Plant Ecology and Evolution 149 (1): 45–58, 2016
http://dx.doi.org/10.5091/plecevo.2016.1128

REGULAR PAPER

Microsatellite analysis of four similar *Euphrasia* (Orobanchaceae) species changes the traditional view of this group

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Background and aims – The genus *Euphrasia* comprises a taxonomically intricate group. In Central Europe, *E. nemorosa* and *E. stricta* are widely accepted species. However, the occurrence of putative intermediate morphotypes considered to be the result of regular hybridization makes identification of populations often difficult. Besides these mostly late-flowering species, two mostly early-flowering species, *E. coerulea* and *E. slovaca*, are distinguished in the Sudeten and in the Carpathians, respectively. Because of the doubtful nature of intermediate forms and difficult distinction of early-flowering morphotypes, the aims of this study were to find genetically supported groups and test morphological differences among them.
Methods and key results – We conducted a survey of the genetical and morphological diversity in 42 populations, which were assigned to four species based on morphology. Using microsatellite analysis, we discovered three genetic groups within our data set. Whereas *E. stricta* and *E. nemorosa* comprised separate clusters, most of the early-flowering populations identified as *E. coerulea* and *E. slovaca* formed one common cluster. Traditional characters such as corolla length, branching and the presence of a long awn on the bracts were identified in multivariate analyses as the most reliable morphological differences between genetically defined *E. stricta* and *E. nemorosa*. Early-flowering populations differed generally by their low number of nodes. In spite of their genetic similarity, they differed morphologically between the two geographical areas. In spite of the assumption of different selfing rates correlated with corolla size, differences in genetic diversity among populations with different corolla sizes were not found.
Conclusions – There are three well supported groups in the studied dataset of *Euphrasia* species. Delimitation of *E. stricta* and *E. nemorosa* is in concert with traditional views, but delimitation of the third group changes the traditional distinction of two mostly early-flowering species in the study area.

Key words – *Euphrasia*, microsatellites, genetic diversity, morphometrics.

INTRODUCTION

The genus *Euphrasia* L. (eyebright) comprises annual hemiparasitic plants (rarely perennials or semishrubs) distributed in temperate areas and in mountainous areas of tropical zones (e.g. von Wettstein 1896, Hartl 1974, Barker 1982, Smejkal & Dvořáková 2000, Gussarova et al. 2008). Because of their diverse breeding systems, interspecific hybridization, rapid and relatively recent radiation (Gusarova et al. 2008), rapid adaptations to specific conditions (Karlsson 1976, Vitek 2011) resulting in high intraspecific variation (Kolseth & Lönn 2005), seasonal dimorphism and phenotypic plasticity, the genus *Euphrasia* represents one of the most taxonomically intricate and challenging genera in the European flora.

In the European flora there are two ploidy levels in *Euphrasia*: diploid (2n = 2x = 22) and tetraploid (2n = 4x = 44) (Yeo 1954, Vitek & Kiehn 1990). The tetraploids are the most widespread and most critical taxonomically.

The breeding system is supposed to depend on flower size and shape. Several types of flowers and prevailing breeding modes can be found in this genus. Each of them differs in the degree of autogamy, which is very frequent in small-flowered species (von Wettstein 1896, Gómez 2002, Vitek 1998). Species with large flowers reproduce mostly by outcrossing, but if no pollinator visits the flower, they can self-pollinate as well. Species with mid-sized flowers reproduce mostly by allogamy, but autogamy occurs more often compared to large-flowered species (Hartl 1974, Vitek 1998).

Seasonal dimorphism is another important source of intraspecific variability in the genus *Euphrasia*, as well as in other genera of hemiparasitic Orobanchaceae (von Wettstein 1895, von Sterneck 1901, Ronniger 1911, Zopfi 1997, 1998a,

1998b, Štech 2000) and in some Gentianaceae (Zopfi 1991). Two (or more) types of phenologically different forms can be found: early flowering (aestival) and late flowering (autumnal). These two types differ in growth habit, which is used as one of the species determination criteria. Plants of the early-flowering type have no branches (or very few), a small number of internodes and their leaves often persist during flowering. The stems of late-flowering plants are usually branched, have many short internodes and there are usually no stem leaves during flowering (von Wettstein 1895, Smejkal & Dvořáková 2000).

In general, hybridization is common in perennial plants (Ellstrand et al. 1996), while in annuals it is quite rare (Solbrig 1970). However, in *Euphrasia*, as well as in the related genus *Rhinanthus* (von Sterneck 1901, Kwak 1978, Ducarme & Wesselingh 2005), hybridization is assumed to be relatively frequent and is considered to be one of the main causes of the current variability in this genus (Smejkal 1960, Karlsson 1976, Yeo 1978). Hybridization in the genus *Euphrasia* is assumed to occur between all species that come into contact with one another (Vitek 1998). While hybridization between diploid species is not reported very often (Vitek 1982, Smejkal & Dvořáková 2000), hybridization between tetraploids is considered to be very common (Yeo 1954) and many hybrids have been described (e.g. von Wettstein 1893–1895).

Euphrasia is a monophyletic genus (Bennet & Mathews 2006, Gussarova et al. 2008, Těšitel et al. 2010) with 100–300 species depending on the authors (Hartl 1974, Smejkal & Dvořáková 2000, Vitek 2002). The original narrow species concept resulted in the description of hundreds of species and intraspecific taxa, which are connected with many names of different taxonomic categories (von Wettstein 1893–1895, Sennen 1930, Rothmaler 1935, Vitek 1985a, 1985b, 1986). Many early taxonomic treatments (Smejkal 1963, Hartl 1974, Yeo 1978) were based on the narrow species concept used by von Wettstein (1893–1895, 1896). Increasing knowledge on different aspects of variability in this genus may reduce the number of recognized taxa. Recent authors prefer a relatively wide species concept (Vitek 1998, 2002, 2011, Krok et al. 2013). On the contrary, a rather narrow species concept has been accepted in the Czech Republic (Smejkal 1963, Smejkal & Dvořáková 2000, Dvořáková 2002), Slovakia (Králik 1997) and in the Ukraine (Peregrym 2010).

Similar to other parts of Europe, the tetraploid taxa are the principal source of taxonomic uncertainties and identification difficulties in the Czech Republic and there has been a strong need to revise their taxonomy in the country. In addition to morphologically distinct *E. micrantha* Rchb., *E. frigida* Pugsley and the extinct *E. corcontica* (Smejkal) Smejkal & Dvořáková, six other taxa are recorded from the Czech Republic at this ploidy level (Smejkal & Dvořáková 2000, Dvořáková 2002). *Euphrasia stricta* J.P.Wolff ex J.F.Lehm. and *E. nemorosa* (Pers.) Wallr. are widely accepted in the recent European literature (Marhold 2011). On the other hand, *E. tatarica* auct. and *E. curta* subsp. *glabrescens* (Wettst.) Smejkal differ from *E. stricta* or *E. nemorosa* by the presence of short eglandular hairs, which are today usually included in this widely accepted species in most studies (Yeo 1971, Vitek 2005, 2011, Marhold 2011). *Euphrasia coerulea* Tausch and *E. slovaca* (Yeo) Holub are taxonomically the most uncertain species. *Euphrasia coerulea* is described from the Jizera Mts in the Sudeten (Tausch 1834, Szeląg 2014). It is usually considered as an early-flowering ecotype of *E. nemorosa*, and this concept is accepted at the species (Yeo 1978, Králik 1997, Smejkal & Dvořáková 2000) or subspecies level (Danihelka et al. 2012). However, this taxon is often considered as a part of morphological variation of *E. nemorosa* to include in this species by some authors (Vitek 2011). *Euphrasia slovaca* was described originally as a subspecies of *E. arctica* Lange ex Rostr. (Yeo 1971), which is morphologically similar to the *E. stricta* group, and some authors do not accept their separation (Hartl 1974, Krok et al. 2013). The *E. arctica* group differs from *E. stricta* by leaf and bract shape, bract teeth shape and capsule width and separation of the two groups was supported especially by Yeo (1971). Until then, the presence of glandular hairs was considered as the most important characteristic and *E. stricta* was regarded as a species without short glandular hairs, in contrast to the short-glandular pubescent *E. brevipila* Burnat & Gremli ex Wettst. (von Wettstein 1896) and *E. slovaca* (Smejkal 1963, Králik 1997).

Thus, in the recent Czech literature, the four main tetraploid species have been distinguished based on flower size, bract characteristics and indumentum, phenology and occurrence in different geographic regions of Central Europe (Smejkal & Dvořáková 2000, Dvořáková 2002). *Euphrasia stricta* and *E. slovaca* belong to species with medium-sized flowers, and *E. nemorosa* and *E. coerulea* belong to small-flowered species. *Euphrasia stricta* and *E. slovaca* have awns at the end of the bract teeth which lack in the other two species. *Euphrasia nemorosa* is more branched than *E. stricta* and its branches are thicker, while *E. slovaca* and *E. coerulea* are early-flowering species with their first flowers on lower nodes (2^{nd}–6^{th}) as well as branches that are short and thin. All of the species may occur without branches, depending on the surrounding vegetation, their vigour and growth stage.

Euphrasia stricta is distributed in most parts of Europe and is a relatively common species throughout the Czech Republic, while *E. nemorosa* can be found in mountains and hills of Atlantic and subatlantic Europe and occurs only in the suboceanic climate regions of the Czech Republic (Smejkal 1963, 1964, Smejkal & Dvořáková 2000). Due to phenotypic plasticity and a broad range of variation, there are many morphologically intermediate populations which make identification of these species problematic. Additionally, a putative hybrid between these two species, *E.* × *haussknechtii* Wettst., was described (von Wettstein 1893–1895, Yeo 1978), and the frequent occurrence of hybrid populations is reported in the Czech Republic (Smejkal 1960). *Euphrasia slovaca* is considered to be endemic to the western and Ukrainian Carpathians and is recorded in the Czech Republic only from the Moravian Carpathians (Yeo 1971, Smejkal & Dvořáková 2000). *Euphrasia coerulea* is a Sudeten-Carpathian endemic and is recorded from mountains and hills of northern Bohemia (incl. Krkonoše Mts) and from Carpathian regions (Smejkal 1964, Smejkal & Dvořáková 2000).

This study is focused on genetic and morphological variation in these four *Euphrasia* species. Molecular data were used (1) to test hypotheses of differentiation among traditionally distinguished morphotypes, (2) to test morphological

differences among genetically defined groups and (3) to test whether the presumed pollination syndromes are reflected in patterns of genetic diversity.

MATERIAL AND METHODS

Plant material

Euphrasia populations were collected in the Czech Republic, Slovakia, Poland, Austria and Germany (table 1 & fig. 1). In total, 42 populations and 398 plants (vouchers are deposited in CBFS herbarium) were sampled. After preliminary identification in the field, based on flowering time, branching, flower size and colour, presence and amount of glandular hairs (table 2) and the region of sampling, *E. stricta*, *E. nemorosa*, *E. coerulea* and *E. slovaca* were equally covered. Populations showing intermediate morphological characters were classified into one of the species based on prevailing similarity. From most of the populations, ten individuals were collected. In four very small populations (VLH2, HAVR, JAV, VKAR), only five plants were collected. From each individual, several leaves and bracts were silica-dried and taken for genetic analyses. One flower and one bract from the middle of the main inflorescence were attached to paper with transparent tape and scanned at a resolution of 600 dpi. Two plants from the BBAR population were too tiny to obtain material for both genetic and morphometric analyses. Thus, material was taken only for microsatellite analysis.

Microsatellite analysis

DNA extraction was performed using Invisorb Spin Plant Mini Kit (Invitek, Berlin, Germany) or the NaOH method (Werner et al. 2002). Eight microsatellite loci were used for this study. Five microsatellite loci (Ene1, Ene2, Ene3, Ene4 and Ene5) were amplified in 5-μL reactions using primers and PCR conditions as described by French et al. (2003), except that 2.5 μL of 2x Plain PP Master Mix (Top-Bio, Prague, Czech Republic) was used. To obtain higher resolution in the data, microsatellite loci developed by Wang et al. (2009) were tested on European *Euphrasia* species. Three loci (En-B, En-G and En-I) showed variability and were selected for this study. These loci were amplified using M13-tailed primers (Schuelke 2000). PCR contained 2.5 μL 2x Plain PP Master Mix, 0.3 μM of fluorescently labelled M13 primer and reverse primer, 0.075 μM of M13-tailed forward

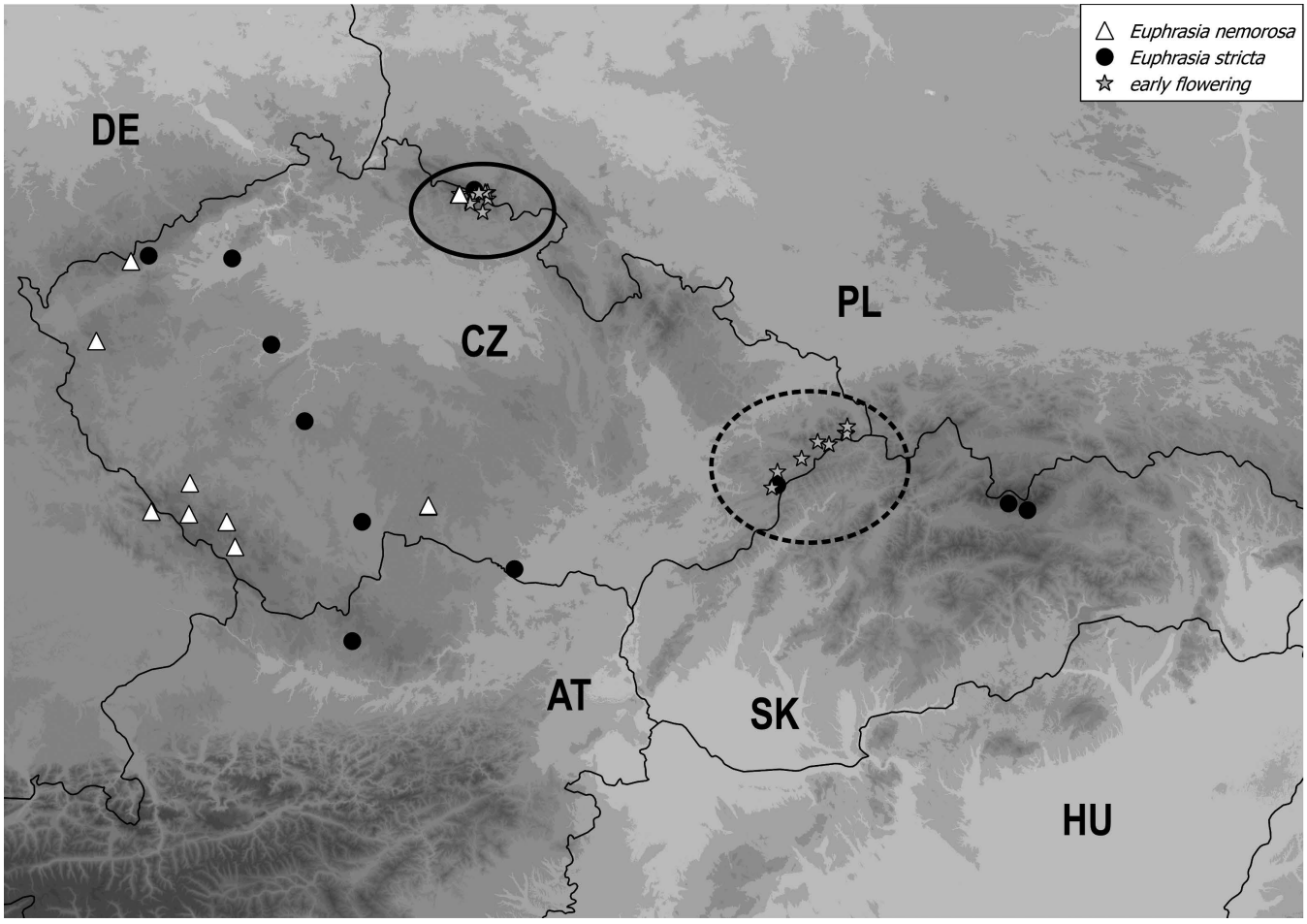

Figure 1 – Localities where the samples used in the present study were collected. *Euphrasia* populations are marked according to the results of genetic analyses. Solid ellipse indicates the Krkonoše Mts (Sudeten Mts), dotted ellipse indicates a part of the Czech Carpathians. Borders of the following central-European countries are displayed: CZ – Czech Republic, AT – Austria, SK – Slovakia, PL – Poland, HU – Hungary, DE – Germany.

Table 1 – List of *Euphrasia* populations used in this study.
Spec. prelim. = species according to preliminary identification, Genet. group = genetic group according to STRUCTURE results, N = number of individuals in population, Ng = number of multilocus haplotypes in population, D_G = diversity index, Na = number of microsatellite alleles in population.

Spec. prelim.	Genet. group	Pop ID	Country	Population	Latitude	Longitude	Altitude (m)	N	Ng	D_G	Na
E. stricta	*E. stricta*	MED	Czech Republic	Měděnec	50°25′28.5″N	13°06′40.8″E	905	10	10	1.000	23
E. stricta	*E. stricta*	BER	Czech Republic	Beroun	49°58′25.1″N	14°04′59.8″E	280	10	10	1.000	20
E. stricta	*E. stricta*	STUD	Czech Republic	Studánky	48°35′24.9″N	14°18′51,0″E	750	10	10	1.000	22
E. stricta	*E. stricta*	POP	Slovakia	Popradské pleso	49°07′24.6″N	20°04′28.6″E	1240	10	7	0.911	19
E. stricta	*E. stricta*	PODB	Slovakia	Podbanské	49°09′28.5″N	19°55′28.3″E	990	10	9	0.978	18
E. stricta	*E. stricta*	SKO	Czech Republic	Skoupý	49°34′56.2″N	14°20′51.6″E	545	10	10	1.000	22
E. stricta	*E. stricta*	RANA	Czech Republic	Raná	50°24′38.7″N	13°46′23.3″E	340	10	10	1.000	26
E. stricta	*E. stricta*	WBW	Austria	Weinsberger Wald	48°26′24.6″N	14°43′29.3″E	660	10	7	0.911	18
E. stricta	*E. stricta*	HAVR	Czech Republic	Havraníky	48°49′01.8″N	16°00′36.3″E	315	5	5	1.000	14
E. nemorosa	*E. stricta*	VO	Czech Republic	Lužnice	49°03′46.8″N	14°48′09.3″E	445	10	8	0.933	23
E. nemorosa	*E. nemorosa*	VLH2	Czech Republic	Velká Lhota 2	49°08′35.3″N	15°19′34.3″E	470	5	2	0.400	13
E. nemorosa	*E. nemorosa*	KLI	Czech Republic	Klínovec	50°23′47.0″N	12°58′15.7″E	1210	10	10	1.000	25
E. nemorosa	*E. nemorosa*	MIS	Czech Republic	Horní Mísečky	50°44′0.8″N	15°34′09.8″E	1000	10	8	0.933	16
E. nemorosa	*E. nemorosa*	HMCP	Czech Republic	Medvědín - late flow.	50°44′0.8″N	15°34′28.9″E	1035	10	3	0.644	12
E. nemorosa	*E. nemorosa*	VIMP	Czech Republic	Vimperk	49°03′44.9″N	13°43′49.2″E	900	10	9	0.978	22
E. nemorosa	*E. nemorosa*	VBOR	Czech Republic	Velký Bor	49°06′09.5″N	13°25′49.3″E	890	11	3	0.378	16
E. nemorosa	*E. nemorosa*	VLH	Czech Republic	Velká Lhota	49°08′50″N	15°19′38.7″E	600	9	2	0.222	13
E. nemorosa	*E. nemorosa*	ML	Czech Republic	Mariánské lázně	49°59′32.5″N	12°41′49.9″E	520	10	5	0.861	13
E. nemorosa	*E. nemorosa*	LEN	Czech Republic	Lenora	48°55′58.7″N	13°47′46.2″E	765	10	10	1.000	23
E. nemorosa	*E. nemorosa*	HMB	Czech Republic	Hory Matky Boží	49°15′46.3″N	13°26′16.9″E	685	10	9	0.978	20
E. nemorosa	*E. nemorosa*	JAV	Germany	Javor	49°06′57″N	13°07′59.1″E	1320	5	1	0.000	13
E. coerulea	early flow.	SML	Czech Republic	Nad Spáleným mlýnem	50°42′36.2″N	15°47′58.4″E	775	10	10	1.000	21
E. coerulea	early flow.	PJE	Czech Republic	Pod Jelenkou	50°44′29.8″N	15°47′45.0″E	1035	10	10	1.000	25
E. coerulea	early flow.	JLN	Czech Republic	Jelenka	50°44′31.5″N	15°46′41.8″E	1255	10	10	1.000	22
E. coerulea	early flow.	CHO	Czech Republic	Černá hora	50°38′33.6″N	15°45′27.8″E	1070	10	10	1.000	25
E. coerulea	early flow.	MOS	Czech Republic	Modré sedlo	50°43′39.7″N	15°41′37.4″E	1410	10	1	0.000	13
E. coerulea	early flow.	JLN2	Czech Republic	Jelenka 2	50°44′31.5″N	15°46′39.3″E	1260	10	8	0.956	20
E. coerulea	early flow.	SB	Czech Republic	Slezská Bouda 1	50°44′21.7″N	15°43′41.5″E	1390	10	1	0.000	12

Table 1 (continued) – List of *Euphrasia* populations used in this study.

Spec. prelim.	Genet. group	Pop ID	Country	Population	Latitude	Longitude	Altitude (m)	N	Ng	D_G	Na
E. coerulea	early flow.	SB2	Czech Republic	Slezská Bouda 2	50°44'20.3"N	15°43'43.1"E	1385	10	3	0.378	17
E. coerulea	early flow.	PRB	Czech Republic	Přední Renerovy boudy	50°41'42.8"N	15°39'22.6"E	1225	10	8	0.933	23
E. coerulea	early flow.	HB	Czech Republic	Husí Boudy	50°41'08.4"N	15°39'32.8"E	1040	10	9	0.978	18
E. coerulea	early flow.	HMCC	Czech Republic	Medvědín - early flow.	50°44'0.8"N	15°34'28.9"E	1036	10	3	0.378	16
E. coerulea	*E. stricta*	VST	Poland	Velký Stav	50°45'20.2"N	15°41'28.4"E	1405	10	3	0.378	15
E. slovaca	*E. stricta*	ZDE	Czech Republic	Zděchov	49°15'10.3"N	18°05'34.0"E	660	10	1	0.000	12
E. slovaca	early flow.	SIV	Czech Republic	U Sivků	49°19'17.5"N	18°05'37.3"E	640	10	3	0.378	14
E. slovaca	early flow.	HLU	Czech Republic	Horní Lomná - Úpalený	49°31'18.3"N	18°37'56.2"E	605	10	8	0.822	17
E. slovaca	early flow.	PREL	Czech Republic	Přelač	49°31'0.7"N	18°38'31.3"E	780	10	4	0.711	15
E. slovaca	early flow.	BBAR	Czech Republic	Baraní	49°27'42.0"N	18°30'5.8"E	575	8	8	1.000	27
E. slovaca	early flow.	KAM	Czech Republic	Pod Kamenitým	49°33'16.9"N	18°38'51.3"E	685	10	9	0.978	19
E. slovaca	early flow.	HUT	Czech Republic	Staré Hamry	49°28'32.2"N	18°24'44.3"E	535	10	10	1.000	24
E. slovaca	early flow.	VKAR	Czech Republic	Velké Karlovice	49°23'20.9"N	18°17'03.5"E	715	5	3	0.700	18
E. slovaca	early flow.	LUZ	Czech Republic	Lužná	49°14'18.5"N	18°2'55.9"E	660	10	5	0.667	16

primer, 0.4 µL of DNA template, and sterile water to a final volume of 5 µL. PCR conditions for all three loci were as follows: 3 min of initial denaturation at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 50°C and 30 s at 72°C, then followed by 8 cycles of 30 s at 94°C, 30 s at 46°C and 30 s at 72°C, and then a final extension step for 10 min at 72°C. PCR products were pooled and subjected to fragment analysis (SEQme s.r.o., Dobříš, Czech Republic).

The lengths of microsatellite alleles were read using GeneMarker ver. 1.80 (SoftGenetics, LLC, USA) and scored as dominant data (0/1) because all studied species are tetraploids and allelic scoring was not possible. The genetic structure was inferred by a Bayesian clustering approach using STRUCTURE 2.3.3 (Pritchard et al. 2000) at MetaCentrum VO (https://metavo.metacentrum.cz/). Both admixture with no prior information and correlated allele frequencies and no admixture models with no prior information and uncorrelated allele frequencies were used. The burn-in period was 200,000 and 1,500,000 iterations were run afterwards. Analyses were performed for K from 1 to 10 with 20 replicate runs for each K. The optimal number of clusters K in the dataset was selected using the methods described by Evanno et al. (2005) using Structure Harvester (Earl & vonHoldt 2012). Moreover, the analysis of population subsets of only the populations morphologically classified as *E. slovaca* and *E. coerulea* was performed.

Jaccard's coefficient was used to calculate the distance matrix for principal coordinate analysis (PCoA), which was computed in Canoco for Windows ver. 5 (ter Braak & Šmilauer 2012). STRUCTURE clustering was independently displayed in PCoA. The number of microsatellite alleles and the number of multilocus genotypes per population and shared genotypes were calculated using Arlequin 3.5.1 (Excoffier & Lischer 2010). Genotype diversity was estimated as a modification of the Simpson's index (Pielou 1969, Berg & Hamrick 1994): $D_G = 1 - \Sigma\, n_i(n_i - 1)/N(N - 1)$, where ni is the number of individuals of genotype i and N is the total number of individuals in population. Differences among groups delimited by STRUCTURE in the genotype diversity index and the number of alleles per population were tested by non-parametric Kruskal-Wallis one-way analysis of variance using Statistica 12 (StatSoft 2001), as well as the correlation between corolla length and the genotype diversity index.

Morphological analysis

In total, twenty characters were measured on each plant (table 3 & fig. 2). Software tpsDig ver. 2.10 (Rohlf 2006) was used for measurements of the scanned material. The normality of traits was checked visually on histograms. The character "number of branching nodes", which distribution markedly deviated from normal, was log-transformed. The correlation between traits was examined with the use of Pearson's correlation coefficients. The main components of variation were evaluated using principal component analysis (PCA). To find out which characters significantly separated groups defined by the microsatellite analysis, canonical discriminant analysis (CDA) with forward selection of traits was applied. The threshold significance level was set to $\alpha = 0.05$ with Bonferroni correction and a Monte-Carlo permutation

Table 2 – Characters used to identify species in the field.

Species	Seasonal type	Branches	Flower size	Flower colour	Glandular hairs
E. stricta	late	erect or divergent	6–10 mm	lilac to white	none
E. nemorosa	late	frequent, thick, ascending	5–7 mm	white to lilac	none
E. slovaca	early	short or none	6–8.5 mm	deep lilac to white	many
E. coerulea	early	short or none	5–7 mm	purple to white with lilac upper lip	infrequent

Table 3 – List of characters studied in the morphometric analysis.
The accuracy of the measurements was on one decimal place.

ID	Description of character	Unit
V2	height of plant to the first flower	cm
nodes	number of nodes up to first flower	count
nodes_br	number of branching nodes	count
CL	corolla length	mm
CTL	corolla tube length	mm
CTW	corolla tube width	mm
CH	corolla height	mm
UCL	length of upper corolla lip	mm
CLU	length of side of lower corolla lip	mm
CLL	length of lower corolla lip	mm
CLW2	1/2 of width of lower corolla lip	mm
LCD	diagonal of lower corolla lip	mm
BL	bract length	mm
BW	bract width	mm
BD	distance from the widest point of the bract to its base	mm
BT3W	width of third tooth on bract	mm
BT3L	length of third tooth on bract	mm
BT3O	length of awn of third tooth on bract	mm
BTLW	width of terminal tooth on bract	mm
BTLL	length of terminal tooth on bract	mm
BTLO	length of last of terminal tooth on bract	mm

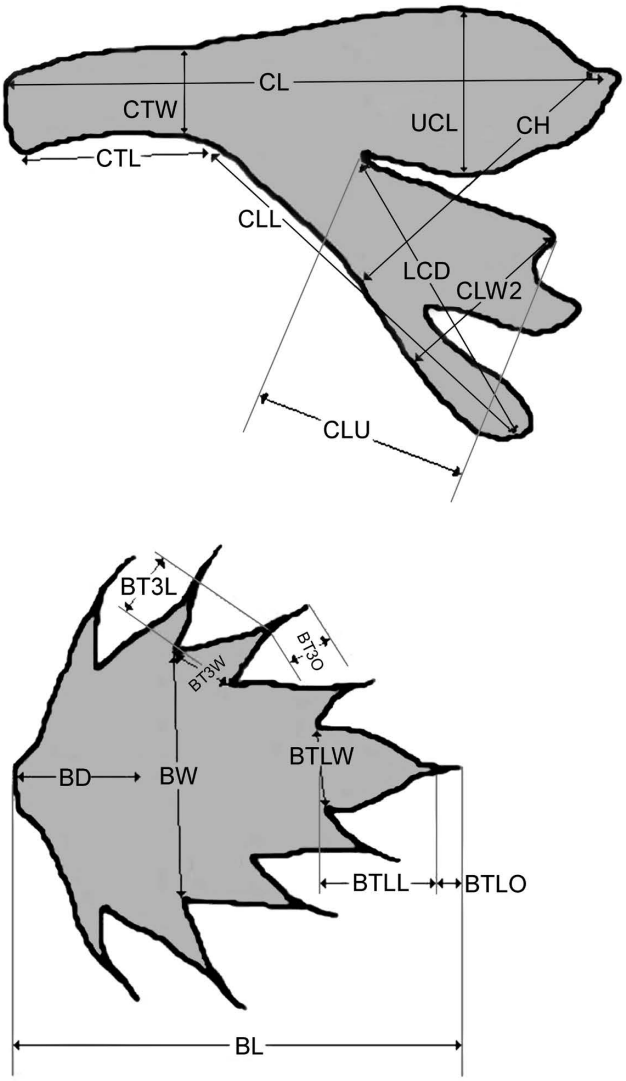

Figure 2 – Characters measured on flowers and bracts. Abbreviations are explained in table 3.

test (999 permutations) was used. Classificatory discriminant analysis was performed with cross-validation using population as the leave-out unit. All analyses were performed using MorfoTools scripts in R 3.1.2 (R Core Team 2014, Koutecký 2015) except for PCA and CDA, which were computed using Canoco for Windows ver. 5 (ter Braak & Šmilauer 2012).

RESULTS

Microsatellite analysis

A total of 63 alleles were detected across the eight analysed loci (twelve from Ene1, eleven from Ene2, twelve from Ene3, four from Ene4, four from Ene5, thirteen from En-B, two from En-G and five from En-I). All microsatellite loci were polymorphic across the whole data set. In one group of populations (*E. stricta*, see the group definition below), all loci were polymorphic. In the other two groups (*E. nemorosa* and early flowering group), the En-G locus was monomorphic with a single exception (LEN).

STRUCTURE clustering (both the no-admixture and admixed models) revealed an optimal separation of the dataset into three groups (fig. 3, data shown for no-admixture model), instead of the expected four (DeltaK for four clusters was considerably lower, electronic appendix 1). Most of the populations that were preliminarily identified as late flowering *E. stricta* and *E. nemorosa* formed separate clusters

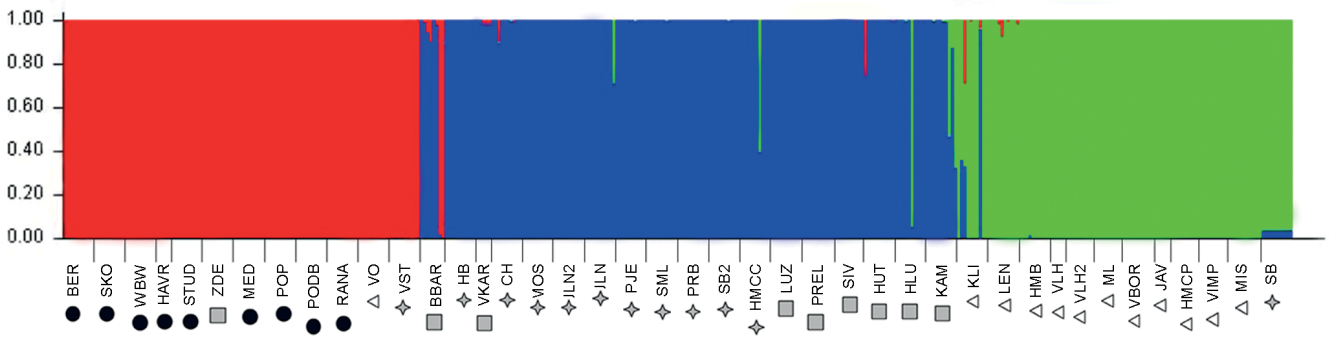

Figure 3 – Bar plot of the three genetic groups (*E. stricta*: red, *E. nemorosa*: green, early flowering populations: blue) detected by STRUCTURE. Original identification of populations is depicted with symbols (circle = *E. stricta*, square = *E. slovaca*, star = *E. coerulea*, triangle = *E. nemorosa*).

while early flowering populations corresponding to *E. coerulea* and *E. slovaca* formed one common cluster despite their provenance from two geographically distant regions. In the analyses resulting in four genetic groups, the early-flowering populations were divided into two subgroups not corresponding to morphology, ecology or geographic distribution. The same result was obtained from the analysis of the subset of early-flowering populations only (results not shown). In *E. stricta*, populations were uniform and all individuals were clearly assigned to *E. stricta* in all STRUCTURE runs. Only few populations of the rest of the *Euphrasia* taxa included in the study comprised individuals from different molecular clusters. In *E. nemorosa*, the population VO was competely assigned to *E. stricta* based on microsatelite analysis, the population KLI was admixed with both the early-flowering group and *E. stricta* group, and three other populations showed minor admixture. In the early-flowering group, more populations were slightly admixed either with *E. stricta* or *E. nemorosa* (fig. 3).

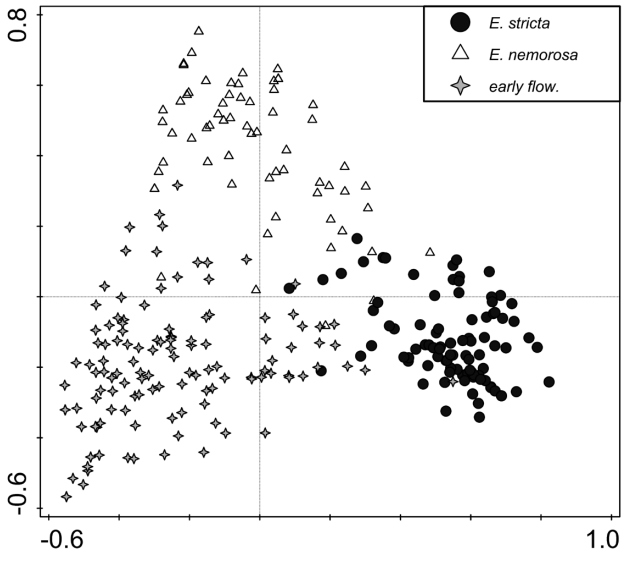

Figure 4 – Principal coordinate analysis (PCoA) of variability in microsatellites of 398 *Euphrasia* individuals based on the Jaccard coefficient. The first, second and third axis explained 15.78%, 11.3% and 6.27%, respectively, of the total observed variability. First and second ordination axes are shown.

The three genetic groups were also distinct in the PCoA plot (fig. 4). The first three axes explained 33.35% of variability in the microsatellite data. *Euphrasia stricta* was placed on the right side of the ordination plot, while *E. nemorosa* and early-flowering populations were on the left. The second axis further separated *E. nemorosa* from the early-flowering populations. The separation of all three clusters was not complete and implied shared alleles in several populations from different genetic groups. Part of this overlap was caused by the admixed populations identified by STRUCTURE (fig. 3). In most cases, individuals from particular populations formed rather compact clusters. However, some populations showed higher intrapopulation variation and individuals were scattered over a larger part of the ordination diagram.

Genetic diversity parameters of populations are shown in table 1. There was apparent variation among populations in a number of genotypes per population as well as genetic diversity.

Kruskal-Wallis one-way ANOVA revealed no significant differences among the three genetic groups in the number of alleles per population and the diversity index ($p > 0.5$). No significant correlation was revealed between corolla length and diversity index (Spearman $r = 0.2$, $p > 0.05$). Most populations of *E. stricta* had a high diversity index (> 0.9), while only two had a low diversity index (0 and 0.67 respectively). Most of the early-flowering populations showed a high diversity index. In contrast, the proportion of populations with a high diversity index and a low diversity index was rather balanced in the *E. nemorosa* group.

Except for a few exceptions, multilocus genotypes were private for all populations except two geographically very close populations of *E. nemorosa* (MIS, HMCP), which shared one multilocus genotype. Another two geographically close "early-flowering" *E. coerulea* populations (PRB and HB) shared two multilocus genotypes. One of these populations from the Krkonoše Mts (PRB) shared one multilocus genotype with a remote *E. slovaca* population from the Carpathians (HUT).

Table 4 – Results of canonical discriminant analysis of the three genetic groups of *Euphrasia* individuals with forward selection of the characters.
Characters with significant conditional effect (i.e. the effect of the variable in addition to other variables already included in the model) are listed. Explained% = part of the total variation explained by the individual variable, P = Bonferonni-corrected significance level, CorE scores = correlations with axes of the canonical discriminant analysis. Marg. – characters with significant marginal effect (i.e. when the variable is alone in the model) but insignificant conditional effect. marg.: nodes_br, CTL, UCL, CLW2, BL, BD, BT3W, BT3L, BTLO.

Character	Explained%	P	CorE scores	
			Axis 1	Axis2
CL	21.0	0.001	0.5923	-0.2625
nodes	20.1	0.001	0.2710	0.5669
BTLW	4.6	0.001	-0.2502	-0.4336
BT3O	3.6	0.001	0.5189	-0.2164
LCD	2.9	0.001	0.4107	-0.4072
BTLL	1.7	0.001	0.0910	-0.2852
CTW	1.7	0.001	0.5563	-0.0797
V2	1.1	0.001	0.1905	0.0819
BW	1.0	0.002	-0.0662	-0.2195

Morphological analysis

Based on the high correlation (Pearson coefficient > 0.85) between characters, three of them (CH, CLU and CLL), which correlated with CL, CLW2 and LCD, respectively, were excluded from further analyses.

Principal component analysis (PCA) of both, populations and individuals, revealed a rather poor structure of morphological variation in our dataset (results not shown). The canonical discriminant analysis (CDA) of populations revealed good morphological separation of the three clusters defined by STRUCTURE when all traits were included (fig. 5). Forward selection identified four characters, corolla length (CL), diagonal of lower corolla lip (LCD), number of nodes up to the first flower (nodes) and the width of the terminal tooth on bract (BTLW), which were sufficient for separation of the groups.

Although CDA of individuals showed morphological separation of the three groups as well, there was some overlap between the groups (fig. 6). The overlap between *E. stricta* and *E. nemorosa* was larger than the overlap of the early-flowering group and *E. stricta* or *E. nemorosa*. Forward selection identified nine characters that contributed most to the separation of the three groups (table 4).

The ability of all of the nine selected traits to make correct identifications was tested by classificatory discriminant analysis. In general, more than 78% of individuals were correctly classified (table 5). The incorrect identification was not evenly spread; most of the populations had more than 90% correct classifications. When there was misclassification in the *E. stricta* or *E. nemorosa* population, they were classified mostly as the early-flowering group. In three populations, PODB, VO and ZDE, classification was very poor and only 30% or less was successful. The detailed results for each population are listed in electronic appendix 2.

Discriminant analysis was also performed for the group of early-flowering populations only, as they come from different mountain ranges. The analysis revealed good morphological separation of plants from these regions (table 6, fig. 7), but this did not correspond to the genetically delimited groups. Characters selected by forward selection as the most suitable for discriminating these two groups of populations were the number of nodes, the number of branching nodes (but in a different range than the one that differentiates between aestival and autumnal taxa), the length of the terminal tooth on bract and corolla length.

DISCUSSION

Genetic structure

Based on preliminary identification, ecology and distribution of studied populations, we expected four genetic groups. However, our results based on microsatellite data revealed a clear division of the studied populations into three groups only, predominantly late-flowering *E. stricta*, late-flowering *E. nemorosa* and the early-flowering group formed by merging *E. coerulea* and *E. slovaca*. Genetically defined groups of *E. stricta* and *E. nemorosa* populations corresponded mostly with the preliminary identification of both species. The presence of short eglandular pubescence recorded in some studied populations of *E. stricta* and *E. nemorosa* have no genetic support and this result is in concert with the recent inclusion of central European populasions named as *E. tatarica* and *E. curta* in *E. stricta* or *E. nemorosa*, respectively (Vitek 2011, Krok et al. 2013).

Table 5 – Classificatory discriminant analysis of the three *Euphrasia* groups.
Number and percentage of correctly classified individuals for each of the three groups are given. Data obtained from CDA with all characters (78.47% correct) and CDA with characters after forward selection (78.75% correct) are shown.

	CDA with all characters			CDA with characters after forward selection		
	early flow.	*E. nemorosa*	*E. stricta*	early flow.	*E. nemorosa*	*E: stricta*
early flow.	141 (91.6%)	8 (5.2%)	5 (3.2%)	138 (89.6%)	9 (5.8%)	7 (4.6%)
E. nemorosa	11 (11.2%)	69 (70.4%)	18 (18.4%)	13 (13.3%)	74 (75.5%)	11 (11.2%)
E. stricta	21 (18.3%)	16 (13.9%)	78 (67.8%)	23 (20%)	15 (13%)	77 (67%)

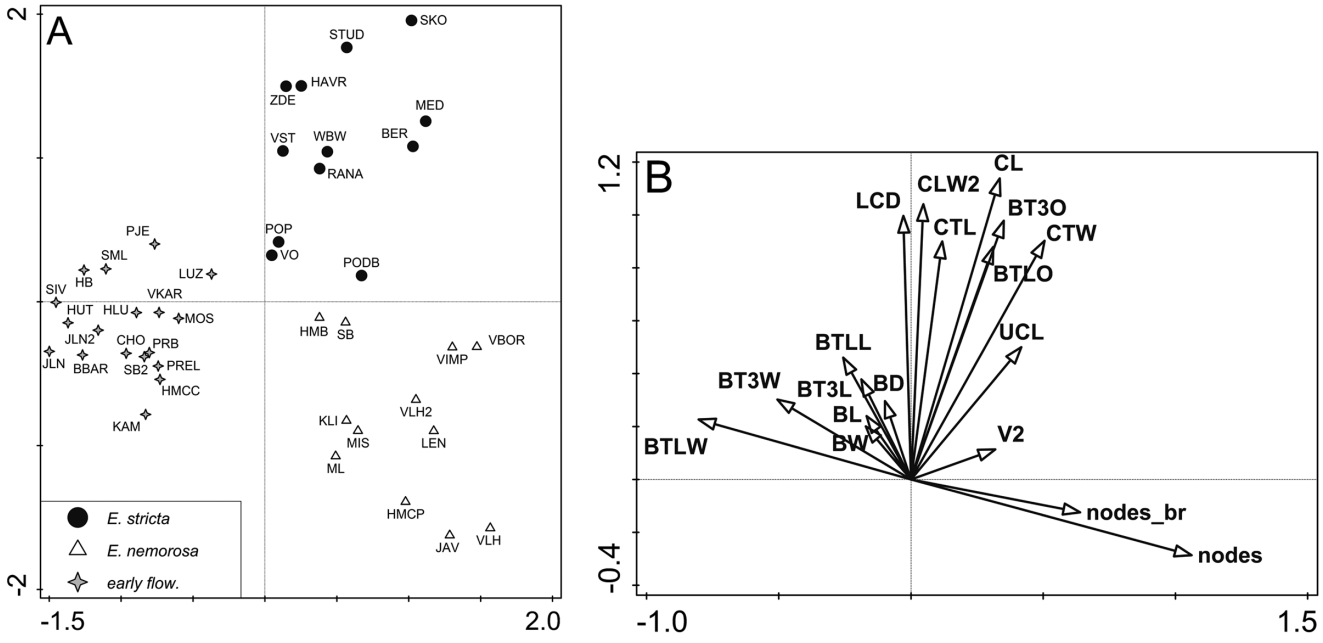

Figure 5 – Results of canonical discriminant analysis (CDA) of populations (A) and all measured characters (B) of the three genetically defined groups of studied *Euphrasia* samples. The first and second axes explained 79.50% of the total observed variability. The first and second ordination axes are shown.

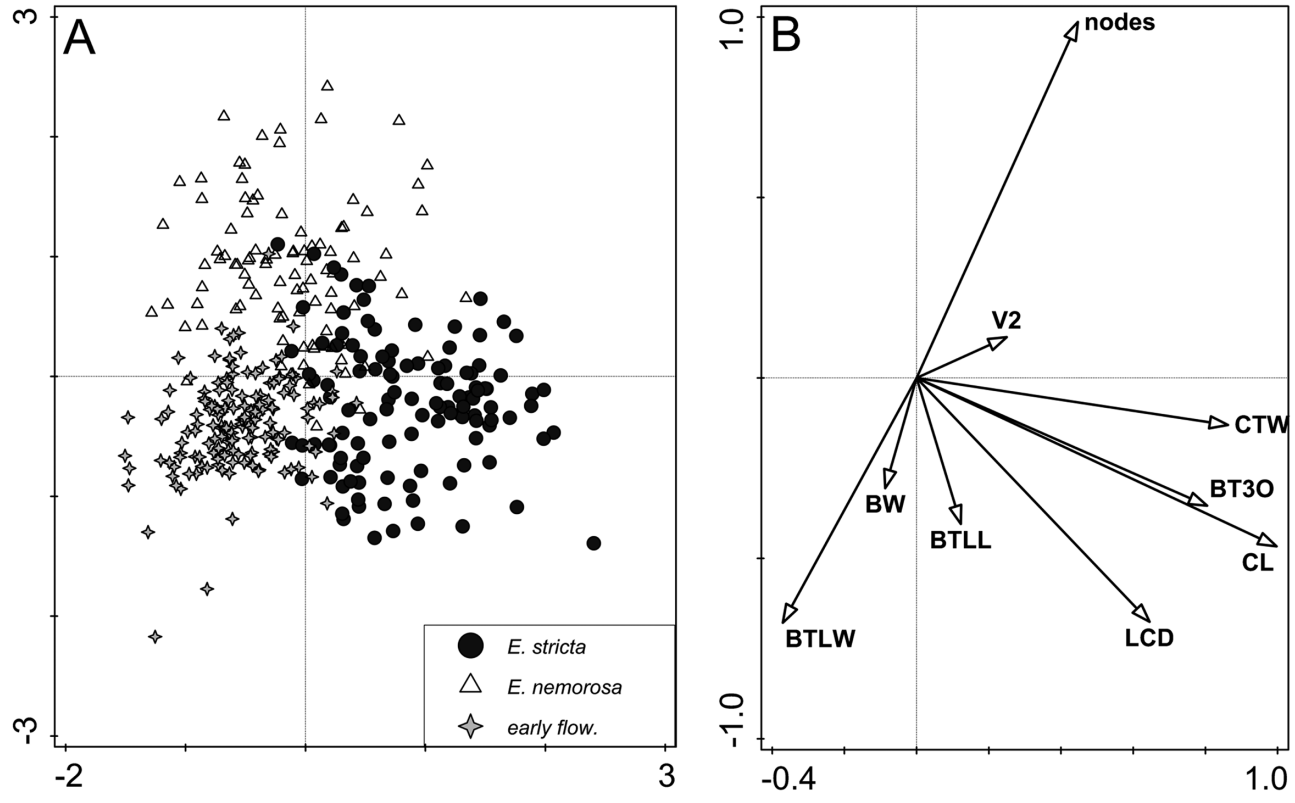

Figure 6 – Results of CDA of individuals (A) and all measured characters (B) of the three genetically defined groups of the studied *Euphrasia* samples. The first and second axes explained 57.67% of the total observed variability.

Table 6 – Classificatory discriminant analysis of the early flowering *Euphrasia* group.
Number and percentage of correctly classified individuals to the mountain range from which they originate are given. Data were obtained from CDA with characters after forward selection (93.54% correct).

	Krkonoše Mts	Carpathians
Krkonoše Mts	95 (95%)	5 (5%)
Carpathians	5 (9.1%)	50 (90.9%)

The third genetic group comprised early-flowering populations both from the Carpathians and most of the populations from the Krkonoše Mts Populations in the Carpathians occur in mowed meadows and along forest paths and are characterized by short glandular pubescence of bracts, although its density may vary considerably. The typical habitat of populations from Krkonoše Mts consists of road edges and subalpine localities. The indumentum of plants differed as well and the occurrence of glandular hairs on bracts was rare.

Differences in the presence of short glandular hairs between Sudeten populations (Krkonoše Mts) and Carpathian populations of *E. coerulea* were observed by Smejkal (1963). Moreover, the rare presence of glandular hairs was observed in many populations of *E. stricta* from different regions included in this study, which indicates variation in this character and the necessity of performing another study in regions where glandular populations classified as taxa from the *E. stricta* assemblage are common (see below). On the other hand, detection of a shared multilocus genotype between Krkonoše Mts and Carpathians suggests close genetic relationships between morphologically diverse populations from geographically separated areas.

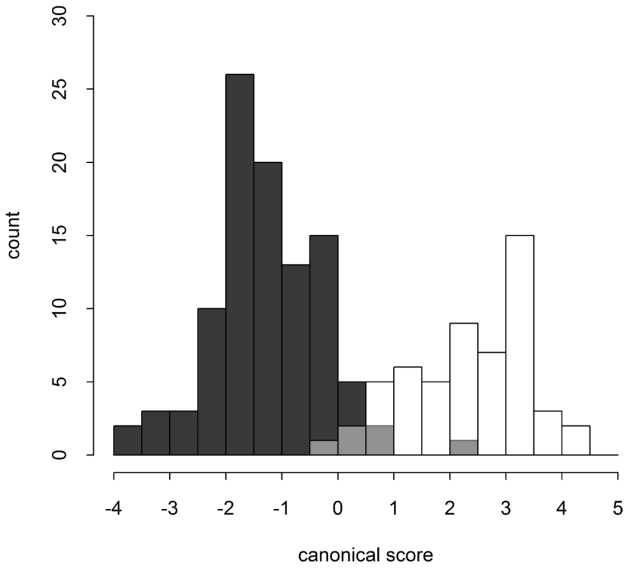

Figure 7 – Histogram of canonical scores of CDA of individuals of the early-flowering genetic group (dark grey = Krkonoše Mts, white = Carpathians; pale grey = overlap of both). Only characters chosen by forward selection were used.

The separate position of early-flowering populations is in opposition to the inclusion of early-flowering populations of *E. coerulea* in *E. nemorosa* in the recent treatment of *Euphrasia* in Germany (Vitek 2011). Smejkal (1963) considered *E. coerulea* as an early-flowering vicariant of *E. nemorosa* as well. However, unification of most of the studied early-flowering populations is an important change for a better taxonomic understanding of this group. Most of the early-flowering populations from the Carpathians have been recently identified as *E. slovaca*. *Euphrasia coerulea* is recorded as an extremely rare species from this region (Dančák 2011). The reality seems to be completely different. Most of the early-flowering populations with relatively small flowers belong to *E. coerulea*. Only one population (ZDE) with early phenology and distinctly larger flowers was classified as *E. stricta*, but was not a separate group. The case of *E. slovaca* is more complex, and our data are not sufficient to resolve this. Although this taxon was described from the Ukraine, the author recorded its occurrence from the Moravian Carpathians (Yeo 1978). Plants from the population ZDE morphologically resemble plants of the type specimen of *E. slovaca* (deposited in herbarium PRC). There is the principal question of the relation between *E. stricta* and *E. arctica* accepted by Yeo (1971) and other similar taxa with glandular hairs – e.g. *E. brevipila*, which also includes *E. slovaca* (Posz 2014). The other taxon that should be studied is *E. chitrovoi* Tzvelev, which was described from northwestern Russia, but is supposed to occur in Central Europe as well (Tzvelev 1980). All these questions must be the topic of separate studies with a large sampling area especially in Northern Europe and in the Carpathians.

Hybridization

Despite general expectations, hybridization was surprisingly rare in the studied populations. Only one population from the *E. nemorosa* group (LEN) contained several alleles typical for *E. stricta*. We should consider possible hybridization with some *E. stricta* populations that grow nearby. A quite good marker of introgression is the En-G locus, which was polymorphic in the *E. stricta* and LEN populations, but monomorphic in the rest of the *E. nemorosa* populations. A trigger of hybridization between populations could be the destruction of natural barriers caused by human activities. This might frequently be the case in mostly ruderal places and on downhill courses in ski areas connecting populations from different altitudes. We suppose this is the case in the most admixed population of the whole dataset, KLI. In spite of this, our results suggest that hybridization between *E. stricta* and *E. nemorosa* occurs much less often in the area of interest than stated in the literature (Smejkal 1963, Smejkal & Dvořáková 2000). Hybridization in the early-flowering group does not seem to be an important phenomenon, because of the relatively small number of populations with an indication of admixture of other species. The presence of markers of *E. stricta* in predominantly early-flowering BBAR population can be explained by the occurrence of non-flowering plants, which probably belonged to *E. stricta*. Actually, if there was a former hybridization event, genes of one paren-

tal species could prevail and populations could be identified rather easily as one of the parental species. Rapid population dynamics and the prevalence of markers of one parental species was detected in hybrid populations of *Rhinanthus minor* and *R. angustifolius* (Ducarme et al. 2010).

Morphological characters separating genetic clusters

The results obtained from classical morphometric analysis of populations showed separation of the three genetic groups (fig. 4). However, there were large overlaps in the morphology of all three groups caused by considerable morphological variability within populations (figs 5 & 6) and phenotypic plasticity (Karlsson 1976). Classificatory discriminant analysis gave a correct morphological identification of most of the populations, although some populations showed very low success of correct identification to genetic clusters.

The most reliable morphological characters delimiting the early-flowering group were the width of the terminal tooth on bract (BTLW) and the number of nodes under the first flower (nodes). These traits were generally used to distinguish early-flowering plants from the late-flowering plants by previous authors (Smejkal & Dvořáková 2000). Populations of *E. stricta* have the largest flowers of all of the studied populations and thus corolla length (CL) and diagonal of lower corolla lip (LCD) were the best characters to discriminate this species. However, plants from several populations of *E. stricta* with unusually small flowers (e.g. PODB, VO) were often misclassified in the classificatory discriminant analysis. The existence of small-flowering populations of *E. stricta* was mentioned by Smejkal (1963) and Karlsson (1976). This is probably the case with population VO, which was wrongly classified in the field. Similarly, misclassification of some plants from two large-flowering populations of *E. nemorosa* was detected.

Another important diagnostic characteristic, especially for *E. stricta* and *E. nemorosa*, is the presence of awns. Although most authors stated that *E. nemorosa* does not have awns (Smejkal 1963, Stace 1997), other authors (e.g. Pugsley 1930, Hartl 1974 or Yeo 1978) admitted that awns might be present. This confusion is caused by a different species concept that includes other species in *E. nemorosa*. However, in spite of following Smejkal's (Smejkal & Dvořáková 2000) species concept, some of the studied *E. nemorosa* populations had awns on bracts. We observed awns most frequently on the lowermost two or three bract teeth. However, awn length in *E. nemorosa* was less than in *E. stricta*.

There were several other populations with very low success of correct identification. The population ZDE was the most challenging. Classification in the early-flowering population is in agreement with a preliminary determination based on general habitat. However, this population belongs to *E. stricta*. Early-flowering populations of *E. stricta* are common in North Europe and some seasonal intraspecific taxa are distinguished there. These taxa are extremely rare in Central Europe, and in spite of the two subspecies (*E. stricta* subsp. *stricta* and *E. stricta* subsp. *suecica* (Murb. & Wettst.) Wettst.) being accepted for the Czech Republic (Smejkal 1963), no subspecies have been recorded in a recent Czech flora (Smejkal & Dvořáková 2000, Dvořáková 2002). However, this issue must be the other topic of a large study of *E. stricta* resemblance.

Morphological convergence in extreme climatic conditions can be another reason for misclassification in some populations. This may be the case in some populations from a mountain ridge (VST, SB), where all populations looked very similar, but they were clustered in different genetic groups than most of the populations from high altitudes of the Krkonoše Mts Gene flow between nearby growing populations can be another reason for morphological differences from typical populations. However, corolla colour seemed to be a useful character distinguishing between *E. nemorosa* and the early-flowering group in this region. On the other hand, the corolla colour is usually stated to be variable in many species (Smejkal 1963, Yeo 1978, Smejkal & Dvořáková 2000) and the situation may be different in other parts of the distribution area of this species.

The apparent morphological separation we found between genetically identical early-flowering populations from the Carpathians and Krkonoše Mts could be explained by the phenomenon of seasonal dimorphism. It is known that different seasonal types of hemiparasitic plants look morphologically distinct in spite of genetic similarity. Different seasonal types can differ not only in the number of nodes and branches but also in bract shape and flower size (von Sterneck 1901, von Soó 1926–1927). One of the important differences between populations from the Carpathians and Krkonoše Mts is the number of nodes which indicates different seasonal types. Populations from Krkonoše Mts had a higher number of nodes, which correlated with a later flowering time in this region. However, it seems that these morphotypes do not differ genetically.

Population diversity

Kolseth & Lönn (2005) observed a stronger divergence among populations than among morphologically and ecologically defined varieties of *E. stricta* in Gottland based on AFLP. The population structure of our dataset showed a similar pattern. Multilocus genotypes were virtually unique for each population. Shared multilocus genotypes were present only in three pairs of populations. In two cases, this was probably caused by seed transport along a road because these populations are in close geographical contact.

We found no significant differences in diversity index values and number of alleles per population among genetic groups of *Euphrasia*, which are likely to differ in their prevailing mode of reproduction. On the other hand, non-significant results might imply a more complex scenario that includes local factors that influence genetic variation. The highest values were found in *E. stricta* (table 1), which has the largest flowers of the species included in our study. It was supposed that this type of flowers favours outcrossing, while small-flowered species rely more often on autogamy (Vitek 1998, Gómez 2002, French et al. 2005). However, there were populations of *E. stricta* with an extremely low diversity index value (ZDE and VST). The population VST was located in the extreme climatic conditions of the mountain ridge, which may cause lack of pollinators and favour autogamy. This phenomenon could underlie the obvious reduction in

multilocus genotype numbers in populations of other species in this area. In addition to a potentially autogamous reproduction, the extremely small population size in the ZDE population caused by a bottleneck or resulting from inbreeding could be a reason for the low variation, as reduction of diversity in small populations is well-known (Young et al. 1996). In most small-flowered *E. nemorosa*, the populations usually had a reduced number of multilocus genotypes, especially in small populations (JAV, VLH2). However, the population size itself is not the only factor influencing variability in the population. There were several small-sized populations with high diversity index values, as well as large populations with low values. Another process reducing genetic diversity could be a strong directional selection pressure (Lacy 1987). This may be the case of early-flowering populations in mowed meadows in the Carpathians. Many populations there were quite homogeneous and consisted of one multilocus genotype. This situation suggests adaptation of local populations to management. If the type of management changes, these populations probably go extinct locally or rapidly change their behaviour. Several populations with large variability were found in the Carpathians as well. These populations were usually found along roads, which enables gene exchange or, as in the case of the BBAR population, were mixed with *E. stricta*.

CONCLUSION

Contrary to the traditional view, we detected only three genetically defined groups of tetraploid *Euphrasia* populations in our dataset. The species *E. stricta* and *E. nemorosa* are rather well morphologically defined and hybrid populations are not as frequent as it has been supposed. The best differences are the traditionally used corolla length and awn presence. Populations with an atypical flower size can also be found, which can complicate identification. On the other hand, there is very good support for the distinction of early-flowering populations as a separate species *Euphrasia coerulea*. In general, the number of nodes is a more useful characteristic for identification than the presence of glandular hairs, which is typical for Carpathian populations only. These populations differ from genetically similar populations from the Krkonoše Mts in the number of nodes, which was lower. However, there are large overlaps in the morphology of all three groups caused by considerable morphological variability within populations and the fact that different species react in the same way to habitat conditions. The correct identification may be very difficult due to the rare occurrence of hybrids or introgression, but especially the parallel origin of morphologically similar types as a reaction to habitat conditions. No significant differences in population genetic diversity among species differing by flower size were found.

More exhaustive research must be performed to understand the relationships and taxonomy of *E. stricta* resemblance outside of study area, where other morphotypes (especially with glandular hairs and aestival characteristics) occur.

SUPPLEMENTARY DATA

Supplementary data are available in pdf at *Plant Ecology and Evolution*, Supplementary Data Site (http://www.ingentaconnect.com/content/botbel/plecevo/supp-data), and consist of: (1) structure Harvester (Earl & vonHoldt 2012) summary of STRUCTURE results for whole microsatellite dataset of *Euphrasia* species, and (2) classificatory discriminant analysis of particular *Euphrasia* populations into the three genetic groups.

ACKNOWLEDGMENTS

We would like to thank our reviewers for considerable improvement of the manuscript. We are also very grateful to Josef and Ludmila Harčarikovi for their help in the field and to the Krkonoše Mountains National Park for enabling us to sample in protected areas. This study was financially supported by Grant Agency of the University of South Bohemia in České Budějovice (project 075/2013/P) and by and the Czech Science Foundation (project 14-36079G, Centre of Excellence PLADIAS). Computational resources were provided by the MetaCentrum under the program LM2010005 and the CERIT-SC under the program Centre CERIT Scientific Cloud, part of the Operational Program Research and Development for Innovations, Reg. no. CZ.1.05/3.2.00/08.0144.

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Manuscript received 4 Apr. 2015; accepted in revised version 6 Jan. 2016.

Communicating Editor: Renate Wesselingh.