Preslia 94: 275–304, 2022
doi: 10.23855/preslia.2022.275

Preslia

Pinguicula vulgaris in central Europe: when does one species turn into another?

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Abstract. *Pinguicula vulgaris* is one of the most common carnivorous plants in the Northern Hemisphere. It is a member of the species complex (*P. vulgaris* agg.) of which in Europe six taxa are recognized at different taxonomic ranks. One of them is *P. bohemica*, a taxon considered endemic to the Czech Republic, for which there are varied reports on its chromosome number (ploidy level) and contrasting opinions on its taxonomic rank. Based on the persistent instability of the taxonomy of this group in central Europe, we investigated the ploidy level, genome size, seed structure and genetic differentiation of taxa of *P. vulgaris* agg. in order to resolve the questions connected with *P. bohemica.* We sampled all taxa currently recognized as members of *P. vulgaris* agg. in Europe. Karyological analysis supported the octoploid level for *P. bohemica* and all other taxa, and flow cytometry further supported cytotype uniformity. Although generally conservative, the absolute genome size differentiates the morphologically similar taxa in *P. vulgaris* agg. Molecular markers suggested the presence of distinct groups within the central European populations of *P. vulgaris* agg., where the groups correspond to already recognized or even unrecognized taxa at the infraspecific level. We identified another lineage from southern Poland within the *P. vulgaris* group (with provisional name *P.* "*polonica*"). Most probably it is a direct descendant of the *P. bohemica* lineage, which diversified under isolation. The taxa *P. bohemica* and *P.* "*polonica*" are genetically well differentiated from *P. vulgaris* s.s. and *P. vulgaris* var. *bicolor*. The molecular-genetic differentiation of *P. bohemica* is comparable to narrowly endemic taxa from central Italy. Spatial isolation seems to be an important force causing the gradual divergence of isolated lineages within the *P. vulgaris* agg. Considering all the distinctive features, we suggest a subspecific category for *P. bohemica*, thus *P. vulgaris* subsp. *bohemica.*

Keywords: AFLP, carnivorous plants, cpDNA, genome size, geographic isolation, ITS, *Pinguicula bohemica*, *Pinguicula vulgaris*

Received: 10 May 2021, Revised: 16 Feb 2022, Accepted: 19 Mar 2021, Published online: 13 Jun 2022

Introduction

Europe is home to almost 30 species of butterworts (*Pinguicula* L.), which is far more than the number of any other genus of carnivorous plants on the continent (Roccia et al. 2016, Crespo et al. 2020). Most of the species are concentrated in the Mediterranean region, with many of them being endemic to a particular area or even to a single locality (Zamora et al. 1996, Bacchetta et al. 2014). Farther north, only widespread species are found (*Pinguicula vulgaris* L. being the most notable example), the endemic species suddenly do not occur and none of them occur in the northern Alps or anywhere in the Carpathians (Mrkvicka 1990, Studnička & Hejný 1992, Conti & Peruzzi 2006). The only exception to this pattern is *P. bohemica* Kraj., which, over time, has sometimes been considered a "good" species.

Pinguicula bohemica belongs to the subgen. *Pinguicula* sect. *Pinguicula* (Casper 1963) and within it to *P. vulgaris* agg. Except for *P. bohemica*, this group consists of the widespread *P. vulgaris* s. str., *P. vulgaris* var. *bicolor* Nordst. ex Fr. (found scattered across the European range of *P. vulgaris*) and three narrowly endemic subspecies of *P. vulgaris* occurring in central Italy (*P. vulgaris* subsp. *anzalonei* Peruzzi et F. Conti, *P. vulgaris* subsp. *ernica* Peruzzi et F. Conti, *P. vulgaris* subsp. *vestina* F. Conti et Peruzzi) (Fig. 1). *Pinguicula bohemica* was described by Vladimír Krajina (Krajina 1927) from the Hrabanovská černava fens near Lysá nad Labem in central Bohemia, Czech Republic. It differs from *P. vulgaris*, the only other species of *Pinguicula* native to Bohemia, in the colour of its flowers, which is whitish with a blue-violet throat. However, flowers of *P. vulgaris* var. *bicolor* are very similar in floral colouration (Fig. 2, Table 1). Therefore, *P. bohemica* has always been considered highly questionable and rarely recognized as a distinct species by foreign authors, including Jost Casper, the monographer of the genus *Pinguicula* (Casper 1962, 1966, Steiger 1998, Roccia et al. 2016). Even Czech botanists do not agree whether it should be treated as a distinct species or not (e.g. Studnička 2005, Uher 2005). There are several reasons for considering *P. bohemica* to be different from *P. vulgaris*. In addition to its morphological dissimilarities (floral colouration, wider and apically blunt sepals, Fig. 2, Table 1), the two taxa reportedly differ in chromosome number. While *P. bohemica* is claimed to be tetraploid (2n = 32) (Studnička 1989, Fig. 2 and photo 3 in Studnička & Hejný 1992, see also Figs. 13–15 on Plate 3 in Casper & Stimper 2009), *P. vulgaris* is octoploid (2n = 64; Casper 1966). Tetraploid species are considered to be evolutionarily older than octoploid species (e.g. Steiger 1998, Legendre 2000, Shimai et al. 2021). The combination of morphological differences and different ploidy levels leaves little doubt that *P. bohemica* is a distinct species. However, the tetraploid chromosome number of *P. bohemica* is disputed, as reliable records of the octoploid level also exist (e.g. Bělohlávková 1989, Krahulcová & Jarolímová 1991, Casper & Stimper 2009). Moreover, the two taxa probably hybridize when growing together, producing the putative hybrid taxon *P.* ×*dostalii* Bárta (Bělohlávková 2000).

The three subspecies of *P. vulgaris* occurring in central Italy, *P. vulgaris* subsp. *anzalonei*, *P. vulgaris* subsp. *ernica* and *P. vulgaris* subsp. *vestina* are an interesting analogy to *P. bohemica*. These three subspecies are very morphologically similar (though still distinguishable) and also similar to *P. bohemica* (Fig. 2, Table 1). These subspecies occupy only a very small area and are geographically well isolated from each other, as well as from populations of *P. vulgaris* s. str. (Conti & Peruzzi 2006). Although there are no chromosome counts for them an octoploid level is assumed (Casper & Stimper 2009).

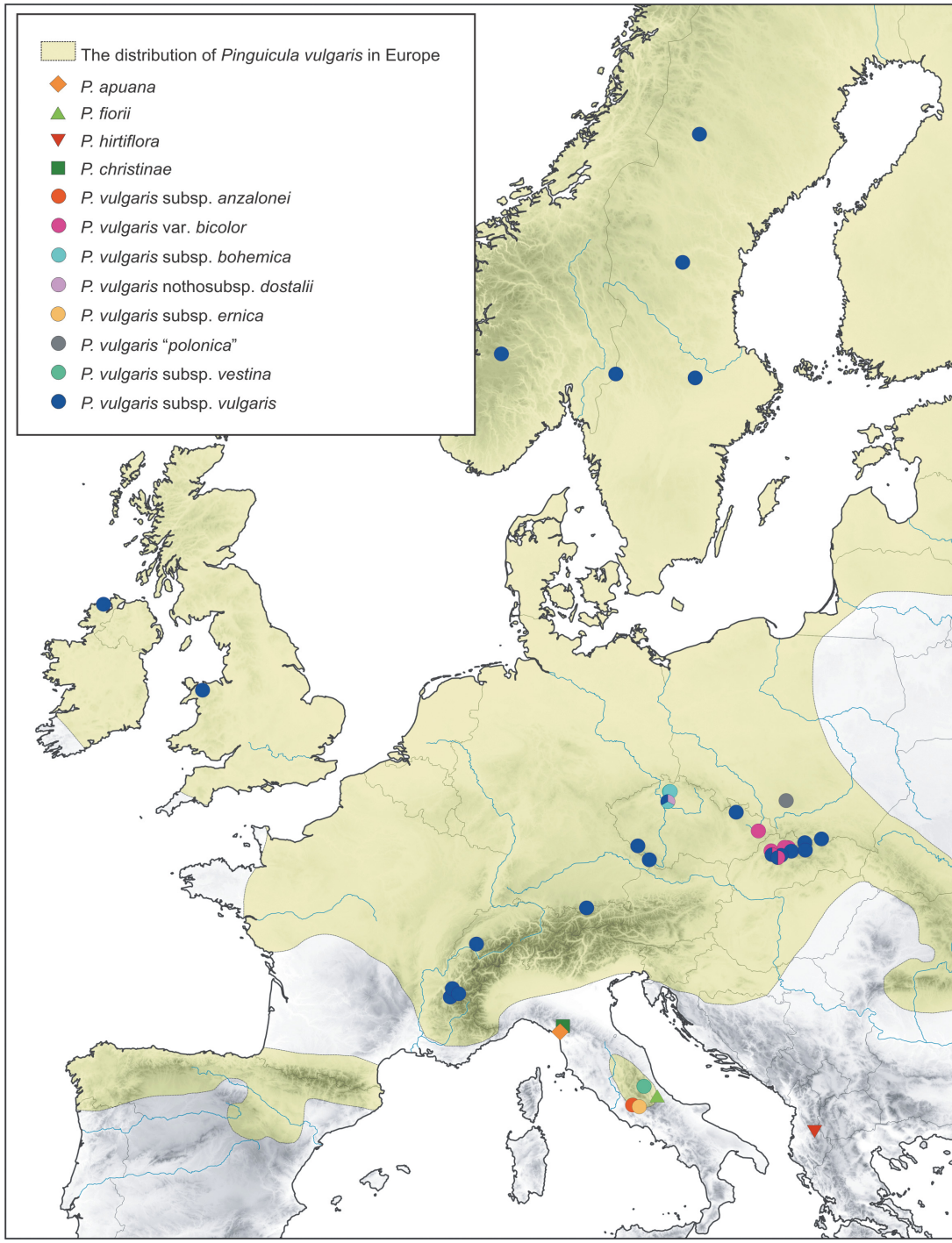

Fig. 1. The distribution of *Pinguicula vulgaris* in Europe with the sites sampled in the present study. Sampled taxa of *P. vulgaris* agg. are denoted by coloured circles, and other taxa by triangles and diamonds. The yellow-green polygon is the distribution of *P. vulgaris* based on Casper (1962) and Conti & Peruzzi (2006).

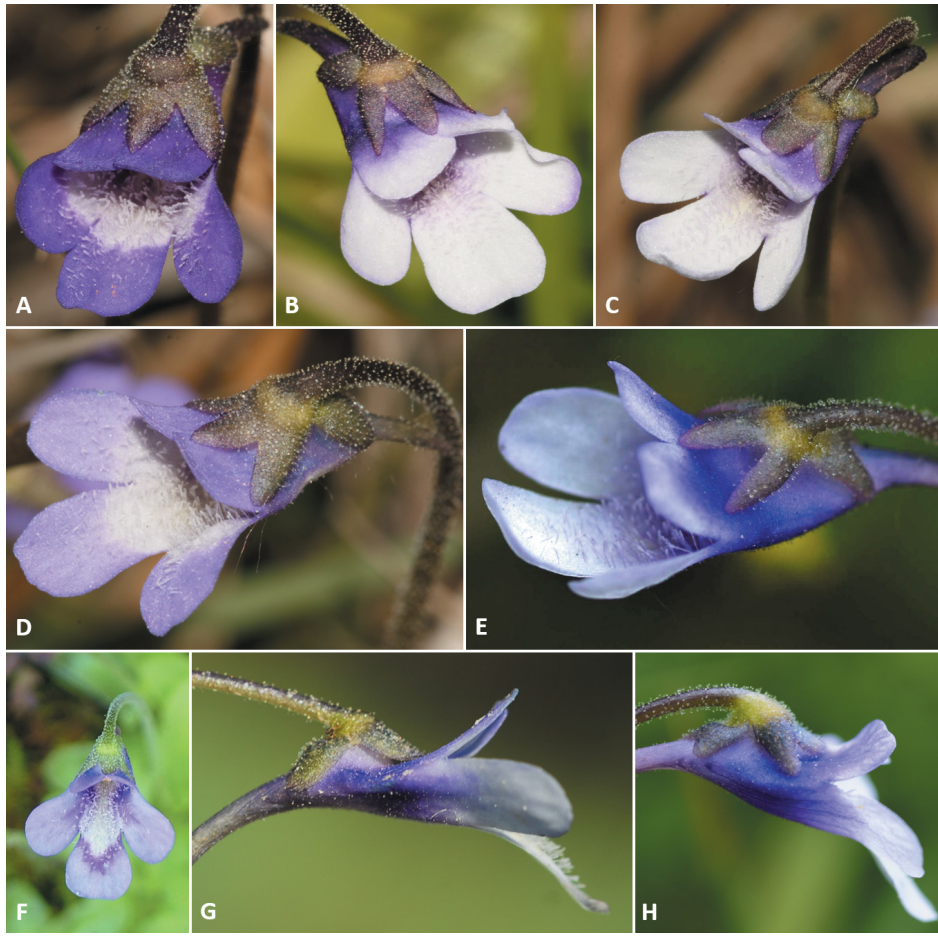

Fig. 2. Photographs of corolla colouration and shape of calyx lobes in the eight investigated taxa of *Pinguicula*. A – *Pinguicula vulgaris* subsp. *vulgaris* (CZ, Mělnická Vrutice); B – *P. vulgaris* var. *bicolor* (CZ, Botanical garden and arboretum Štramberk), C – *P. vulgaris* subsp. *bohemica* (CZ, Mělnická Vrutice), D – *P. vulgaris* nothosubsp. *dostalii* ined. (CZ, Mělnická Vrutice), E – *P.* "*polonica*" ined. (PL, Dąbrowa Górnicza); F – *P. vulgaris* subsp. *anzalonei* (IT, between Subiaco and Jenne); G – *P. vulgaris* subsp. *ernica* (IT, Zompo lo Schioppo); H – *P. vulgaris* subsp. *vestina* (IT, Bivacco Lubrano). Photos: A, B, C, D – F. Trnka, E – B. J. Plachno, F – F. Bartolucci, G, H – F. Conti.

In the present study, we employed four different approaches commonly used in plant biosystematics to investigate *P. bohemica* in relation to other morphologically similar taxa (*P. vulgaris* subsp. *vulgaris*, *P. vulgaris* var. *bicolor*, *P. vulgaris* subsp. *anzalonei*, *P. vulgaris* subsp. *ernica*, *P. vulgaris* subsp. *vestina*) in order to shed more light on the status of this disputed taxon. We performed flow cytometric measurements (FCM) of ploidy level and genome size. To confirm the estimated DNA-ploidy level, we also counted chromosomes for *P. bohemica*, *P. vulgaris* subsp. *anzalonei* and *P. fiorii*. We used scanning electron microscopy (SEM) for detailed imaging of the seeds of the three most similar taxa (*P. bohemica*, *P. vulgaris* subsp. *vulgaris*, *P. vulgaris* var. *bicolor*).

Table 1. Main characteristics of the investigated taxa of *Pinguicula vulgaris* agg. [1]This taxon needs further study to determine its distribution, morphology and ecology.

Taxon	Geographic distribution	Supposed origin	Flower morphology	Ecology
P. vulgaris subsp. *vulgaris*	widespread, temperate parts of Northern Hemisphere	autopolyploid	blue-violet with white hairs, white spot on lower lip, sepals narrow and pointed	acidic to calcareous fens, wet rocks, alpine and subalpine springs from lowlands up to 2600 m a.s.l.
P. vulgaris var. *bicolor*	scattered, Scandinavia, Alps, Carpathians	simple mutation changing pattern of flower coloration	whitish with blue-violet spot on lower lip, white hairs, sepals narrow and pointed	usually calcareous fens, mountains and their foothills
P. vulgaris subsp. *bohemica*	endemic, central Bohemia	hybrid (southern "*vulgaris*" × northern "*vulgaris*" lineage)	whitish with blue-violet throat, white hairs, wide and apically blunt sepals	calcareous fens in lowlands (alt. 180-260 m a.s.l.)
P. vulgaris nothosubsp. *dostalii*	endemic, central Bohemia	hybrid (*vulgaris*♀ × *bohemica*♂)	pale blue-violet, whitish spot on lower lip, white hairs, sepals ± narrow and pointed	calcareous fens alt. 180 m a.s.l.
[1]*P.* "*polonica*"	southern Poland	direct descendant of *bohemica* lineage, geographic isolation	whitish with blue-violet upper lobes and throat, white hairs, sepals narrow and ± pointed	calcareous fens on sand alt. 270 m a.s.l.
P. vulgaris subsp. *anzalonei*	endemic, central Italy	descendant of southern "*vulgaris*" lineage, geographic isolation	blue-violet with white hairs/dot near the throat, white V-shaped dot on lower lip	dripping cliffs alt. 700 m a.s.l.
P. vulgaris subsp. *ernica*	endemic, central Italy	descendant of southern "*vulgaris*" lineage, geographic isolation	pale violet, white hairs, whitish-rose lower lip with a yellowish stipe	dripping calcareous cliffs alt. 700 m a.s.l.
P. vulgaris subsp. *vestina*	endemic, central Italy	descendant of southern "*vulgaris*" lineage, geographic isolation, hybridization?	pale violet, white hairs, whitish-rose lower lip with a yellowish stipe	marshes, bogs, humid grasslands on calcareous substrate alt. above 1000 m a.s.l.

Finally, we used molecular markers, namely, AFLPs and sequenced chloroplast and nuclear loci to investigate genetic variation and the presence of boundaries between these taxa. The main objectives of this study were to (i) reassess the status of *P. bohemica*; (ii) investigate the amount and distribution of genetic variation within *P. vulgaris* agg.; (iii) find diagnostic molecular characters, if available, for taxa delimitation within *P. vulgaris* agg.; and (iv) draw infraspecific boundaries between taxa within *P. vulgaris* agg.

Materials and methods

Plant material

We sampled seven taxa of the *P. vulgaris* complex, referred to as *P. vulgaris* agg. Samples of *P. vulgaris* subsp. *vulgaris* (further abbreviated as VUL/*P.* **vulgaris*) were sampled from natural populations in the Czech Republic, France, Ireland, Germany, Great Britain, Norway, Poland, Slovakia, Sweden and Switzerland (Fig. 1, Table 2). Samples of

Table 2. Details of the samples of *Pinguicula vulgaris* agg. Country of origin: AL – Albania; CH – Switzerland; CZ – Czech Republic; DE – Germany; FR – France; IE – Ireland; IT – Italy; NO – Norway; PL – Poland; SK – Slovakia; SE – Sweden; UK – the United Kingdom. N(AFLP) – number of individuals used for AFLP analysis; N(cp/ITS) – number of individuals used for sequencing; N(PL) – number of individuals used for ploidy level measurement/relative genome size; N(GS) – number of individuals used for absolute genome size measurement; * samples used for SEM; ** sample of *P. vulgaris* subsp. *vulgaris* with pure white flowers.

Taxon/code	Country	Locality	N (AFLP)	N (cp/ITS)	N (PL)	N (GS)
P. vulgaris subsp. *vulgaris*						
VUL_SE_1	SE	Central Sweden, along lake Havern	3	2	-	-
VUL_SE_2	SE	Northeast Sweden, near Högland village	4	2	-	2
VUL_SE_3	SE	South Sweden, near Fagersta	4	2	-	-
VUL_NO_1	NO	South Norway, between Haugastøl and Ustaostat	4	2	-	-
VUL_NO_2	NO	Southeast Norway, along lake Varaldsjøen	4	2	-	-
VUL_CZ_1	CZ	Šumava Mts, near Nové Údolí	3	2	6	-
*VUL_CZ_2	CZ	Šumava Mt., near Keply	4	2	-	3
VUL_CZ_3	CZ	Central Bohemia, Mělnická Vrutice, Polabská Černava	-	-	12	-
VUL_CZ_4	CZ	Hrubý Jeseník Mts, Malá Morávka, Ovčárna challet	-	-	1	-
VUL_SK	SK	North Slovakia, Važecké lúky lawns, near Važec village	4	2	-	-
VUL_SK_2	SK	Malá Fatra Mts, lawns near Vrícko village	-	-	13	-
VUL_SK_3	SK	Velká Fatra Mts, E of Rakša village	-	-	1	-
VUL_SK_4	SK	Veľká Fatra Mts, Blatnica, Dedošova dolina	-	-	-	2
VUL_SK_5	SK	Lubovnianska vrchovina Mts, near Litmanová village	-	-	-	2
VUL_DE_1696	DE	Bavaria, near Wallgau village	6	1	-	3
VUL_PL	PL	Tatry Mts, Zakopane	6	2	-	3
VUL_UK_686	UK	Wales, Snowdonia	3	1	-	1
VUL_CH_667	CH	Canton of Vaud, near Vevey	2	1	-	2
VUL_FR_706	FR	Cottian Alps, Col de l'Agnelin	1	1	-	1
VUL_FR_709	FR	Isère, near lake 'Lac Fourchu'	1	1	-	1
VUL_FR_713	FR	Isère, Theyes, peat bog	1	1	-	1
VUL_IE_1338	IE	Downings, Co. Donegal	5	1	-	3
**VUL_SK_1253	SK	Velká Fatra Mts, near Ružomberok	1	1	-	1
P. vulgaris var. *bicolor*						
BIC_SK_1	SK	Malá Fatra Mts, lawns near Vrícko village	3	2	18	-
BIC_SK_2	SK	Malá Fatra Mts, near village Stankovany	3	2	5	-
BIC_SK_3	SK	Chočské vrchy Mts, near village Valaská Dubová	3	2	-	-
BIC_SK_4	SK	Velká Fatra Mts, E of Rakša village	-	-	5	-
BIC_SK_5	SK	Strážovské vrchy Mts, near village Šuja	-	-	5	-
BIC_SK_6	SK	Malá Fatra Mts, Klačno	-	-	-	1
*BIC_CZ	CZ	Štramberk town, abandoned stone-pit 'Dolní Kamenárka'	10	4	16	2
P. vulgaris subsp. *bohemica*						
BOH_SH	CZ	NW Bohemia, Shnilé louky	10	2	13	9
*BOH_BR	CZ	NW Bohemia, Baronský rybník	5	2	-	2
BOH_PO	CZ	Central Bohemia, Mělnická Vrutice, Polabská Černava	-	-	1	-
P. vulgaris nothosubsp. *dostalii* (DOS)	CZ	Central Bohemia, Mělnická Vrutice, Polabská Černava	2	2	-	2
P. "*polonica*" (POL)	PL	Woj. śląskie, Dąbrowa Górnicza, abandoned sandpit	5	3	-	2
P. vulgaris subsp. *anzalonei* (ANZ)	IT	Simbruini Mts, between Subiaco and Jenne	3	2	-	3
P. vulgaris subsp. *ernica* (ERN)	IT	Ernici Mts, Zompo Lo Schioppo	4	3	-	4
P. vulgaris subsp. *vestina* (VES)	IT	Gran Sasso Mts, Fonte Rionne	3	2	-	1
P. apuana (APU_1081)	IT	Alpi Apuane, Mt. Corchia	6	2	-	3
P. fiorii (FIO)	IT	Maiella Mts, Mt. Focalone	5	2	-	3
P. christinae (CHR)	IT	Tuscany, Lama Rossa	5	2	-	4
P. hirtiflora (HI)	AL	Shkumbin river valley, Librazhd	2	-	-	-

P. vulgaris var. *bicolor* (abbreviated BIC/*P. *bicolor*) originated from natural populations in central Slovakia, one natural population in Poland, and one possibly non-natural population in north-eastern Moravia (Czech Republic). Molecular genetic analyses revealed an unexpected result in the samples from the Polish locality Dąbrowa Górnicza, which were determined during sampling as *P. *bicolor*; therefore, samples from this site were treated separately as a distinct taxon under the provisional name *P.* "*polonica*" (abbreviated as POL). Samples of *P. bohemica* (abbreviated as BOH) originated from the last three remaining natural populations in central and northern Bohemia (*P. bohemica* is considered extinct at the "Mělnická Vrutice" locality; however, we found a few plants there, apparently of one genet). We also sampled a putative hybrid *P.* ×*dostalii* (*P. vulgaris* × *P. bohemica*, abbreviated DOS/*P. *dostalii*) from Mělnická Vrutice. The remaining three taxa belonging to the *P. vulgaris* agg. (*P. vulgaris* subsp. *anzalonei* – abbreviated as ANZ/*P. *anzalonei*; *P. vulgaris* subsp. *ernica* – abbreviated as ERN/*P. *ernica*; *P. vulgaris* subsp. *vestina* – abbreviated as VES/*P. *vestina*) were sampled from central Italy. We included three additional taxa endemic to Italy in some analyses, namely, *P. apuana* Casper et Ansaldi (abbreviated as APU), *P. fiorii* Tammaro et Pace (abbreviated as FIO) and *P. christinae* Peruzzi et Gestri (abbreviated as CHR), to better link the observed genetic variation within the section *Pinguicula*. Although these species belong to the section *Pinguicula*, they are not considered to be a part of *P. vulgaris* agg., but occasionally grow sympatrically. *Pinguicula hirtiflora* (abbreviated as HIR), which belongs to a different evolutionary lineage (sect. *Cardiophyllum*) not related to *P. vulgaris* agg. (Shimai et al. 2021), was used as an outgroup for AFLP analyses. All the samples studied were documented by vouchers deposited in the OL (herbarium of Department of Botany, Faculty of Science, Palacký University in Olomouc) or by photographic documentation.

Karyological analysis

For karyological analyses, root tip meristems of potted plants were used (*P. bohemica*, *P.* "*polonica*", *P. *anzalonei*, *P. fiorii*). The root tips were pre-treated in a 0.002 M water solution of 8-hydroxyquinoline at 4 °C for approximately 16 h (overnight), fixed in a 1:3 mixture of 98% acetic acid and 96% ethanol for 1–24 h, washed in distilled water, macerated in 1 N HCl at 60 °C for 6 min and washed in distilled water. Squashes were made using the cellophane square technique (Murín 1960). The slides were stained with a 7% solution of Giemsa stain (Sigma-Aldrich®) in Sorensen phosphate buffer (pH 7.0), dried and observed in a drop of immersion oil using a Leica DM 1000 microscope equipped with HDCE-X5 and software ScopeImage 9.0.

Flow cytometry: genome size measurements and ploidy level

The absolute DNA content (genome size, AGS) of 61 individuals of the eleven taxa studied (Table 2) was determined using a BD Accuri C6 flow cytometer (BD Biosciences, San Jose, USA) equipped with a blue laser (488 nm, 20 mW, BD Accuri™; BD Biosciences, San Jose, USA). Sample preparation followed the protocol given in Doležel et al. (2007) and described in full in Kobrlová & Hroneš (2019). Before analysis, the samples were incubated at 4 °C for approximately 4 hours and occasionally shaken. A fluorescence intensity of at least 5000 particles was recorded. *Glycine max* (L.) Merr. 'Polanka' (2C = 2.50 pg, Doležel et al. 1994) served as a primary internal reference standard. On several

occasions, *Zea mays* CE-‘777’ (2C = 5.67 pg, recalculated against *G. max*) was used as the secondary internal standard. As material of species of *Pinguicula* present a challeng for flow cytometry, histograms with coefficients of variation (CVs) of the G0/G1 peaks of both the sample and the standard of less than 5% were accepted. Each individual was analysed at least three times on three different days and the average value used for the calculation of the AGS. If the absolute differences of the three measurements exceeded the 5% threshold, then the most outlying measurement was discarded and a new measurement recorded. The within-measurement variation of one sample was calculated as [(maximum value – minimum value)/average value of all three measurements] *100. The monoploid genome size, 1Cx value (Greilhuber et al. 2005), was calculated by dividing the 2C value by the ploidy derived from published chromosome counts. Data analysis was performed in NCSS 9 (Hintze 2013). Pairwise differences in AGS were tested using the Tukey HSD test. However, due to low and unevenly distributed sample sizes, only particular pairwise comparisons were undertaken (BIC vs. BOH, BOH vs. VUL).

An analysis of ploidy level was used to assess the cytological diversity of a broader sample of taxa VUL (33 individuals from five populations), BOH (14 individuals from two populations) and BIC (49 individuals from five populations) from central Europe (Table 2). The DNA-ploidy level (Suda et al. 2006) of the field-collected fresh plant tissue was established using an ML CyFlow flow cytometer (Partec GmbH, Münster, Germany) equipped with a diode-pumped solid-state green laser (532 nm, 100 mW, Cobolt Samba; Cobolt AB, Stockholm, Sweden). Sample preparation and analysis followed the same procedure as in the GS analysis, but only one measurement per individual was recorded and *Zea mays* was used as an internal standard. The DNA-ploidy level and relative genome size (RGS) of each sample were determined separately by the position of its G0/G1 peak relative to the G0/G1 peak of the internal standard. Data analyses (ANOVA, Tukey HSD test) were performed in NCSS 9.

Scanning electron microscopy

For SEM imaging, seeds of BIC, BOH and VUL were sputter-coated with gold (using a JEOL-JFC 1100E sputter coater) and examined at an accelerating voltage of 20 kV using a Hitachi S-4700 scanning electron microscope (Hitachi, Tokyo, Japan) housed at the Institute of Geological Sciences, Jagiellonian University in Kraków.

DNA extraction and AFLP fingerprinting

Total genomic DNA was extracted from 122 individuals of 11 taxa: VUL – 56 individuals; BIC – 18, POL – 5; BOH – 15; DOS – 2; ANZ – 3; ERN – 4; VES – 3; APU – 6; FIO – 5; CHR – 5 and HIR – 2 individuals (Table 2). DNA was extracted from fresh leaves according to the protocol of Doyle & Doyle (1987) with minor modifications. AFLP analyses were performed according to Vos et al. (1995) with modifications as described in Ali et al. (2017) using three primer combinations (Supplementary Table S1) selected by preliminary testing. Amplified fragments were separated by capillary electrophoresis (GenomeLab CEQ/GeXP DNA, Beckman Coulter, USA). Raw data were analysed with CEQ 8000 software using an incorporated dominant scoring algorithm (bin 1–2 bp, threshold 100), where peaks ranging from 70 to 600 bp were scored as either present (1) or absent (0). To avoid genotyping errors and retain reproducibility of the analyses,

controls consisting of blind samples, double samples and repetitions were integrated into our analysis.

AFLP data analysis

For all the subsequent analyses, samples were grouped based on taxonomy; thus, the designated groups are the taxa investigated not the populations sampled. Since some groups consisted of fewer than five individuals, fragments present in only one sample were excluded from the final binary matrix to avoid excluding a great proportion of rare bands, as discussed by Bonin et al. (2004). Monomorphic fragments were also excluded. The final binary matrix (comprising of bands characteristic of *P. vulgaris* agg. and excluding private bands for *P. hirtiflora*) was used to compute overall AFLP statistics, i.e. the mean number of bands (NB), number of polymorphic bands (N_{POL}) at the 5% level, number of private markers (N_{PRI}; marker restricted to a given taxon) and number of diagnostic bands (N_{DG}; monomorphic marker restricted to a given taxon) were calculated using Famd (Schlüter & Harris 2006). To investigate the genotypic diversity of the taxa studied, the number of different genotypes (NG), genotype diversity (GD), Nei's gene diversity (He) and overall polymorphism (PLP) were calculated using the R script AFLPdat (Ehrich 2006, R 3.0.2 environment – R Core Team 2021). To assess the possible relict status of the taxa investigated, the rarity of AFLP markers (frequency-down-weighted marker values; DW; Schönswetter & Tribsch 2005) was computed for each taxon using the R script AFLPdat. Greater DW values imply a greater number of rare markers, as expected for relic populations that have been isolated for a long time. To investigate the effect of unequal sample size, rarefaction was used, as implemented in the AFLPdiv program (Petit et al. 1998). The genetic diversity was measured as band richness (Br) for a standardized sample size (n = 3, i.e. three randomly selected samples). To determine the amount of differentiation between the investigated taxa, pairwise F_{ST} was calculated, and significance was tested by permutations (1000 repetitions) using the program Arlequin ver. 3.5 (Excoffier & Lischer 2010).

To investigate differentiation of the studied taxa, AFLP data were subjected to several clustering methods. The neighbour-joining analysis was performed in DARwin 6.0.21 (Perrier & Jacquemoud-Collet 2006) based on the genetic distance calculated based on the Jaccard similarity coefficient. The robustness of the clustering was tested using 1000 bootstrap replications. The resulting dendrogram was visualized in FigTree (freely available at http://tree.bio.ed.ac.uk/software/figtree/) and rooted using *P. hirtiflora*. To compare neighbour-joining analysis with the network-based method, neighbour-network analysis (based on the Jaccard similarity coefficient) was performed in SplitsTree 4 (Huson & Bryant 2006). Finally, we used Bayesian clustering, implemented in Structure (Falush et al. 2007). Computation was carried out using the recessive allele model and the admixture model with correlated allele frequencies. Because of the overall low differentiation of the investigated taxa, the effect of turning the LOCPRIOR option on and off was also tested. In our case, LOCPRIOR provided information about taxon determination. K was set to 1–11 with 10 replicate runs for each value of K, using 500,000 MCMC iterations following 50,000 burn-in iterations. To summarize the results and prepare the graphical output programs, Structure Harvester (Earl & von Holdt 2012), Clumpp (Jakobsson & Rosenberg 2007) and Distruct (Rosenberg 2004) were used. Principal

coordinate analysis (PCoA) was performed using Famd (Schlüter & Harris 2006) (Jaccard similarity matrix) and inspected on 2D and 3D plots.

Sequencing and sequence alignment

Based on a survey of the literature, 15 cpDNA loci and four ncDNA loci were selected (Supplementary Table S2) and screened for polymorphisms in a subset of 8 samples representing taxa VUL, BIC and BOH. Due to low variation or nonspecific amplification (Supplementary Table S2), only four chloroplast loci (cpDNA: *trn*T-*trn*F, *trn*C-*psb*M, *trn*Q-*rps*16 and *trn*K-*mat*K) and one nuclear locus (ncDNA: ITS1-5.8S rDNA-ITS2) were selected for further investigation.

Based on the AFLP data, 52 individuals (Table 2) were chosen for sequencing of selected loci. PCR was performed in a total volume of 25 µl with 20 ng of template DNA, 2 µM of forwards and reverse primers, 0.2 mM of each dNTP, 1X PCR buffer (containing 2 mM of $MgCl_2$) and 1 U of *Pfu* DNA polymerase (Fermentas). The reaction conditions were as follows: 95 °C for 3 min; 36 cycles with 95 °C for 30 s; an experimentally adjusted annealing temperature (see Supplementary Table S2) for 45 s; and 72 °C for 1 min; followed by 10 min at 72 °C. The PCR products were purified using a GeneElute PCR Clean-Up Kit (Sigma–Aldrich®) and sequenced commercially by Macrogen Europe (Amsterdam, The Netherlands) from both forward and reverse directions. To acquire a high-quality and the longest possible alignment of the entire *trn*T–*trn*F and *trn*C-*psb*M regions, PCR products were sequenced with several internal primers. Sequences were visually checked and edited in Geneious® 7.1.7 (Kearse et al. 2012). Ambiguous (heterozygous) positions were recognized in the ITS region only when overlapping peaks, corresponding to two different nucleotides, were present at a given position in electrophoretograms from both sides. Alignment and haplotype/ribotype identification were carried out using MEGA 5. (Tamura et al. 2011). Sequence data were deposited in GenBank (see Supplementary Table S3). We compared ITS and *trn*K-*mat*K sequences with sequences of VUL, BOH, ANZ, VES, ERN and FIO available in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/). There was a total of nine records: eight records contained the full ITS1-5.8S-ITS2 sequence (DQ438093, DQ438086, DQ222949, DQ441597 – Degtjareva et al. 2006; AB198361, AB198343 – Kondo & Shimai 2006; LN887946, LN887945, LN887944, LN887943, LN887942 – De Castro et al. 2016) and four records JN999374 – JN999377 contained only a partial sequence of ITS2 (Kuzmina et al. 2012). For *trn*K-*mat*K, there were two relevant records (LC348435, and AF531806 – Shimai et al. 2021).

Results

Karyology, genome size and ploidy level

Karyological analyses of all four taxa, ANZ, BOH, POL and FIO, revealed 2n = 64 chromosomes (Fig. 3). Ploidy level estimation by flow cytometry supported the same ploidy level for all the plants analysed (Table 3, Fig. 4A). The observed small difference in genome size between VUL and BOH does not correspond to different ploidy levels. The measured AGS ranged from 1.31 pg in BOH and ERN to 1.96 pg in FIO (Table 4, Fig. 4B). BOH differed significantly in both AGS and RGS from VUL and BIC (AGS:

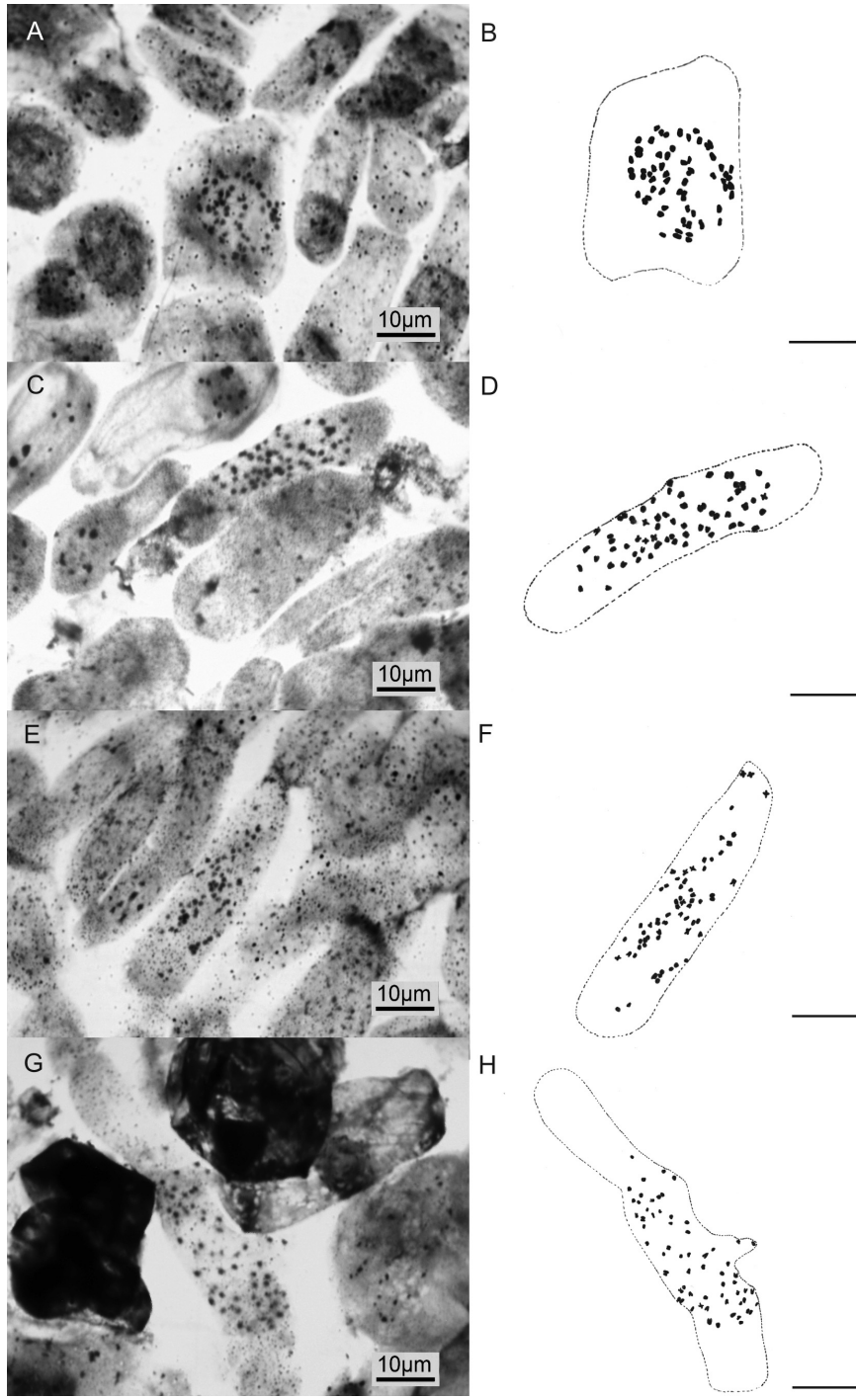

Fig. 3. Mitotic metaphase plates of four karyologically investigated taxa: *P. vulgaris* subsp. *bohemica* (A/B), *P.* "*polonica*" (C/D), *P. vulgaris* subsp. *anzalonei* (E/F) and *P. fiorii* (G/H), showing 2n = 8x = 64 chromosomes.

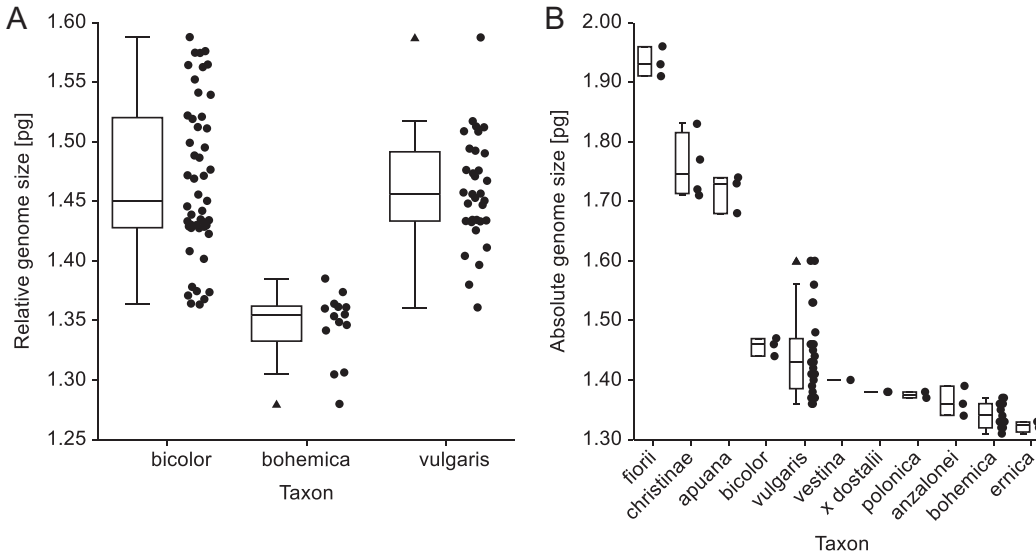

Fig. 4. Cytological variation recorded for *Pinguicula vulgaris* agg. A – Relative genome size of three central European taxa; B – Absolute genome size of taxa from sect. *Pinguicula* included in this study. Dots represent each measurement, the body of the box plot defines the 25th and 75th percentiles, horizontal lines show the median, whiskers are from the 10th to 90th percentiles and triangles show outlier values.

Table 3. Relative genome size of selected taxa of *Pinguicula vulgaris* agg. determined by flow cytometry. N – sample size; RGS – relative genome size; RGS and ratio of sample to the standard *Zea mays* are given as min–(mean ± standard error)–max; variation is calculated as the difference between the most extreme values expressed in % of the mean value; * is used here to replace full taxon names, for further explanation see Materials and methods.

Taxon	N	sample/standard ratio	RGS (pg)	RGS variation (%)
*P. *vulgaris*	33	0.24–(0.258±0.001)–0.28	1.36–(1.46±0.01)–1.59	15.8
*P. *bicolor*	49	0.24–(0.259±0.002)–0.28	1.36–(1.47±0.01)–1.59	15.6
*P. *bohemica*	14	0.23–(0.237±0.002)–0.24	1.28–(1.35±0.01)–1.39	8.1

Table 4. Absolute genome sizes of the investigated species of *Pinguicula vulgaris* agg. * is used here to replace full taxon names, for further explanation see Materials and methods.

Taxon	N	2C (pg)	SE	Min–max (pg)	Mean 1Cx value (pg)
P. fiori	3	1.93	0.010	1.91–1.96	0.24
P. christinae	4	1.76	0.030	1.71–1.84	0.22
P. apuana	3	1.72	0.030	1.66–1.75	0.22
*P. *bicolor*	3	1.46	0.010	1.44–1.47	0.18
*P. *vulgaris*	25	1.44	0.010	1.36–1.60	0.18
*P. *vestina*	1	1.40	n.a.	n.a.	0.18
*P. *dostalii*	2	1.38	n.a.	n.a.	0.17
P. "polonica"	2	1.38	0.005	1.37–1.38	0.17
*P. *anzalonei*	3	1.36	0.010	1.34–1.39	0.17
*P. *bohemica*	11	1.34	0.010	1.31–1.37	0.17
*P. *ernica*	4	1.32	0.004	1.31–1.33	0.17

ANOVA – DF = 2, F = 12.06, P < 0.001; Tukey HSD – DF = 36, MSE = 0.0035, critical value = 3.45; RGS: ANOVA – DF = 2, F = 27.78, P < 0.001; Tukey HSD – DF = 93, MSE = 0.0030, critical value = 3.38). According to AGS, two groups of species may be recognized, one comprised of taxa of *P. vulgaris* agg., the second of outgroup taxa (APU, CHR, FIO, Fig. 4B, Table 4). The intraspecific variation in genome size was low, with the exception of VUL, which varied 1.18-fold. The quality of the measurements was generally good; the CVs of the internal standard and sample ranged between 0.96 and 3.90 (mean 1.96, median 1.91) and 1.29 and 4.75 (mean 2.82, median 2.80), respectively, and the within-measurement variation of a sample ranged between 0.02% and 2.82% (mean 1.04%, median 0.79%).

Scanning electron microscopy

In all of the three taxa studied, no essential differences in the shape and general morphology of seeds were observed (Fig. 5). The seeds were tiny; ~620–934 μm long, ~220–314 μm wide for VUL; ~660–754 μm long, ~160–290 μm wide for BIC; and ~660–815 μm long, ~275–310 μm wide for BOH. Their shape was mostly regular cylindrical to ellipsoidal with one micropylar appendage. In all the studied taxa, the micromorphology of the seed coat was similar. The outer parts of the anticlinal walls of adjacent epidermal cells were divided by a furrow.

Genotype diversity of Pinguicula vulgaris agg.

Fingerprinting of 122 individuals yielded 230 polymorphic AFLP markers. The overall increased tendency for selfing, accompanied by decreased variability in *Pinguicula vulgaris* agg., was reflected in the number of different genotypes/AFLP phenotypes in fingerprinted individuals. VUL was represented by 45 different AFLP phenotypes (56 individuals analysed), BIC was represented by 15 different AFLP phenotypes (18 individuals analysed), and BOH was represented by 12 different AFLP phenotypes (15 individuals analysed). POL was represented by two different AFLP phenotypes in the five individuals investigated and the putative hybrid taxon DOS was represented by two AFLP phenotypes. In the rest of the investigated taxa (ANZ, ERN, VES, APU, FIO, CHR), the number of recovered AFLP phenotypes was identical to the number of investigated individuals (Table 5). The increased clonality of the investigated taxa (especially in the case of VUL, BIC, BOH) was even more pronounced when AFLP phenotypes differing in up to two markers were considered identical (this represents a 2% error rate calculated as the difference in the number of markers observed in the investigated individuals in repeated analyses).

Genotype diversity (GD) (calculated without the incorporation of the error rate of 2%) was high, ranging from $GD^{ANZ/ERN/VES/APU/FIO/CHR/DOS} = 1$ to $GD^{POL} = 0.4$; $GD^{BOH} = 0.97$, which was the same as for BIC (Table 5). Gene diversity (or heterozygosity; He) was lowest for $POL^{He = 0.005}$, while $BOH^{He = 0.034}$ showed a similar level of heterozygosity as $APU^{He = 0.032}$. The highest heterozygosity was recorded for $VUL^{He = 0.058}$ and $FIO^{He = 0.052}$. The three Italian narrowly endemic taxa ANZ, ERN and VES had a similar level of heterozygosity. The level of polymorphism followed the gradient of heterozygosity. The least polymorphic taxa were DOS, POL, ERN, ANZ and VES, while the percentage polymorphism reached 9.0% in BOH, 7.3% in APU, 11.3% in FIO and 13.9% in BIC. The highest polymorphism

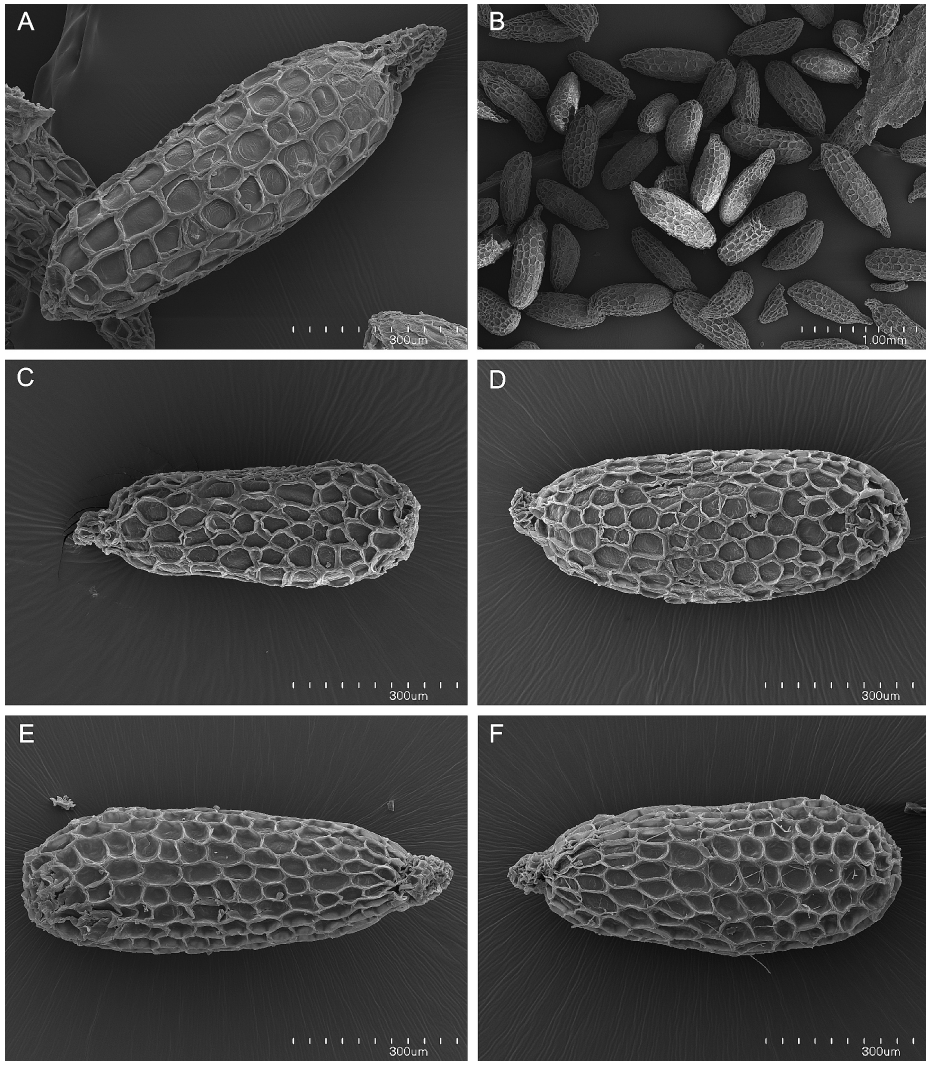

Fig. 5. Scanning electron microscope pictures of seeds of the three investigated species of *Pinguicula*. A-B – *P. vulgaris* subsp. *vulgaris;* C-D – *P. vulgaris* subsp. *bohemica;* E-F – *P. vulgaris* var. *bicolor*.

was detected in VUL *(*26%) and CHR *(*30%). Unequal sample size did not affect the computed statistics and band richness (Br) (computed for standardized sample size n = 3), which was in concordance with the percentage of polymorphic loci (PLP) (Table 5). The presence of private markers in the majority of the taxa investigated (missing only for BIC, POL, DOS) points to the presence of interspecific differentiation (mainly among the Italian taxa). The differentiation is highlighted by the presence of diagnostic markers encountered for these taxa (except CHR; Table 5). The pattern of pronounced differentiation of the Italian taxa and their relict status is also reflected by DW reaching values of over two (highest DW^{VES} = 15.8), while the values for VUL, BOH, BIC, POL and DOS

Table 5. Summary statistics for the AFLPs of the studied taxa of *Pinguicula vulgaris* agg. N – sample size; NG – number of different genotypes/AFLP phenotypes (the first number is NG without allowing for the difference of two markers/second number is NG with the difference of two markers incorporated); GD – genotype diversity; He – gene diversity; NB – number of bands; Br[3] – band richness for a standardized sample size n = 3; N_{POL} – number of polymorphic bands; PLP – polymorphism; N_{PRI} – number of private markers; N_{DG} – number of diagnostic markers; DW – frequency-downweighted marker values; [1]computed in AFLPdat; [2] computed in Famd; [3]computed in AFLPdiv. For taxon abbreviations, see Table 2.

Indices/taxon	VUL	BOH	POL	BIC	DOS	ANZ	ERN	VES	APU	FIO	CHR
N	56	15	5	18	2	3	4	3	6	5	5
NG[1]	45/25	12/8	2/2	15/11	2/1	3/3	4/3	3/3	6/6	5/5	5/5
GD[1]	0.99/0.96	0.97/0.88	0.4/0.4	0.97/0.94	1	1/1	1/0.83	1/1	1/1	1/1	1/1
He[1]	0.058	0.034	0.005	0.046	0.009	0.029	0.017	0.029	0.032	0.052	0.170
NB[2]	66.60	72.80	70.60	71.89	67.00	72.67	73.25	92.67	63.00	77.00	63.00
Br[3][3]	1.086	1.051	1.008	1.070	n.a.	1.043	1.025	1.043	1.048	1.078	1.254
N_{POL}[2]	60	21	3	32	2	10	7	10	17	26	69
PLP(%)[1]	26.00	9.10	1.30	13.90	0.87	4.35	3.00	4.35	7.39	11.30	30.00
N_{PRI}[2]	7	2	0	0	0	2	3	40	5	2	21
N_{DG}[2]	0	0	0	0	0	1	2	37	2	11	0
DW[1]	0.96	1.20	0.95	0.97	1.10	2.90	2.40	15.80	2.10	5.20	5.90

were between 1.2^{BOH} and 0.95^{POL}. The level of molecular differentiation, expressed by pairwise F_{ST}, is comparable between the Italian vs. central European taxa and is thought to be greater for the former group (Supplementary Table S4, Supplementary Fig. S1). Within the central European taxa, BIC and DOS are the least differentiated from VUL, while POL and BOH show similar levels of differentiation from VUL and BIC. The higher differentiation of BOH from POL and DOS is very interesting, although the latter two taxa are presumed to be descendants of BOH. These results may be due to an uneven sample size.

Ordination and clustering analyses

The interpretation of the results of the Structure analysis was straightforward. The mean L(K) increased up to K = 6 and then decreased significantly (Fig. 6A). Maximum values for ΔK were present for K = 2, K = 5 and K = 6 (Fig. 6B). For K = 2, two different genetic clusters were recognized, clearly showing a different genetic pool of Italian taxa (FIO, VES) and BOH, POL, BIC and VUL. The taxa ANZ, ERN, DOS, CHR and APU showed a different level of admixture of the two genetic clusters (Fig. 6B). Clustering for higher Ks revealed further differentiation of the investigated taxa and the results were highly similar to those of the neighbour-joining and PCoA analyses (Fig. 7). Taxa BOH and VUL represented two distinct genetic clusters, while BIC showed an admixture of BOH and VUL clusters (Fig. 6B). The situation is similar for POL and DOS, which, however, show an admixture of another genetic cluster, which is not specific for BOH or VUL, but is identified in Italian taxa.

The result of the neighbour-joining clustering corresponds to the PCoA. Visualization of the clustering in a dendrogram shows taxon-specific clusters for all the investigated taxa (except one) (Fig. 7B). Only samples of *P. christinae* appeared in two separate clusters, two samples as sister to *P. apuana*, while the remaining three samples clustered even more basally as sister to all the rest of the investigated taxa except VES and HIR. This het-

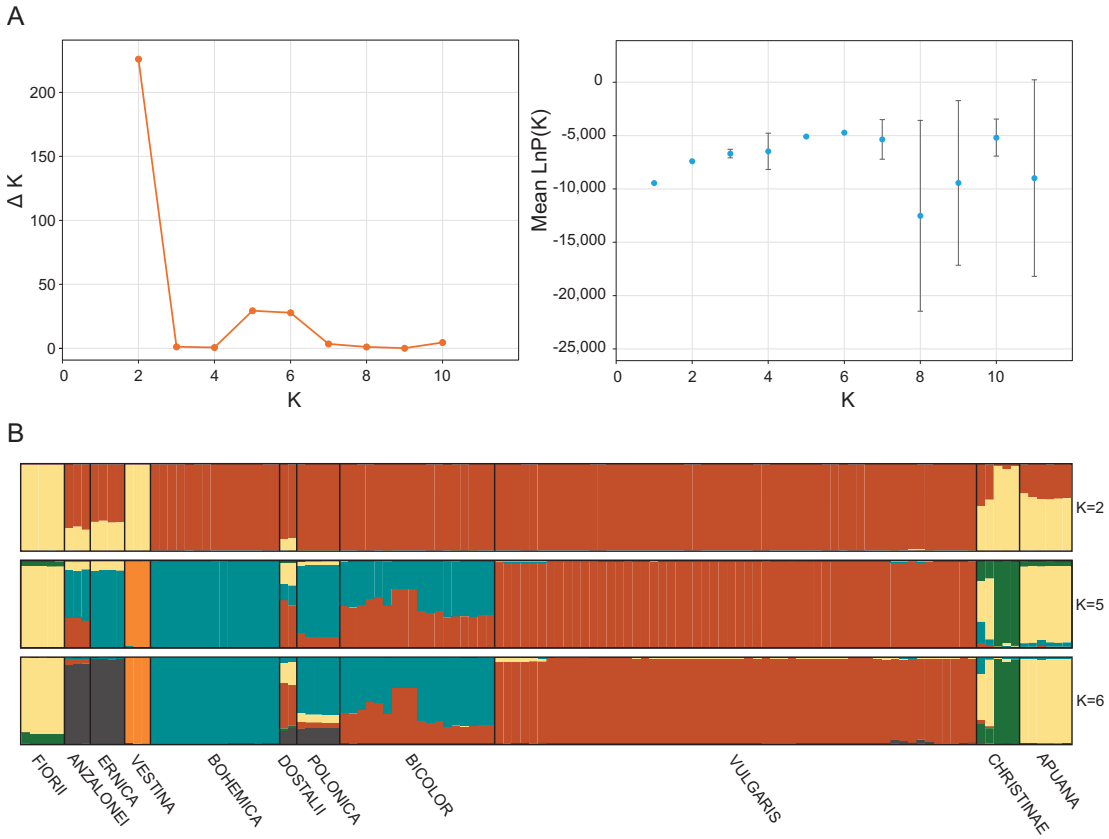

Fig. 6. Results of STRUCTURE clustering used for analysing AFLP data (230 markers) of 11 taxa of the *P. vulgaris* species complex (altogether 122 individuals). A – Structure analysis result. The plot of mean likelihood L(K) and variance per K value and plot of ΔK for detecting the number of K best fitting the data analysed. B – Structure clustering for K = 2, K = 3 and K = 5. Each individual is represented by a vertical bar, the colour representing the probability of assignment to different clusters is shown, and each clustering is based on 10 replicate runs.

erogeneity of CHR was also recorded in the PCoA analysis and is reflected in the diversity indices (Table 5). The large cluster of *P. vulgaris* agg. taxa can be divided into seven sub clusters. The most basal is the cluster of ANZ-ERN taxa, both representing well-differentiated groups. Interestingly, there was clear and exceptional separation of the third taxon, VES, which appeared in a well-supported cluster and was sister to all of the other taxa investigated. The rest of the *P. vulgaris* agg. was separated by neighbour-joining analysis into two groups. One consisted of BOH and POL, which are sister to each other. All the investigated VUL and BIC samples created two distinct clusters sister to each other. Last, two samples of DOS were positioned by the analysis basally to the BIC/VUL cluster and, at the same time, in-between the BOH/POL and BIC/VUL clusters. Such a placement corresponds to the presumed hybrid nature of DOS. The neighbour-network analysis provided a very similar view (Fig. 7A).

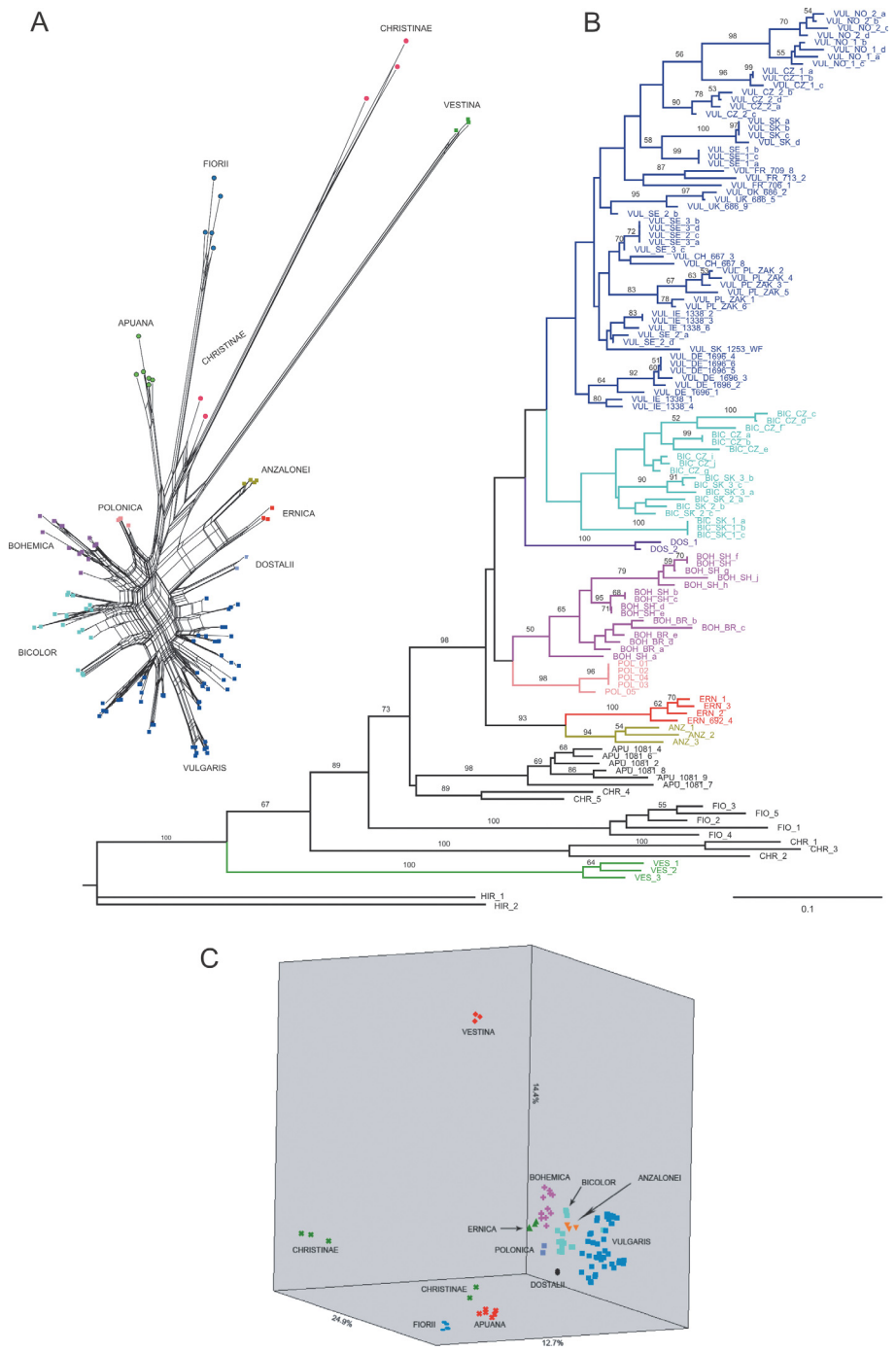

Fig. 7. Results of clustering methods used for analysing AFLP data (230 markers) of 11 taxa of the *P. vulgaris* species complex (altogether 122 individuals). A – Neighbour network showing all possible (congruent and incongruent) relationships among the investigated *Pinguicula* taxa. The analysis is based on the Jaccard similarity coefficient; B – Neighbour-joining analysis based on Jaccard coefficient of similarity showing the division of the sampled population into separate taxon-specific clusters. Note the distinct clustering of *P. vulgaris* subsp. *vestina* and *P. christinae*; C – Principal coordinate analysis (PCoA 3D plot, based on Jaccard's similarity coefficient). The first three axes explain 52% of the total variation.

Table 6. Summary of the detected polymorphisms within the investigated loci. Length – length of the final alignment in the number of nucleotides; Pi – the number of parsimony-informative sites; INDEL – presence of insertion/deletion, the number and length (in parentheses) of nucleotides (nt); n.a. – not applied; polyX – variable position within polyA/T/C stretch.

Locus	Length	Conserved sites	Variable sites	Pi	INDEL	polyX
*trn*T-*trn*F	1581	1576	5	5	0	polyA
*trn*C-*psb*M	1828	1826	2	2	0	polyT
*trn*Q-*rps*16	618	618	0	0	1 (7nt)	polyT
*trn*K-*mat*K	1082	1080	2	2	0	polyT
ITS1-5.8S-ITS2	617	607	10	10	0	polyC
Together	5726	5707	19	19	n.a.	n.a.

PcoA separated the taxa of *P. vulgaris* agg. from APU, CHR and FIO, which clustered separately. Interestingly, VES was clearly separated from the cluster of *P. vulgaris* agg. The remaining seven taxa of this species aggregate formed one cluster within which there were subgroups, which corresponded to defined taxa. The first three axes explain 52% of the total variation (Fig. 7C).

Sequence variation

The detailed characterization of the sequenced loci is presented in Table 6. The overall sequence divergence among the investigated taxa was very low, with only 19 variable positions in the investigated 5726 bp concatenated alignment. Within 52 sequenced individuals, five different ITS ribotypes and four within *P. vulgaris* agg. were identified (Table 7). The majority of VUL and one DOS sample had ribotype-A, and only two samples from Sweden and three from France had ribotype-B (differing in 3 SNPs). The three taxa ANZ, ERN and VES had ribotype-C, differing by one SNP from ribotype-A. All the samples of BOH and POL had ribotype-A*, which differed from ribotype-A in one heterozygous position (position no. 441 in the final alignment). Since the ITS region is present in multiple copies in a given genome, there should be two variants of ITS in the BOH and POL genomes that differ by one nucleotide (A/G). The presence of ambiguous nucleotides in all the sequenced individuals in both reads (forwards, reverse) at the same position convinced us of the equal frequency of the presence of both variants within BOH. A comparison of our samples with GenBank records showed both consistencies and discrepancies (for explanation see Supplementary Data S1).

Multiple alignment of the three sequenced cp-DNA loci revealed eight different multilocus haplotypes (Table 7). Sequence divergence in the analysed chloroplast loci was higher than that in the nuclear ITS locus. Haplotypes a-c were recorded only for VUL and BIC. Within haplotype-a, four (a, a1, a2, a3) variants and within haplotype-b, two (b, b1) variants differed in the number of nucleotides in polyA/T stretches (Table 7). All BIC except for one individual shared the same haplotype-a3, which was unique for this taxon and differed from the main haplotype-a at the polyA stretch. All the sequenced individuals of POL and BOH had the same haplotype with a unique 7 nt long duplicated motif (TAGTTAG) in the *trn*Q-*rps*16 locus, which was not recorded in any other species inves-

Table 7. Comparison of the haplotypes and ribotypes recorded in sequenced individuals of *Pinguicula vulgaris* agg. and *P. fiorii*. For population/taxon abbreviations, see Table 2. Numbers refer to a variable position in the final alignment; • – a match with haplotype a/ribotype A; - – an absence of particular nucleotide at a particular position; x – missing data (due to unsuccessful PCR); position in bold and italic indicates variation in polyA/T/C stretch; * – marks sample of *P. vulgaris* subsp. *vulgaris* with pure white flowers.

Locus	*trn*T-*trn*F								*trn*C-*psb*M				*trn*Q-*rps*16										*trn*K-*mat*K		Haplotype	ITS1-5.8S-ITS2										Ribotype
Position / taxon	1 4 8	***3*** ***7*** ***0***	5 1 4	5 5 8	***8*** ***3*** ***3***	***8*** ***3*** ***4***	1 3 5 9	1 5 0 3	***5*** ***2*** ***9***	***5*** ***3*** ***0***	5 3 7	1 6 0	1 1 9 9 7	1 9 8	1 9 9	2 0 0	2 0 1	2 0 2	2 0 3	***2*** ***5*** ***5***	***2*** ***5*** ***6***	***4*** ***8*** ***0***	7 1 3		4 3	5 6	***1*** ***7*** ***3***	2 0 2	2 2 7	2 6 1	4 3 2	4 4 1	5 2 5	5 8 5	5 8 7	
*VUL SK 1253	G	-	C	A	-	***A***	A	A	-	***T***	T	G	-	-	-	-	-	-	-	-	***T***	-	C	a	C	C	***C***	G	A	C	A	G	A	G	T	A
VUL CZ 1b	.	-	.	.	-	.	.	.	-	.	.	.	-	-	-	-	-	-	-	-	.	-	.	a	.	.	.	.	.	.	.	.	.	.	.	A
VUL CZ 1c	.	-	.	.	-	.	.	.	-	.	.	.	-	-	-	-	-	-	-	-	.	-	.	a	.	.	.	.	.	.	.	.	.	.	.	A
VUL CZ 2a	.	-	.	.	-	.	.	.	-	.	.	.	-	-	-	-	-	-	-	-	.	-	.	a	.	.	.	.	.	.	.	.	.	.	.	A
VUL CZ 2b	.	-	.	.	-	.	.	.	-	.	.	.	-	-	-	-	-	-	-	-	.	-	.	a	.	.	.	.	.	.	.	.	.	.	.	A
VUL SK a	.	-	.	.	-	.	.	.	-	.	.	.	-	-	-	-	-	-	-	-	.	-	.	a	.	.	.	.	.	.	.	.	.	.	.	A
VUL SK c	.	-	.	.	-	.	.	.	-	.	.	.	-	-	-	-	-	-	-	-	.	-	.	a	.	.	.	.	.	.	.	.	.	.	.	A
VUL SWE 3d	.	-	.	.	-	.	.	.	-	.	.	.	-	-	-	-	-	-	-	-	.	-	.	a	.	.	.	.	.	.	.	.	.	.	.	A
VUL PL ZAK 2	.	-	.	.	-	.	.	.	-	.	.	.	-	-	-	-	-	-	-	-	.	-	.	a	.	.	.	.	.	.	.	.	.	.	.	A
VUL PL ZAK 6	.	-	.	.	-	.	.	.	-	.	.	.	-	-	-	-	-	-	-	-	.	-	.	a	.	.	.	.	.	.	.	.	.	.	.	A
VUL CH 667 3	.	-	.	.	-	.	.	.	-	.	.	.	-	-	-	-	-	-	-	***T***	.	-	.	a1	.	.	.	.	.	.	.	.	.	.	.	A
VUL DE 2	.	-	.	T	-	.	.	.	-	.	.	.	-	-	-	-	-	-	-	-	-	-	.	b1	.	.	.	.	.	.	.	.	.	.	.	A
VUL UK 686 9	.	-	.	T	-	.	.	.	-	.	.	.	-	-	-	-	-	-	-	-	.	-	.	b	.	.	.	.	.	.	.	.	.	.	.	A
VUL NO 1a	.	-	.	T	-	.	.	.	-	.	.	.	-	-	-	-	-	-	-	-	.	-	.	b	.	.	.	.	.	.	.	.	.	.	.	A
VUL NO 1d	.	-	.	T	-	.	.	.	-	.	.	.	-	-	-	-	-	-	-	-	.	-	.	b	.	.	.	.	.	.	.	.	.	.	.	A
VUL SE 2a	.	-	.	T	-	.	.	.	-	.	.	.	-	-	-	-	-	-	-	-	.	-	.	b	.	.	.	.	.	.	.	.	.	.	.	A
VUL SE 2d	.	-	.	T	-	.	.	.	-	.	.	.	-	-	-	-	-	-	-	-	.	-	.	b	.	.	.	.	.	.	.	.	.	.	.	A
VUL SE 3c	.	-	.	T	-	.	.	.	-	.	.	.	-	-	-	-	-	-	-	-	.	-	.	b	.	.	.	.	.	.	.	.	.	.	.	A
VUL SE 1a	.	-	.	T	-	.	.	.	-	.	.	.	-	-	-	-	-	-	-	-	.	-	.	b	.	.	.	.	T	.	A	.	T	.	.	B
VUL SE 1c	.	-	.	T	-	.	.	.	-	.	.	.	-	-	-	-	-	-	-	-	.	-	.	b	.	.	.	.	T	.	A	.	T	.	.	B
VUL FR 706 1	.	-	.	.	-	.	.	.	-	.	.	.	-	-	-	-	-	-	-	-	.	-	.	a	.	.	.	.	T	.	A	.	T	.	.	B
VUL FR 709 8	.	-	.	.	-	.	.	.	-	.	.	.	-	-	-	-	-	-	-	-	.	-	.	a	.	.	.	.	T	.	A	.	T	.	.	B
VU FR 713 2	.	-	.	.	-	-	.	.	-	.	.	.	-	-	-	-	-	-	-	-	.	-	.	a2	.	.	.	.	T	.	A	.	T	.	.	B
VUL FR 706 2	.	-	.	.	-	-	.	.	-	.	.	.	-	-	-	-	-	-	-	-	.	-	.	a2	x	x	x	x	x	x	x	x	x	x	x	x
VUL NO 2a	.	-	.	.	-	.	.	.	-	.	G	.	-	-	-	-	-	-	-	-	.	***T***	.	c	.	.	.	.	.	.	.	.	.	.	.	A
VUL NO 2d	.	-	.	.	-	.	.	.	-	.	G	.	-	-	-	-	-	-	-	-	.	***T***	.	c	.	.	.	.	.	.	.	.	.	.	.	A
DOS_1	x	x	x	x	x	x	x	x	-	.	.	.	-	-	-	-	-	-	-	-	.	-	.	?	.	.	.	.	.	.	.	.	.	.	.	A
DOS_2	x	x	x	x	x	x	x	x	-	.	.	.	-	-	-	-	-	-	-	-	.	-	.	?	.	.	.	.	.	.	.	.	.	.	.	A
BIC CZ c	.	-	.	.	-	.	.	.	-	.	.	.	-	-	-	-	-	-	-	-	.	-	.	a	.	.	.	.	.	.	.	.	.	.	.	A
BIC CZ b	.	-	.	.	***A***	.	.	.	-	.	.	.	-	-	-	-	-	-	-	-	.	-	.	a3	.	.	.	.	.	.	.	.	.	.	.	A
BIC SK 1a	.	-	.	.	***A***	.	.	.	-	.	.	.	-	-	-	-	-	-	-	-	.	-	.	a3	.	.	.	.	.	.	.	.	.	.	.	A
BIC SK 1b	.	-	.	.	***A***	.	.	.	-	.	.	.	-	-	-	-	-	-	-	-	.	-	.	a3	.	.	.	.	.	.	.	.	.	.	.	A
BIC SK 2a	.	-	.	.	***A***	.	.	.	-	.	.	.	-	-	-	-	-	-	-	-	.	-	.	a3	.	.	.	.	.	.	.	.	.	.	.	A
BIC SK 2c	.	-	.	.	***A***	.	.	.	-	.	.	.	-	-	-	-	-	-	-	-	.	-	.	a3	.	.	.	.	.	.	.	.	.	.	.	A
BIC SK 3a	.	-	.	.	***A***	.	.	.	-	.	.	.	-	-	-	-	-	-	-	-	.	-	.	a3	.	.	.	.	.	.	.	.	.	.	.	A
BIC SK 3c	.	-	.	.	***A***	.	.	.	-	.	.	.	-	-	-	-	-	-	-	-	.	-	.	a3	.	.	.	.	.	.	.	.	.	.	.	A
BIC CZ h	.	-	.	.	***A***	.	.	.	-	.	.	.	-	-	-	-	-	-	-	-	.	-	.	a3	.	.	.	.	.	.	.	.	.	.	.	A
POL 1	.	***A***	A	T	-	-	.	T	-	-	.	A	T	A	G	T	T	A	G	-	-	-	.	d	.	.	.	.	.	.	R	.	.	.	A*
POL 3	.	***A***	A	T	-	-	.	T	-	-	.	A	T	A	G	T	T	A	G	-	-	-	.	d	.	.	.	.	.	.	R	.	.	.	A*
POL 5	.	***A***	A	T	-	-	.	T	-	-	.	A	T	A	G	T	T	A	G	-	-	-	.	d	.	.	.	.	.	.	R	.	.	.	A*
BOH BR a	.	***A***	.	T	-	-	.	T	-	-	.	A	T	A	G	T	T	A	G	-	-	***T***	.	e	.	.	.	.	.	.	R	.	.	.	A*
BOH BR b	.	***A***	.	T	-	-	.	T	-	-	.	A	T	A	G	T	T	A	G	-	-	***T***	.	e	.	.	.	.	.	.	R	.	.	.	A*
BOH BR e	.	***A***	.	T	-	-	.	T	-	-	.	A	T	A	G	T	T	A	G	-	-	***T***	.	e	.	.	.	.	.	.	R	.	.	.	A*
BOH SH d	.	***A***	.	T	-	-	.	T	-	-	.	A	T	A	G	T	T	A	G	-	-	***T***	.	e	.	.	.	.	.	.	R	.	.	.	A*
BOH SH i	.	***A***	.	T	-	-	.	T	-	-	.	A	T	A	G	T	T	A	G	-	-	***T***	.	e	.	.	.	.	.	.	R	.	.	.	A*
BOH SH a	.	***A***	.	T	-	-	.	T	-	-	.	A	T	A	G	T	T	A	G	-	-	***T***	.	e	.	.	.	.	.	.	R	.	.	.	A*
ANZ 1	A	-	.	.	-	.	.	T	***T***	.	.	A	-	-	-	-	-	-	-	***T***	.	-	.	f	.	.	.	.	.	.	A	.	.	.	C
ANZ 2	A	-	.	.	-	.	.	T	***T***	.	.	A	-	-	-	-	-	-	-	***T***	.	-	.	f	.	.	.	.	.	.	A	.	.	.	C
ERN 2	A	-	.	.	-	.	.	T	***T***	.	.	A	-	-	-	-	-	-	-	***T***	.	***T***	.	f1	.	.	.	.	.	.	A	.	.	.	C
ERN 692/4	A	-	.	.	-	.	.	T	***T***	.	.	A	-	-	-	-	-	-	-	***T***	.	***T***	.	f1	.	.	.	.	.	.	A	.	.	.	C
VES 1	A	-	.	T	-	-	.	T	-	-	.	A	-	-	-	-	-	-	-	***T***	.	-	A	g	.	.	.	.	.	.	A	.	.	.	C
VES 2	A	-	.	T	-	-	.	T	-	-	.	A	-	-	-	-	-	-	-	***T***	.	-	A	g	.	.	.	.	.	.	A	.	.	.	C
FIO 1	.	-	.	.	-	.	G	T	***T***	.	.	A	-	-	-	-	-	-	-	-	-	-	.	h	T	A	-	T	T	.	T	.	C	A	C	D
FIO 5	.	-	.	.	-	.	G	T	***T***	.	.	A	-	-	-	-	-	-	-	-	-	-	.	h	T	A	-	T	T	.	T	.	C	A	C	D

tigated. For the *trn*C-*psb*M locus, these two taxa and interestingly VES, also shared the same haplotype, which differed in the polyT stretch and by one transition (1660:G›A) from the most common type recorded for VUL and BIC. This transition was also present in ANZ, ERN and FIO, but these taxa differed by one position in the polyT stretch. For the *trn*T-*trn*F locus, the haplotypes of BOH and POL were the most similar differing by only one transversion (514:C›A). Despite repeated attempts, we failed to amplify the *trn*T-*trn*F locus in samples of DOS; however, the two other loci were identical to those of the most common haplotype-a. The presence of a unique 7 nt duplication at the *trn*Q-*rps*16 locus and specific transversion at the *trn*T-*trn*F locus (Table 7) indicate that POL was derived from BOH. Although locus *trn*K-*mat*K contains two variable positions, neither of them was useful for the unambiguous discrimination of BOH due to the pattern in the polyT stretch at position 480. The transversion (713:C›A) differentiated VES from the rest of the investigated samples (Table 7, for details see Supplementary Data S2).

Discussion

Karyological and cytological differentiation

The somatic chromosome number 2n = 64 for *P. vulgaris*, corresponding to an octoploid cytotype, is repeatedly reported and confirmed for individuals from nearly its entire distribution, i.e. from the USA (Wood Jr. & Godfrey 1957), throughout Greenland (Jørgensen et al. 1958) and Spain (Zamora et al. 1996) and up to Slovakia (Murín 1976a, b; for more works see Heslop-Harrison 2004). The octoploid level and Holarctic distribution imply that *P. vulgaris* is among the evolutionarily youngest species within the whole genus (Casper 1966, Casper & Stimper 2009, Shimai et al. 2021). The reported chromosome count for *P.* **anzalonei* is the first confirmation of its presumed octoploid level (Casper & Stimper 2009). This indicates that there is only one ploidy level, 2n = 8x, for the *P. vulgaris* agg., even for individuals from the southern relictual part of its broad distribution. On the other hand, there is persistent uncertainty about the ploidy level of *P. bohemica* (tetraploid vs. octoploid: Bělohlávková 1989, Studnička 1989, Krahulcová & Jarolímová 1991, Studnička & Hejný 1992). In an extensive karyological study of the genus *Pinguicula*, both counts (2n = 32 and 2n = 64) are reported (Casper & Stimper 2009). The tetraploid chromosome number is viewed as the main evidence for the delimitation of *P. bohemica* as a distinct species not conspecific with *P.* **vulgaris*. Our karyological analysis confirmed 2n = 64 chromosomes (Fig. 3) for *P. bohemica* and *P.* "*polonica*". In addition, FCM measurements confirmed that all the individuals of BIC, BOH and VUL analysed were of the same DNA-ploidy level, i.e. 2n ≈ 8x (Table 3). Due to the rarity of *P. bohemica*, only a few individuals from natural populations were analysed; thus, if any other ploidy levels occur it is possible we did not detect them. Nevertheless, it seems that the populations of *P. bohemica* are at least predominantly, if not exclusively, octoploid. The determined chromosome count of 2n = 64 for *P. fiorii* is consistent with previous findings reported by Casper & Stimper (2009). These two independent counts contradict the 2n = 32 reported by Tammaro & Pace (1987, p. 432).

Genome size, as an intrinsic feature of all living organisms, often reveals infra-taxonomic diversity, sometimes even in the absence of morphological variation (e.g. Štubňová et al. 2017, Kolarčik et al. 2018, Prančl et al. 2018) and this also applies to the *P. vulgaris* agg.

Very similar genome sizes of the investigated taxa of *P. vulgaris* agg. points to their close evolutionary relationship, which is also supported by molecular analyses. The genome sizes of nine species of *P.* sect. *Pinguicula* has been recorded, including *P. *vulgaris* and *P. bohemica*. Veleba et al. (2014) report almost identical genome sizes for both taxa, which does not agree with our results. The reported values are also lower than our values. This could be attributed to a different method of genome size derivation based on internal standards (see Šmarda et al. 2019 for explanation) and the use of different internal standards (i.e. *Solanum lycopersicum* by Veleba et al. 2014). After recalculation of the values to *Glycine* (the standard we used), the genome size of *P. bohemica* corresponded well with our data, but the estimate for *P. vulgaris* was still lower.

Although the investigated taxa of *P. vulgaris* agg. have similar genome sizes, they can be clearly differentiated (e.g. *P. *bicolor* vs. *P. bohemica*; Table 4, Fig. 4B). Therefore, the genome size may help with the identification of taxa in sect. *Pinguicula* independent of their morphology and molecular data.

Genetic differentiation within the Pinguicula vulgaris agg.

Even though AFLPs are dominant, the method is very useful for population genetic studies and the discrimination of evolutionarily young and closely related taxa (e.g. Kolarčik et al. 2010, Hodálová et al. 2015, Kitner et al. 2015, Kolář et al. 2015, Majeský et al. 2015). In the present study, AFLP analysis revealed clear differentiation of all the investigated taxa from *P. vulgaris* agg. The surprising distinctiveness of *P. *vestina* relative to the *P. vulgaris* agg. (Fig. 7) may point to a possible hybrid origin of this taxon, although data from both sequenced loci (nuclear and chloroplast) did not suggest this. A possible explanation for this may be the homogenization of the ITS locus (through the process of concerted evolution; e.g. Wendel et al. 1995, Alvarez & Wendel 2003, Harpke & Peterson 2006) to the maternal type represented by *P. *vulgaris*. However, AFLPs that target the whole genome may uncover a hybrid origin more easily than the sequencing of a single nuclear locus. The intermediate morphology of many Mexican and European butterwort taxa probably stems from introgressive hybridization, which influenced the genus diversification (Shimai et al. 2021). On the other hand, AFLPs also point to another process that markedly shaped the diversity of this genus. The process of geographic isolation may have contributed to development of unique molecular and morphological characteristics in butterworts (Conti & Peruzzi 2006, Crespo et al. 2018, 2020, Shimai et al. 2021). The level of differentiation within the central European representatives of *P. vulgaris* agg. is less pronounced than that between the Italian taxa (Supplementary Table S4, Supplementary Fig. S1). This most likely points to a more recent divergence of central European taxa, as well as recent and imperfect isolation due to their ongoing contact (especially between VUL and BIC).

Data from the sequenced loci supported AFLP data and confirmed that the investigated taxa within *P. vulgaris* agg. represent distinct lineages, including *P. bohemica*. The presence of different ITS variants in *P. bohemica* may point to an incomplete concerted evolution of ITS variants (in contrast to *P. *vestina*) after hybridization, followed by heterozygosity fixation due to restricted gene flow and small effective population size. The identified ribotype-C, specific for *P. *anzalonei*, *P. *ernica* and *P. *vestina*, together with the most common ribotype-A, represent a fully compatible combination for

ribotype-A* specific for *P. bohemica*. It is possible that ribotypes A and C were once more common at higher latitudes in central Europe and became extinct or retreated only recently (during the Holocene) to refugia in the central Apennines. This is interesting in light of the possible origin of *P. bohemica*, which could represent a hybrid line between the relict southern (ribotype-C) and common widespread northern (ribotype-A) lineages of *P. vulgaris*. During the Quaternary climatic oscillations, distinct genetic lineages of *P. vulgaris* had repeated opportunities to meet and hybridize in central Europe. Considering the data from sequenced cpDNA loci, this scenario seems to be even more plausible due to the higher similarity of haplotype-e (specific for *P. bohemica*) to haplotypes-f/f1 and -g (specific for ANZ, ERN and VES). However, such a hybrid origin of *P. bohemica* is only speculative at this time, but cannot be ruled out.

One highly interesting and unexpected finding was the discovery of a pronounced molecular differentiation of samples of *P.* "*polonica*" from BIC samples and a high similarity to BOH samples. The locality Dąbrowa Górnicza is an abandoned sandpit with relictual flora and vegetation of calcium-rich groundwaters, which support many endangered species such as *Liparis loeselii* (L.) Rich., *Epipactis palustris* (L.) Crantz, *Malaxis monophyllos* (L.) Sw., *Eleocharis quinqueflora* (Hartmann) O. Schwarz, *Cladium marsicus* (L.) Pohl, *Utricularia bremii* Heer (Czylok et al. 2008, Krajewski 2011, Krajewski & Płachno 2015) and the rare arctic-alpine moss species *Drepanocladus turgescens* (T. Jensen) Broth. (Krajewski 2017). The area was heavily exploited for sand, which was used to fill space in coal mines (e.g. Czylok et al. 2008), but the exploitation ceased in approximately 1985 (Rahmonov & Szymczyk 2010). The vegetation in parts of sandpits that did not undergo reclamation has characteristics of primary succession with spontaneous introduction/regeneration of vegetation cover. The recent introduction of *Pinguicula* to this locality is not very likely, although the oldest specimens from this locality deposited in the KTU herbarium date back only to 1981 (personal communication). Its natural introduction from neighbouring localities is more probable.

Morphological and ecological differentiation within Pinguicula vulgaris agg.

Butterworts, in general, lack conspicuous morphological differentiation (Legendre 2000, Roccia et al. 2016). This is especially true for the temperate species of sect. *Pinguicula*. The most useful traits for species recognition are generative structures, including differences mainly in the size and position of corolla lobes, corolla size and colour, shape of calyx, shape of fruits, etc. (e.g. Conti & Peruzzi 2006, Ansaldi & Casper 2009, Crespo et al. 2018, 2020). However, the plasticity of these traits is high in central European populations of *P. vulgaris* agg. (Bernátová 2010). Morphological differentiation between the four taxa (BOH/BIC/POL/VUL) defined in our molecular analyses is very weak. In practice, only the shape of sepal lobes is a useful trait for the differentiation of living plants of *P. bohemica* from *P. *bicolor* (Fig. 2, Table 1). Other morphological traits differentiating *P. bohemica* from *P. *vulgaris* and/or *P. *bicolor* (see Studnička 1981, Studnička & Hejný 1992) are unreliable. However, the shape of the calyx lobes of none of the plants of *P.* "*polonica*" was wide and obtuse (thus bohemica-like) but narrow and pointed (thus vulgaris/bicolor-like). It is possible that this trait became unique and diagnostic for *P. bohemica* by drift only after the separation of these two lineages. On the other hand, the lack of private morphological traits in *P. *bicolor*, other than bicolour colouration, may

be explained by the absence of spatial isolation (and possible ongoing gene flow). Although seeds of all species from sect. *Pinguicula* are very similar, they are sometimes useful for taxa differentiation (e.g. see Zamora et al. 1996, Degtjareva et al. 2004, Conti & Peruzzi 2006, Shuka et al. 2007, Ansaldi & Casper 2009, Bacchetta et al. 2014, Crespo et al. 2018, 2020, Shimai 2021). Scanning electron microscopy of seeds showed a high degree of similarity among the three investigated taxa (BIC, BOH, VUL), with the only remarkable difference being an overall higher variation in the size and shape of VUL seeds (Fig. 5). This is in line with the detected variation in genome size, which is expected to correlate with seed mass (Bennett 1987).

Diversification of habitat preferences and/or resource utilization in geographically peripheral populations is common in closely related taxa and is one of the key features responsible for the evolutionary divergence between endemic species and their widespread congeners (e.g. Lavergne et al. 2004, Buira et al. 2020). Investigation of the environmental differentiation between two sympatric species, endemic *P. arvetii* Genty and widespread *P. vulgaris*, in the southwestern Alps showed significant environmental differences, with altitude being the most significant indicator of those measured. The overall reproductive and population performance is influenced differently by the individual and population characteristics of the two species (Bertolini et al. 2016). The ecological differentiation of *P. bohemica* and *P. *vulgaris* is discussed in detail in Studnička (2013), where *P. bohemica* is described as more basiphilous than *P. *vulgaris*, although this differentiation is not strong. In addition, there seems to be no ecological differentiation between these two taxa and *P. *bicolor* and *P.* "*polonica*" (Table 1). *Pinguicula *bicolor* is frequently found growing sympatrically with the typical blue-violet-flowered *P. *vulgaris*. A similar preference for different substrates (siliceous versus calcareous) is also reported between *P. *vulgaris* versus *P. fiorii*, *P. *ernica*, *P. *anzalonei* and *P. *vestina* (e.g. Conti & Peruzzi 2006).

The role of isolation in speciation within Pinguicula vulgaris agg.

Geographic isolation seems to play an important role in speciation within the genus *Pinguicula* (Shimai et al. 2021) and *P. vulgaris* agg. The more pronounced the isolation the more distinct the characteristics (morphological and molecular/genetic) that develop and become fixed, e.g. as represented by *P. *anzalonei*, *P. *ernica* and *P.*vestina*. However, if the spatial isolation is not perfect and populations are in contact, then the divergence is slowed by the homogenization effect of occasional gene flow.

Basically, there are two contrasting opinions on the origin of *P. bohemica*. One of the hypotheses is based on the concept that *P. bohemica* is a tetraploid (Studnička1981, Studnička & Hejný 1992). This hypothesis supposes migration of an ancestor of *P. bohemica* to Bohemia from southern Europe, which inhabited lowland areas corresponding to the present range of *P. bohemica*. This would imply that *P. bohemica* is evolutionarily older than the octoploid taxa of *P. vulgaris* agg. and should express greater differentiation from *P. vulgaris* than we recorded. Another hypothesis supposes that *P. bohemica* evolved from isolated populations of *P. vulgaris* that survived the early postglacial period at suitable sites in lowlands (whereas the other populations were at higher altitudes) and developed into a stabilized lineage, whereas the current sympatric occurrence with *P. vulgaris* represents a secondary contact zone (Hadač 1977).

Our results favour the latter hypothesis and suggest that *P. bohemica* is probably a relict lineage, which arose via hybridization between southern (represented by ribotype-C) and northern (represented by ribotype-A) lineages (Table 7). Data from the sequenced cpDNA loci support this finding. Further divergence of *P. bohemica* could be directed by differences in the spectrum of pollinators (due to different patterns of flower colouration) or predominant autogamy, which led to assortative mating. This strengthened the differentiation between flower morphs and resulted in a gradual ecological differentiation and speciation. Hybridization is common among European butterworts (Roccia et al. 2016) and the absence of an effective reproductive barrier suggests recent and rapid radiation of this genus in Europe (Shimai et al. 2021). This also supports hybridization between *P. bohemica* and *P. vulgaris* (as already pointed out by Krahulcová & Jarolímová 1991). The putative hybrid taxon, *P. *dostalii*, is known only from two sites in central Bohemia (Polabská černava near Mělník and Hrabanovská černava near Lysá nad Labem), where both parental taxa co-occurred in the past (Bělohlávková 2000). The hybrid origin of *P. *dostalii* is supported by our results, but the very limited amount of material renders this result inconclusive. Assuming maternal inheritance of cpDNA and the observed haplotype, *P. *vulgaris* was the seed parent.

Gradual differentiation of relic populations would also explain the origin of *P.* "*polonica*". We assumed that lineage haplotype-e, specific to *P. bohemica*, was more common in the past (through the Pleistocene up to the early to middle Holocene) in the area of northern Bohemia and southern Poland. The onset of the rapid spread of dense forests during the early Holocene, which culminated in the middle Holocene, might have caused the fragmentation of suitable habitats and the retreat of species of calcium-rich fens and bogs to relic sites maintaining open vegetation during the late Holocene. The subsequent diversification was directed mainly by isolation and genetic drift.

Taxonomic conclusion

The delimitation of species, subspecies and varieties of the majority of European butterworts is hampered by a low level of structural differentiation (Legendre 2000, Roccia et al. 2016). Characteristics of their vegetative parts (leaves and scapes) are of limited use, serving only to distinguish between isophyllous and anisophyllous species. Although further traits are found in the reproductive organs, they are frequently rather variable, with a high overlap between-species (Conti & Peruzzi 2006, Ansaldi & Casper 2009, Crespo et al. 2020). Chromosome numbers divide European butterworts into only three major groups corresponding to diploid, tetraploid and octoploid levels (Legendre 2000, Casper & Stimper 2009). Therefore, individual taxa are frequently distinguished by their geographic distribution, which is often rather restricted and well separated from that of the other taxa (e.g. Conti & Peruzzi 2006, Crespo et al. 2018). Molecular markers play an important role in modern plant taxonomy, with butterworts being no exception (e.g. Crespo et al. 2020). Single locus sequence analysis is not suitable for recognizing closely related species (e.g. Table 7, or De Castro et al. 2016). However, the combination of multilocus sequence analysis with a genome-wide skimming technique (AFLP in our case) seems to have enough resolution power for discrimination of different genetic lineages within polymorphic species complexes, such as *P. vulgaris* agg. Correspondingly, genome size appears also to offer taxonomically important information.

What, however, remains questionable is the taxonomic value and implication of the observed differences? Distinctive morphology that is useful for recognition should be a guide for the delimitation of taxa. However, the rapid development of genuine molecular techniques in recent decades has revealed the presence of cryptic species complexes, in which particular taxa are distinguishable only on a molecular basis (e.g. Carstens & Satler 2013). However, the taxonomic value of such taxa may remain questionable. The level of genetic differentiation of *P. bohemica* is somewhat lower but comparable to the differentiation of the three *P. vulgaris* agg. taxa from central Italy (*P. *anzalonei*, *P. *ernica*, *P. *vestina*). Although the Italian taxa are accepted as distinct subspecies, the central European taxa are subject to endless debates concerning their taxonomic value. They are sometimes classified as different species or forms or are not recognized as different at all (*P.* "*polonica*"). Among them, the most distinctive are *P. bohemica* and *P.* "*polonica*", and the latter seems to have diversified rather recently in isolation directly from *P. bohemica*. *Pinguicula *dostalii* seems to be a hybrid of *P. *vulgaris*♀ × *P. bohemica*♂. Although *P. *bicolor* is considered to be only a morphological variety of *P. vulgaris*, it is a genetically distinct unit within *P. *vulgaris*.

There are two possible taxonomic approaches to address this situation. The first is to treat *P. vulgaris* agg. as a single highly variable and polymorphic species (*P. vulgaris*) without recognition of any infraspecific taxa. This approach would stabilize the taxonomy of the group and remove the difficulties with the identification of extremely similar taxa. On the other hand, the existing unique lineages would remain formally unrecognized and might receive less attention and legal protection, eventually facing extinction. In contrast, the distinct units within the *P. vulgaris* agg. with a similar degree of morphological and genetic differentiation from typical *P. vulgaris* can be treated as separate taxa, preferably at the same taxonomic rank. This approach would ensure that these units receive formal recognition that better reflects the evolutionary history of the group being classified; therefore, we strongly prefer this approach. Concerning the current taxonomy of European butterworts and the degree of morphological and genetic differentiation within *P. vulgaris* agg., we suggest the following treatment. *Pinguicula bohemica* should be treated as *P. vulgaris* subsp. *bohemica* (Kraj.) Domin. Morphologically, it can be readily distinguished by the shape of its sepals from VUL, POL and BIC and has a clearly defined geographical range. *Pinguicula *bicolor* should be treated as a variety *P. vulgaris* var. *bicolor* Nordst. ex Fr. This name should be used for individuals with whitish blue-violet corollas found often sympatrically with the typical blue-violet-flowered morphotype of *P. *vulgaris*. Although our molecular analyses revealed some degree of differentiation, the differences seem to be under simple genetic control and arise repeatedly. The taxon *P.* "*polonica*" requires further study, which would involve broader sampling of *P. *vulgaris* within Poland. Although the results of genetic analyses seem to be conclusive, the individuals investigated lack morphological characters pointing to their direct connection with *P. bohemica* and/or differentiating it from *P. *bicolor*. Thus, at the moment, and based on current knowledge, we do not suggest its formal recognition. Finally, *P. *dostalii* should be treated as *P. vulgaris* nothosubsp. *dostalii* (Bárta) ined.

We are convinced that classification should reflect the evolutionary processes that shaped the taxonomic diversity of a particular group and that hybridization and spatial isolation seem to be the most important evolutionary forces responsible for diversification/speciation at the same ploidy level in butterworts.

Supplementary materials

Table S1. – AFLP primer combinations used for selective and preselective amplification.
Table S2. – Loci tested in the present study, annealing temperature, primers used, length of the aligned sequences, note about the detected sequence polymorphisms and reference for the source of the primers.
Table S3. – GenBank accession numbers for the investigated loci and samples.
Table S4. – Molecular differentiation based on pairwise F_{ST} computed from AFLP data.
Fig. S1. – Pairwise F_{ST} differentiation based on AFLP data.
Data S1. – Final alignment of the ITS region of the investigated species of *Pinguicula* and GenBank records.
Data S2. – Final alignment of the *trn*K-*mat*K region of the investigated species of *Pinguicula* and GenBank records.

Supplementary materials are available at www.preslia.cz

Acknowledgements

We would like to thank F. Trnka and F. Bartolucci for kindly providing pictures for Fig. 2. The research was supported by the European Social Fund, The Education for Competitiveness Operational Programme (CZ.1.07/2.3.00/30.0004). This research was also partially financially supported by the Ministry of Science and Higher Education of Poland within the statutory activities of the Department of Plant Cytology and Embryology, Institute of Botany, Faculty of Biology, Jagiellonian University in Kraków (N18/DBS/000002). L. Kobrlová and M. Jandová are acknowledged for their assistance with flow cytometry and L. Vaculná for preparation of Figure 1. Finally, we would like to acknowledge the Ministry of the Environment of the Czech Republic, Regional Authority of Liberec Region, and Nature Conservation Agency of the Czech Republic for providing exceptions from nature conservation guidelines to facilitate the collection of wild plant material in the Czech Republic. We would also like to acknowledge both The Regional Directorate for Environmental Protection in Kraków and The Regional Directorate for Environmental Protection in Katowice for providing exceptions from nature conservation guidelines for the collection of protected species in Poland.

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Pinguicula vulgaris ve střední Evropě – kdy se jeden druh mění v jiný?

Pinguicula vulgaris agg. tvoří v Evropě šest blízce příbuzných a navzájem velmi podobných taxonů. Kromě široce rozšířené *P. vulgaris* var. *vulgaris* jsou to *P. vulgaris* var. *bicolor,* vyskytující se roztroušeně v části areálu nominátní variety, *P. bohemica*, endemický taxon Polabí, a tři stenoendemické poddruhy *P. vulgaris* subsp. *anzalonei*, *P. vulgaris* subsp. *ernica* a *P. vulgaris* subsp. *vestina*, které rostou ve střední Itálii. Taxonomická hodnota jednotlivých taxonů je některými autory zpochybňována nebo naopak nadsazována. Příkladem je *P. bohemica*, která je některými autory považována za samostatný druh, zatímco jinými není jako samostatný taxon uznávána vůbec. Pojetí *P. bohemica* jako samostatného druhu podporuje zejména uváděný tetraploidní počet chromozomů, zatímco pro *P. vulgaris* s. str. je to počet oktoploidní. Tetraploidie je však u *P. bohemica* zpochybňována studiemi, které zjistily oktoploidní počet chromozomů. V naší studii jsme se zaměřili na rozlišení taxonů v rámci *P. vulgaris* agg. za pomoci molekulárních metod (AFLP, sekvenování úseků jaderné a chloroplastové DNA), průtokové cytometrie (stanovení ploidie a velikosti jaderného genomu), počítání chromozomů a studia morfologie semen pomocí skenovací elektronové mikroskopie. Všech šest studovaných taxonů mělo stejnou ploidii, včetně *P. bohemica*, u které byla plodie potvrzena také karyologicky počítáním chromozomů. Některé taxony (např. *P. vulgaris* var. *bicolor* vs. *P. bohemica*) se však lišily velikostí genomu. Rozdíly ve velikosti a povrchové struktuře semen studovaných druhů nebyly zjištěny. Molekulární markery ukázaly na velmi blízkou příbuznost všech studovaných taxonů. Všech šest taxonů však vytváří samostatné a jednoznačně odlišitelné skupiny. Poněkud překvapivé bylo zjištění, že populace z okolí obce Dąbrowa Górnicza v jižním Polsku se jasně vyčleňuje jako další samostatná linie. Rostliny této populace jsou morfologicky velmi podobné jedincům *P. vulgaris* var. *bicolor*, avšak na molekulární úrovni vykazují podobnost s *P. bohemica*. V evoluci celé skupiny pravděpodobně hrají významnou roli hybridizace a geografická izolace, které způsobují rozrůznění jednotlivých linií. Míra rozrůznění na molekulární i cytologické úrovni u jednotlivých taxonů v *P. vulgaris* agg. je nejvýraznější u *P. vulgaris* subsp. *anzalonei*, *P. vulgaris* subsp. *ernica*, *P. vulgaris* subsp. *vestina* a *P. bohemica*. Proto navrhujeme hodnotit tyto taxony na stejné taxonomické úrovni, tedy jako poddruhy. Linie, zde provizorně označována jako *P.* "*polonica*", vyžaduje další výzkum. Prozatím jí nepřiřazujeme žádnou taxonomickou kategorii. Pro taxon *P. vulgaris* var. *bicolor* navrhujeme zachování dosavadního ranku variety.

How to cite: Majeský Ľ., Hroneš M., Kitner M., Válová L., Mártonfiová L., Płachno B. J., Conti F. & Dančák M. (2022) *Pinguicula vulgaris* in central Europe: when does one species turn into another? – Preslia 94: 275–304.

Preslia, a journal of the Czech Botanical Society

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