the south-western part of the Hercynian Massif (Štech & Drábková 2005) and no considerable high-scale variation in the *M. sylvaticum* group has ever been reported from Western Europe.

Unfortunately, we managed to sample only a very low and definitely not sufficient number of populations belonging to the type occurring in the southern part of the Eastern Carpathians which prevents us from any exact taxonomic delimitation here. Nonetheless, we can make an approximate preliminary characterization of this morphotype. Big, deeply convexly curved corollas with relatively narrow lips seems to be very typical (Table 3). Anther length values appear variable and considerably lower compared to the *M. herbichii/saxosum* type in general, except for the northernmost population where a potential gene flow from the northern populations could explain the observation of extremely long anthers. Description of the geographical distribution of this type is not possible at the

current time and is certainly beyond the scope of the present work which is focused on the Eastern Carpathians. Additional sampling is required in the Southern Carpathians and Transsilvania to solve this issue.

Biogeographical context

The geographical distribution of the *M. herbichii/saxosum* type appears restricted to those of the Eastern Carpathian mountain ranges that are high enough to reach and substantially exceed the tree-line. Although we observed occasional descends to lower altitude, it does never grow in forests but seem to strictly prefer sunny sites at margins of meadows, pathways or roads, i. e. man-made habitats (this is quite similar in *M. sylvaticum* s. str., but in this type it is even more pronounced). These habitat preferences can explain the fact that this species does not also occur in the Western Carpathians in its pure form. The most western parts of the Eastern Carpathians do not reach above the tree-line, which did not allow for a recent migration of numerous Eastern Carpathian (sub-)alpine species to the Western Carpathian region. Such a distribution limit is well documented for several diploid species of *Hieracium* (Mráz & Szeląg 2004), a mountain spring-area species *Cochlearia borzeana* (Kochjarová et al. 2006) and is well known e. g. for *Rhododendron kotschyi* and *Alnus viridis* (Polívka, Domin & Podpěra 1928). Unfortunately, the biogeography of (sub-)alpine flora of the Eastern Carpathians did not receive much attention in the last decades, otherwise there would be more examples demonstrating this phenomenon.

The geographic range of the *M. herbichii/saxosum* type advocates for the Pleistocene origin of this type, although some recent evolutionary processes (hybridization with the other types, introgression etc.) affecting its populations are possible and quite likely. It probably survived the Weichsel (Würm) glacial period in a refuge located somewhere in the Eastern Carpathian region (or Transsilvania). Growing predominantly in communities near the tree-line, the *M. herbichii/saxosum* type features ecological traits that allow for survival at sites with relatively cold climate and short vegetative season. It also displays a hemiparasitic life style as do all related taxa. Hence, any biogeographical and palaeoecological data referring to its host species (predominantly *Pinus mugo*, *Vaccinium myrtillus* and *Picea abies*) can provide a useful reference source of information for the biogeography of *M. herbichii/saxosum* itself. Recently published studies of *Pinus mugo*, vegetation of which seems to provide the most favorable conditions for *M. herbichii/saxosum* plants, have probably the most important implications for the biogeography of *M. herbichii/saxosum* presenting an evidence that *Pinus mugo* vegetation persisted in a refuge in the Eastern Carpathian region or a proximate area.

An investigation of *Pinus mugo* variation (Boratyńska, Muchewicz & Drojma 2004) has revealed a considerable differentiation between north Eastern Carpathian populations and other Central European and Balkan populations of this species (unfortunately no Southern Carpathians populations were included). Such variation pattern suggests a long-term isolated evolution with no or very limited contact with other populations of the species (Boratyńska, Muchewicz & Drojma 2004). This seems possible if there was a refuge in the Eastern Carpathians which persisted during whole Weichsel glacial period (or at least its major part). Existence of such a refuge is moreover supported by a palynological sequence from the

Iezerul Calimani Lake (1650 m a. s. l.) in the Calimani Mts (Farcas et al. 1999) which revealed a strong deposition of *Pinus* (probably *Pinus mugo*) pollen even in the oldest layer analyzed (dated to 14800 ± 1100 BP by non-calibrated ¹⁴C chronology) which corresponds to the late Weichsel glacial period. Similar study (Feurdean 2004) was performed in Gutai Mts ca 100 km on NE of Calimani, which detected high deposition of *Pinus* and *Betula* pollen in a layer around Weichsel/Holocene boundary (11500 BP by calibrated ¹⁴C chronology) in a palynological sequence from a crater lake (peat bog nowadays) at ca 750 m a. s. l.. These are definitely not evidences that *Pinus mugo* survived the pleniglacial period at such elevated sites (it hardly could), especially if we consider the long-distance *Pinus* pollen dispersal which can significantly bias palynological data (Lang 1994). Nonetheless, such a strong pollen deposition certainly supports the possibility of *Pinus mugo* persistence in local refuges located perhaps at more favorable sites at the feet of the mountains. In summer, such a refuge could have been also well supplied by water coming from glaciers located at higher altitude. Questions on exact placement and extent of the potential glacial refuge in the Eastern Carpathian region remain to be solved. We expect that comparison between the populations of species growing in both North-Eastern and Southern Carpathians could reveal interesting indices in this respect. Unfortunately, no data on *Melampyrum*, *Pinus mugo* nor other species are available for such a comparison at the moment.

The mechanism of the origin and development of the transitional zone between *M. sylvaticum* s. str. and the *M. herbichii/saxosum* type is still not clear. We however suppose the following scenario to be possible. Some populations of the north-eastern morphotype could migrate to the west in the first phase of the Holocene. The tree-line was at substantially lower in that time and *Picea abies* probably dominated local forests (Lang 1994), which could have allowed for migration over the Eastern and Western Carpathian boundary. These populations could have become widespread especially at the northern feet and slopes of the Western Carpathians and Hercynian Massif but after subsequent climate warming and forest development became extinct there except for high mountains which reach the tree-line. *M. sylvaticum* s. str could have started its immigration to the Central Europe from western or (and) southern regions at the same time. This species appears more shadow-tolerant and sometimes it grows even in a deep forest, thus it could have followed the immigration of *Picea abies*. It could have migrated to mountain valleys and then ascended to higher altitude where it met relict populations of the North-Eastern Carpathian type and these types have undergone a hybridization. Such scenario can explain enormous morphological variation in the Western Carpathian and northern Hercynian regions by different contribution of the mother types to the genotype of the assumed hybrids.

The taxonomic status and geographical distribution of the *M. sylvaticum* group type growing in the southern part of the Eastern Carpathians is another interesting question arising from our work. We detected this type in forests only but this can be obviously caused by general deficiency of sites above tree-line in the area southwards of the Calimani Mts and Ceahlau Massif in the Eastern Carpathians. It is possible that this type is widespread in the Southern Carpathians, where it is also present in the subalpine zone, or conversely it could be a specific forest type immigrated from more southern areas of the Balkans.

Further study of the *M. sylvaticum* group in the Southern Carpathians and in the Balkans appears very promising. It is likely that resolution of relationships among populations of the Carpathian regions and the Balkans will provide the key basis for the ultimate phylogeographical study of the group. Comparison between Southern and North-Eastern Carpathian populations could be also helpful in the general reconstruction of the development of Carpathian flora and vegetation in the Holocene.

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Appendix 1: List of localities

Eastern Carpathians – the Ukraine

1 – Chernogora Mts, Rakhiv: montane forest on slope ca 2 km ESE of the town; 48°02'36"N, 24°15'13"E, 950 m a. s. l.; 30th June 2005; 27 specimens analyzed.

2 – Svydovets Mts, Yasinya: forest edge by ski slopes at tourist base ca 8 km W of the town; 48°14'25"N, 24°14'24"E, 1410 m a. s. l.; 12th July 2005; 30 specimens analyzed.

Eastern Carpathians, northern part – Romania

3 – Ceahlau Massif, Stațiunea Durău resort: margin of path between Fîntînele and Dochia chalets on northern slope of Mt Ceahlau ca 1 km N of its top, ca 3 km ESE of the mountain resort; 46°59'03"N, 25°57'25"E, 1610 m a. s. l.; 2nd July 2006; 30 specimens analyzed.

4 – Rărau-Giumalau Mts, Vatra Dornei: rocky massif Piatrele Doamnei ca 1 km SW of Mt Rărau, ca 20 km NE of the town; 47°26'51"N, 25°33'53"E, 1590 m a. s. l.; 3rd July 2006; 30 specimens analyzed.

5 - Rărau-Giumalau Mts, Vatra Dornei: path margin in a spruce forest on the ridge between Mt Rărau and Mt Giumalau ca 13 km NE of the town; 47°27'01"N, 25°29'60"E, 1420 m a. s. l.; 4th July 2006; 20 specimens analyzed.

6 - Rărau-Giumalau Mts, Vatra Dornei, Mt. Giumalau: *Pinus mugo* vegetation on the eastern slope of the mountain ca 250 m E of the summit, ca 11 km NE of the town; 47°26'13"N; 25°29'04"E, 1790 m a. s. l.; 4th July 2006; 27 specimens analyzed.

7 - Rărau-Giumalau Mts, Vatra Dornei: mountain meadows of Poiană Obcina Mică ca 5 km NE of the town; 47°22'46"N, 25°22'39"E, 1250 m a. s. l.; 5th July 2006; 29 specimens analyzed.

8 - Rărau-Giumalau Mts, Vatra Dornei: track margin in meadows at the NE boundary of the town; 47°21'38"N, 25°22'14"E, 950 m a. s. l.; 5th July 2006; 22 specimens analyzed.

9 – Caliman Mts, Gura Haitii: *Pinus mugo* vegetaion around a path ca 1 km S of the rocky massif Stîncile doisprezece apoștolii; 47°13'04"N, 25°13'28"E, 1590 m a. s. l.; 5th July 2006; 30 specimens analyzed.

10 – Suhard Mts, Iacobeni: spruce forest edge on southern slope of Mt Tarnița ca 5 km W of the village; 47°24'49"N, 25°14'13"E, 1420 m a. s. l.; 6th July 2006; 27 specimens analyzed.

11 – Suhard Mts, Rotunda settlement: *Pinus mugo* vegetation around a path ca 1.5 km SE of the of Mt Omu peak, ca 8 km SE of the settlement; 47°29'18"N, 25°06'23"E, 1740 m a. s. l.; 7th July 2006; 30 specimens analyzed.

12 – Suhard Mts, Rotunda settlement: margin of the road between the Rotunda settlement and the Pasul Rotunda saddle ca 2 km SW of the settlement; 47°33'27"N, 25°01'14"E, 1130 m a. s. l.; 8th July 2006; 29 specimens analyzed.

13 – Suhard Mts, Rotunda settlement: margin of the road between the Rotunda settlement and the Pasul Rotunda saddle ca 1.5 km SW of the settlement; 47°33'33"N, 25°01'52"E, 1080 m a. s. l.; 8th July 2006; 27 specimens analyzed.

Eastern Carpathians, southern part – Romania

14 – Harghita Mts, Danești (= Izvoru Oltului): spruce forest and spring area on northern slope (just below top) of a hill ca 1 km NW of the village; 46°34'46"N, 25°46'47"E, 910 m a. s. l.; 1st July 2006; 29 specimens analyzed.

15 – Harghita Mts, Baile Tușnad: margin of a spruce forest at the western boundary of the town ca 1 km SW of the railway station; 46°08'42"N, 25°51'09"E; 680 m a. s. l.; 30th June 2006; 31 specimens analyzed.

16 – Piatra Mare Massif, Timișu de Jos: path margin in the valley of the Șipaia creek ca 2 km SE of the railway station; 45°34'41"N, 25°38'13"E, 840 m a. s. l.; 28th June 2006; 24 specimens analyzed.

Western Carpathians - Slovakia

17 – Bukovské Vrchy Mts, Runina: alpine pastures at Sedlo pod Ďurkovcom Saddle, 3.2 km NNE of the village; 49°05'08"N, 22°25'24"E, 1130 m a. s. l.; 8th July 2005, 29 specimens analyzed.

Hercynian Massif – Czech Republic

18 – Brdy Massif, Zalány: spruce forest at the N border of the village; 49°38'35"N, 13°51'25"E, 650 m a. s. l.; 12th July 2005; 26 specimens analyzed.

19 – Bohemian Forest Mts, Ovesná: spruce forest next to the railway station; 48°48'26"N, 13°56'21"E, 740 m a. s. l.; 15th June 2006; 30 specimens analyzed.

20 – Bohemian Forest Mts, Volary: Forest margin 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 m a. s. l.; 21st June 2006; 30 specimens analyzed.

21 – Bohemian Forest Mts, Javorník: meadow margin ca 700 m S of the village; 49°07'58"N, 13°39'37"E; 900 m a. s. l.; 21st June 2006; 15 specimens analyzed.

22 – Krkonoše Mts, Pec pod Sněžkou: : forest margin near the lower station of the cableway on Mt Sněžka; 17th June 2006; 24 specimens analyzed.