Cirsium greimleri: a new species of thistle endemic to the Eastern Alps and Dinarides

***Cirsium greimleri* – nový endemický druh pcháče pro východní Alpy a Dinárské hory**

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Bureš P., Šmerda J., Michálková E., Šmarda P., Knoll A. & Vavrinec M. (2018): *Cirsium greimleri*: a new species of thistle endemic to the Eastern Alps and Dinarides. – Preslia 90: 105–134.

The newly described diploid species *Cirsium greimleri* (2n = 34; 2C = 1929.1±60.6 Mbp) belongs to *Cirsium* sect. *Cirsium* and is distributed sparsely throughout the Eastern Alps and Dinarides, whereas the closely related tetraploid vicarious species *C. waldsteinii* (2n = 68; 2C = 3682.3±69.8 Mbp) is endemic to the south-eastern Carpathians. The ploidy, genetic and morphological separation of both taxa is confirmed using flow cytometry, AFLP and morphometric analyses of 169 plants from 27 populations covering representatively distribution ranges of the respective species. The species differ in flower colour, with those of *C. greimleri* ruby red to brownish-purple and those of *C. waldsteinii* pinkish-purple to purple. The colour difference remains consistent when both species are cultivated together under the same conditions. Differences between the species in the size of the stomata, achenes, corollas, styles and pappus are statistically significant and congruent with differences in the ploidy level. Because both species are gynodioecious (their populations contain female and hermaphrodite plants), the generative features should be compared carefully with respect to gender (e.g. females with females) because the between-gender differences within the same species could be larger than the between-species differences. The basal and median cauline leaves of *C. waldsteinii* are narrower and more deeply lobed than those of *C. greimleri*. A slight difference in the flowering period is detected when both species are cultivated together, with *C. waldsteinii* flowering two weeks earlier than *C. greimleri*. Both species share ecological/habitat preferences for subalpine woodland tall-forb vegetation. While *C. waldsteinii* hybridizes extremely rarely with co-occurring diploid congeners, *C. greimleri* produce hybrids very often, which increase the risk of its extinction via genetic erosion.

Keywords: AFLP, allopatric speciation, Alpine endemics, *Asteraceae*, *Carduoideae*, Carpathian endemics, *Compositae*, *Cynareae*, interspecific hybridization, plant taxonomy, polyploid speciation

Introduction

With 250–300 species, *Cirsium* is considered one of the most species-rich genera of *Asteraceae* (Zomlefer 1994, Susanna & Garcia-Jacas 2007). Based on recent critical taxonomic revisions (e.g. Menitskij 1996, Keil 2006, Zhu & Greuter 2011, Yildiz et al. 2016) and the new species described in recent decades (particularly from Turkey and Japan), the estimated richness could be between 400 and 450 species (Roskov et al. 2017). *Cirsium* species occupy a wide range of open habitats in the Northern Hemisphere, where they are

doi: 10.23855/preslia.2018.105

relatively well protected against large herbivores by prickles on leaf lobes and involucral bracts (phyllaries). During the postglacial era, the natural range of *Cirsium* species was often substantially (positively or negatively) influenced by human activities, particularly in the temperate zone. An expanded distribution is most apparent in cosmopolitan weedy species, such as *C. arvense* and *C. vulgare*, as well as in meadow species, such as *C. palustre*, *C. oleraceum*, *C. heterophyllum* and *C. canum*. Although, these common thistles are closely associated with the overall image of the genus, the overwhelming majority of *Cirsium* species have much narrower natural ranges, and stenoendemic taxa are not exceptional in this genus (cf. Werner 1976, Wagenitz 1987, Meusel & Jäger 1992, Keil 2006, Zhu & Greuter 2011). Across the range of this genus, species diversity is highest in the mountains of Europe, the Mediterranean, south-western Asia (Caucasus and Anatolia), Central Asia, China, Japan and North America (Meusel & Jäger 1992). Because these "evolutionary hotspots" differ in their thistle species compositions, many *Cirsium* species are endemic to mountain areas, in which allopatric speciation has been promoted by extensive long-term isolation among mountain ranges. In Europe, this is the case of some species from *Cirsium* sect. *Eriolepis,* which are endemic to the mountains on the Iberian, Apennine or Balkan Peninsulas, and species from *Cirsium* sect. *Cirsium*, such as Alpine *C. spinosissimum,* which has close relatives in the Pyrenees (*C. glabrum*), Apuan Alps (*C. bertolonii*) and Alpe della Luna Mts (*C. alpis-lunae*). Similarly, the Eastern Alpine species *C. carniolicum* has a vicariant species in the Pyrenees (*C. rufescens*). In contrast, taxonomically evaluated differentiation has not been recognized in the disjunctively distributed mountain species *C. alsophilum* (= *C. montanum*; Southern Alps, Appenines and Dinarides) or in *C. waldsteinii*, the natural range of which is divided into three even more isolated parts – the Eastern Alps, Dinarides and Carpathians. The study of speciation processes in these taxa is partly limited by their rare occurrence in nature as well as by their low representation in herbaria.

Among the Old World *Cirsium* species, three basic ploidy levels can be distinguished: diploid (2n = 34), tetraploid (2n = 68) and hexaploid (2n = 102; Rice et al. 2015, Watanabe 2017). Because diploid species strongly prevail (≈80%), polyploidy has a limited effect on speciation within this genus compared with other species-rich genera of *Asteraceae* (e.g. *Artemisia*, *Aster*, *Achillea*, *Centaurea*, *Hieracium*, *Pilosella*, *Senecio*, *Taraxacum*). The notably low abundance of polyploids in *Cirsium* is rather inconsistent with the frequent natural interspecific hybridization, particularly among European species (Wagenitz 1987, Bureš et al. 2004, 2010, Segarra-Moragues et al. 2007). Fifty-one of 73 recently accepted species in Europe have been investigated karyologically, and 43 of these species are diploid, two are tetraploid (*C. appendiculatum*, *C. vulgare*) and two dysploid (*C. baytopae*, 2n = 24; and *C. tymphaeum*, 2n = 32), after excluding exceptional polyploid metaphases or individuals and clear determination errors (Czapik 1958, Skalińska et al. 1974, Bureš et al. 2004, Rotreklová et al. 2004, Rice et al. 2015, Watanabe 2017). In the remaining four species, *C. ciliatum*, *C. italicum*, *C. ligulare* and *C. waldsteinii*, both diploid and tetraploid levels are recognized (Rice et al. 2015, Watanabe 2017). In the first case, chromosomes were counted in plants outside Europe (Tonian 1981, Özcan et al. 2011), and the reliability of the next two is rather questionable (Kuzmanov et al. 1991, Romano et al. 1994, Yuksel et al. 2013). However, in *C. waldsteinii*, the presence of both ploidy levels was clearly demonstrated, with tetraploidy (2n = 68) initially reported by Czapik (1958) and later Mizianti & Frey (1973) from the Polish

Carpathians (Bieszczady Zachodnie) and then confirmed by Pashuk (1987) in the Carpathians in western Ukraine (Chernogora). The only count from the separated Alpine part of this species distribution range (Dobeš et al. 1996) is surprisingly diploid (2n = 34). Karyological data are lacking for the species from the Dinaric part of its distribution (Rice et al. 2015, Watanabe 2017).

In this study, we addressed the following questions. (i) Do the diploids and tetraploids of *C. waldsteinii* differ in their geographic distributions? If so (ii), are the cytotypes separated genetically or morphologically? (iii) Do these differences support their taxonomical separation?

Material and methods

Sampling and herbarium study

The plant material used for the morphological, genetic and flow cytometric analyses was collected in the Eastern Alps of Austria and Slovenia, the Dinarides of Serbia, Bosnia and Herzegovina and the Eastern and Southern Carpathians in Slovakia, Poland, Ukraine and Romania from 2015 to 2017 (Appendix 1). Interspecific hybrids, easily recognizable morphologically, recorded in some Alpine and Slovenian populations were not included in the study but collected for further analysis of hybridization in *Cirsium* (Michálková et al., in prep.). Voucher specimens were deposited in the Herbarium of Masaryk University in Brno, Czech Republic (BRNU), from which some specimens of other collectors were used for the morphometric analyses (as indicated in respective electronic appendices by BRNU specimen numbers). The type material of *C. waldsteinii* was studied in Kitaibel's collection kept in the Herbarium of the Hungarian Natural History Museum in Budapest (BP) in October 2016, and live plants were studied at the locus classicus in the Rodna Mts in June 2017. Type specimens of the newly distinguished species were deposited in herbaria in Brno, Vienna, Prague and Budapest (BRNU, PRC, WU and BP). Acronyms for the herbaria follow Index herbariorum (Thiers 2017), nomenclature of European *Cirsium* species follows Flora Europaea (Werner 1976) and English toponyms of Eastern Alpine mountains follow the main units and subgroups of division made by Grassler (1984).

Cultivation experiment

To test flower colour stability, several samples from populations no. 3, 5, 6, 17, 18, 19 and 24 (Appendix 1) were transferred to the experimental garden of the Department of Botany and Zoology, Masaryk University, Brno, Czech Republic (49°10'44.2"N, 16°34'20.4"E, 268 m a.s.l.) and cultivated starting in 2015. Five ramets of one genet transferred from population 3 (Appendix 1) were used for preparation of type material of the newly distinguished species in June 2017.

Flow cytometry: ploidy level, genome size and genomic GC estimation

For the flow cytometric analyses, the samples were prepared according to the protocol of Šmarda et al. (2008) and measured on two CyFlow flow cytometers (Partec GmbH; recently Sysmex) using the internal standards *Bellis perennis* (2C = 3089.89 Mbp, 39.54% GC; Šmarda et al. 2014) and *Solanum lycopersicum* 'Stupické polní tyčkové

rané' (2C = 1696.81 Mbp, 38.72% GC; Veselý et al. 2012), whose genome sizes and genomic GC contents were derived by comparisons with the completely sequenced *Oryza sativa* subsp. *japonica* 'Nipponbare' (International Rice Genome Sequencing Project 2005). For the genome size or ploidy level estimations, propidium iodide was used as a fluorochrome, and for the genomic GC content estimation, the samples were co-processed with DAPI (4',6-diamidino-2-phenylindole) fluorochrome. The genomic GC content was calculated using an ATGCFlow spreadsheet (http://www.sci.muni.cz/botany/systemgr/ download/Festuca/ATGCFlow.xls; Šmarda et al. 2008). Each sample was analysed once. These individual measurements were consequently averaged per species. For the primary dataset, including the cytometer settings, see Electronic Appendices 1 (genome size) and 2 (genomic GC percentage).

Genetic relationships among populations and individuals

Genomic DNA was extracted from deep-frozen leaves using the commercial kit NucleoSpin Plant II (Marchery-Nagel) with extraction buffer PL2 according to the manufacturer's instructions. AFLP fingerprinting was performed according to the protocol in the AFLP™ plant mapping kit (Applied Biosystems) with the following modifications and specifications. The restriction-ligation reaction was prepared with genomic DNA, EcoRI and MseI restriction enzymes and EcoRI and MseI adaptors at 37 °C for 4 h. The preselective amplification was done with EcoRI+A and MseI+C primers in a GenePro thermocycler (Bioer). Four selective primer pairs were chosen: 6-FAM-EcoRI+ACT/MseI+CTC, VIC-EcoRI+AGG/MseI+CAG, NED-EcoRI+AGC/MseI+CAT and PET-EcoRI+ACA/MseI+CTA. Selective amplification products were mixed with a GS500 size standard and Hi-DiTM Formamide (Applied Biosystems) for the fragment analysis on an ABI 3500 Genetic Analyzer (Life Technologies). Sizing and scoring of the raw data were performed automatically using GeneMarker 2.4.0 (SoftGenetics). A panel of scorable peaks (loci) for each primer combination was created manually. Fragments from 60 to 600 bp were scored automatically and then manually checked and corrected. Data reproducibility was assessed from 23 replicate samples and estimated according to Bonin et al. (2004). Nonreproducible fragments and fragments present in only one individual were removed from the dataset.

The genetic relationships among the samples were visualized as a NeighborNet network using the SplitsTree software 4.14.4 (Huson & Bryant 2006) based on uncorrected p-distances. An analysis of molecular variance (AMOVA) was processed using Arlequin v. 3.11 software (Excoffier et al. 2005) to evaluate the distribution of genetic variation within and among populations and among cytotypes. The significance of the respective variance components were tested using 10,100 random permutations. For the purpose of AMOVA, populations less than 2 km apart were merged (3+4, 17+18, 23+24) and populations 2 and 26 containing single individuals were omitted (Electronic Appendix 3). For each population, the genetic diversity (Nei 1987) was calculated (Electronic Appendix 3) using the R-script AFLPdat (Ehrich 2006).

Flower colour detection

The overall differences in the flower colours were estimated based on field observations and photographs. For more exact colour descriptions, the codes and terms from the Methuen Handbook of colour (Kornerup & Wanscher 1989) were independently applied to the plants cultivated in the experimental garden by three people (first three authors of this paper) in dispersed afternoon light. Each person selected 2–6 colour codes for each of two cytotypes: initially for flowers in full anthesis and subsequently for dying flowers. Codes were summarized with respect to cytotypes/species and phenophases with the same summary weight for each evaluator independent of how many codes were selected by a particular person (Electronic Appendix 4 contains the primary dataset).

Measurements of stomatal guard cells

The stomatal size (guard cell length, stomatal width, stomatal area) was measured from surface impressions via the microrelief method: a thin layer of transparent nail polish was applied to the leaf surface and after it dried the layer was removed with adhesive tape and placed on a microscope slide. The slides were observed with an Olympus BX-51 microscope under 400× magnification. Digitally documented slides were analysed manually in the Olympus Cell^F program. Only the middle part of the abaxial (under) and adaxial (upper) surfaces of fully developed/mature median leaves were used for the surface impressions. On each slide 20–40 stomata were measured (Electronic Appendix 5).

Morphometrical analyses

The generative features of the corolla (length, narrow corolla tube length, length of broad "campanular" corolla tube, length of tips of corolla), anther tube (= synantherium; length) style + stigma (length), pappus (length) and achenes (length, incl. umbo) were measured using the Olympus SZX-AS microscope in combination with a corpus of measuring magnifier (Brinell-lupe) with a resolution of 0.01 mm. Corollas and pappi were fixed between two Petri dishes for this purpose. Usually, 3–5 corollas, pappi or achenes were measured per terminal flower head (= capitulum). Because generative features were thought to be influenced by gender (cf. Kawakubo 1994), the sex of each plant was determined before the measurement, and both sexes (i.e. females and hermaphrodites) were included in a balanced measurement design (Electronic Appendices 6–9 contain the primary datasets, and the measured characters are schematically depicted in Electronic Appendix 10).

The vegetative features of the leaves (length, width, relative position of the broadest part, petiole length, blade length/width ratio, and relative lobe length) were measured on photographs of the basal, median or upper cauline leaves of herbarium specimens (for primary datasets, see Electronic Appendices 11, 12).

Statistical analyses

Differences between cytotypes in measured morphological and genomic characters were tested using t-tests. When between-gender difference or difference between leaf surfaces was expected within species, factorial ANOVA was applied. The proportional variables (the position of the broadest part of a leaf, relative leaf lobe length, relative length of the narrow part of corolla tube, relative length of the campanular part of corolla tube, relative

length of corolla tips 1 and 2, genomic GC content) were logit transformed, log-normally distributed characters (guard cell length, width and blade shape) were ln transformed or square-root transformed (stomatal area) prior to statistical analyses to meet data quality requirements. Probabilities of the statistical tests are given after Bonferroni correction for multiple comparison error (40 tests performed in total). Within graphs, differences among groups were additionally tested also using Tukey HSD (post-hoc) test for illustrative purposes. All statistical tests were calculated and graphs prepared in Statistica 13 program (www.statsoft.com).

Results

Distribution of ploidy levels and variation in genome size

Among the 27 populations still considered to be *Cirsium waldsteinii*, two cytotypes were recognized: diploid and tetraploid. These cytotypes differ in their geographical distributions and have clearly separated vicariant ranges. All 16 Alpine and Dinaric populations (106 plants) were consistently diploid, whereas all 11 Carpathian populations (63 analysed plants) were consistently tetraploid (Appendix 1, Figs 1, 2; Electronic Appendix 1). Ploidy variations were not detected within the populations. The genome size/somatic nuclear DNA content (2C) varied between 1827 and 2125 Mbp in the diploids (Fig. 2; mean 2C = 1929.1 Mbp; standard deviation [S.D.] = 60.6 Mbp) and between 3534 and 3932 Mbp in the tetraploids (Fig. 2; mean 2C = 3682.3 Mbp; S.D. = 69.8 Mbp). No overlap in genome size (2C) was detected between the Alpine-Dinaric and Carpathian samples (Fig. 2).

The detected genomic GC content (mean±S.D.) was 38.51±0.27% for diploids and 38.61±0.29% for tetraploids; the difference was not statistically significant (t-test, Electronic Appendix 2).

Genetic relationships among populations and individuals

The four selective AFLP primers generated 333 polymorphic markers (from 366 scored loci) in 138 individuals. The overall error rate was 0.4% for the compared AFLP replicates. Relationships among samples visualized using NeighborNet indicated two clearly genetically separated lineages corresponding to the detected cytotypes: the Alpine-Dinaric diploid and Carpathian tetraploid (Fig. 3). For the Alpine-Dinaric diploid cytotype, the samples tended to cluster in separate populations), whereas for the Carpathian tetraploid cytotype, the samples tended to intermingle and do not show a clear separation of populations (Fig. 3).

The Alpine-Dinaric diploid cytotype included 212 polymorphic loci (with 58 private alleles) in 86 individuals, and the Carpathian tetraploid cytotype included 265 polymorphic loci (with 106 private alleles) in 52 individuals. The genetic diversity (Nei 1987) varied from 0.0018 to 0.1026 in populations of the Alpine-Dinaric diploid cytotype and from 0.0997 to 0.1949 in populations of the Carpathian tetraploid cytotype, which is congruent with the lower mean number of fragments per individual detected in populations of the Alpine-Dinaric diploid cytotype than in the Carpathian tetraploid cytotype (Electronic Appendix 3).

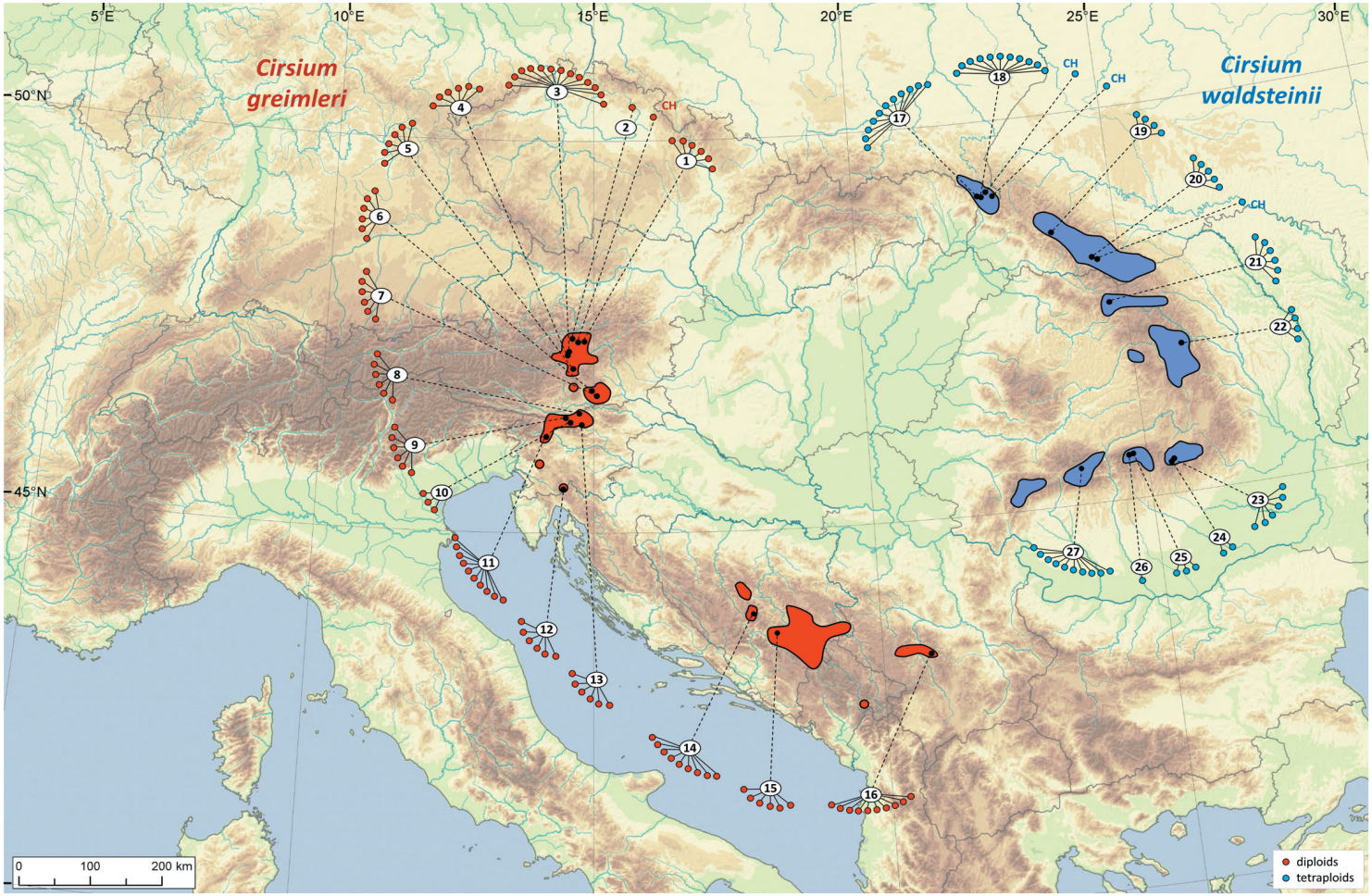

Fig. 1. – Distribution of cytotypes: diploid in the Alps and Dinarides (red circles) and tetraploid in the Carpathians (blue circles). Analysed populations and previously published karyological data (black circles) are mapped on the distribution range of *Cirsium waldsteinii* obtained from Meusel & Jäger (1992), modified according to Stevanović et al. (1991) and coloured according to the newly recognized taxa, with red for *C. greimleri* and blue for *C. waldsteinii*. Published chromosome counts (CH): 2n = 34 for *C. greimleri* (Dobeš et al. 1996) and 2n = 68 for *C. waldsteinii* (Czapik 1958, Mizianti & Frey 1973, Pashuk 1987 from West to East; for more detailed locations, see Electronic Appendix 14).

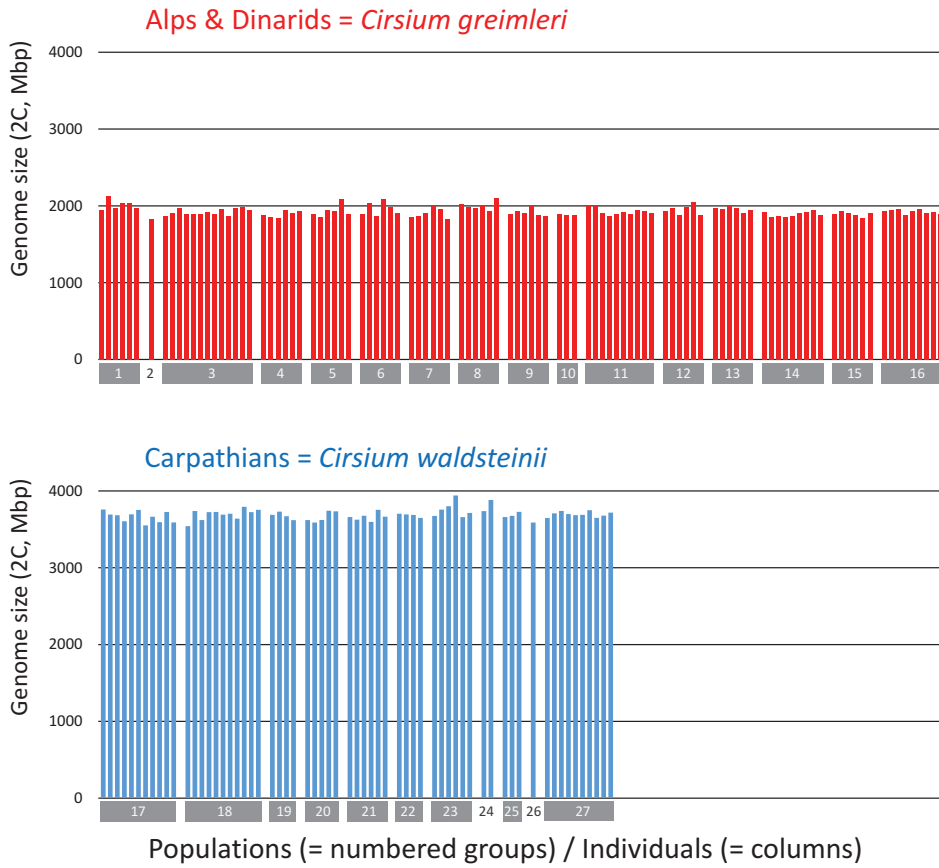

Fig. 2. – Variability in genome size among individuals and populations: diploid populations/individuals (red columns; recognized as *C. greimleri*) and tetraploid populations/individuals (blue columns; recognized as *C. waldsteinii*). The numbers below the column diagram correspond to the populations analysed (Appendix 1, each sample was measured only once – for primary genome size data, see Electronic Appendix 1).

Table 1. – Analysis of molecular variance (AMOVA) of the Alpine-Dinaric diploid and Carpathian tetraploid cytotypes. * All p-values were < 0.001

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among cytotypes	1	830.58	11.45	29.98*
Among populations within cytotypes	19	1310.94	8.17	21.42*
Within populations	115	2134.80	18.56	48.60*
Total	135	4276.32	38.19	
Among diploid populations	13	838.34	8.70	41.45*
Within diploid populations	71	872.81	12.29	58.55*
Total in diploids	84	1711.15	20.99	
Among tetraploid populations	6	472.60	7.67	21.10*
Within tetraploid populations	44	1261.99	28.68	78.90*
Total in tetraploids	50	1734.59	36.35	

The AMOVA showed that 30.7% of the variation occurred between the Alpine-Dinaric diploid and Carpathian tetraploid cytotypes, 21.9% occurred among populations within cytotypes and 47.4% occurred within populations (Table 1). Nested AMOVA tests indicated a higher differentiation among the Alpine-Dinaric diploid populations (41.5%) than in the Carpathian tetraploid populations (22.6%; Table 1), which is congruent with the pattern present in NeighborNet (Fig. 3).

Morphological and phenological variation

The diploid and tetraploid plants differed consistently in the colour of their flowers/corollas: Alpine-Dinaric diploid plants had ruby red or brownish-purple (= deep crimson) corollas (Fig. 4), whereas the Carpathian tetraploids had pinkish-purple or purple corollas (Fig. 4; for more accurate definition/delimitation of colours see Electronic Appendix 4, where the colour terminology follows the Methuen Handbook of colour). The difference in the flower colours remained consistent when both cytotypes were cultivated under the same conditions in an experimental garden (Fig. 4).

Statistically significant differences between the Carpathian tetraploids and Alpine-Dinaric diploids were also found in certain quantitative micromorphological features, particularly in (i) the length of the stomatal guard cells on the abaxial surface of median cauline leaves, 16.5±1.7 μm (mean±S.D.) in the Alpine-Dinaric diploids and 20.9±2.5 μm in the Carpathian tetraploids (Table 2; Fig. 5, in which the differences between the stomata on the adaxial leaf surface of median cauline leaves are also shown). The primary data and statistics of the stomatal parameters (Electronic Appendix 5) showed similar correlated differences in the (ii) width of the stomatal guard cells and (iii) stomatal area. Differences between the Alpine-Dinaric diploids and Carpathian tetraploids were also recorded in the (iv) length of the corolla (Electronic Appendix 10): 17.8±1.6 mm and 19.8±1.9 mm, respectively (Table 2; Figs 5, 6, where the between-gender differences within the species and the between-species differences within particular sexes are shown; see Electronic Appendix 6 for the primary data and statistics); (v) length of the style + stigma (Electronic Appendix 10): 21.3±1.9 mm and 23.4±2.3 mm, respectively (Figs 5, 6, where the between-gender differences within the species and the between-species differences within particular sexes are shown; see Electronic Appendix 6 for the primary data and statistics); (vi) length of the pappus: 16.9±1.3 mm and 18.2±1.5 mm), respectively (Table 2; Figs 5, 6, where the between-gender differences within the species and the between-species differences within particular sexes are shown; see Electronic Appendix 7 for the primary data and statistics); and (vii) length of an achene: 4.4±0.4 mm and 4.9±0.4 mm), respectively (Table 2; Figs 5, 6, where between-gender differences within the species and between-species differences within particular sexes are shown; see Electronic Appendix 8 for the primary data and statistics).

The diploid-tetraploid difference in (viii) the length of the synantherium (Electronic Appendix 10) of the hermaphrodite plants, 8.0±0.8 vs. 8.4±0.6 mm, was not statistically significant (Table 2; Fig. 5B, which shows considerable intraspecific differences between females and hermaphrodites; see Electronic Appendix 9 for primary data and statistics). No between-species differences were found in the relative features of the corolla (relative to the whole length of the corolla), such as the (ix) relative length of the tips of the corolla; (x) relative length of the narrow tube; and (xi) relative length of the broad tube (Electronic Appendix 10; for primary data and statistics for viii–xi Electronic Appendix 6).

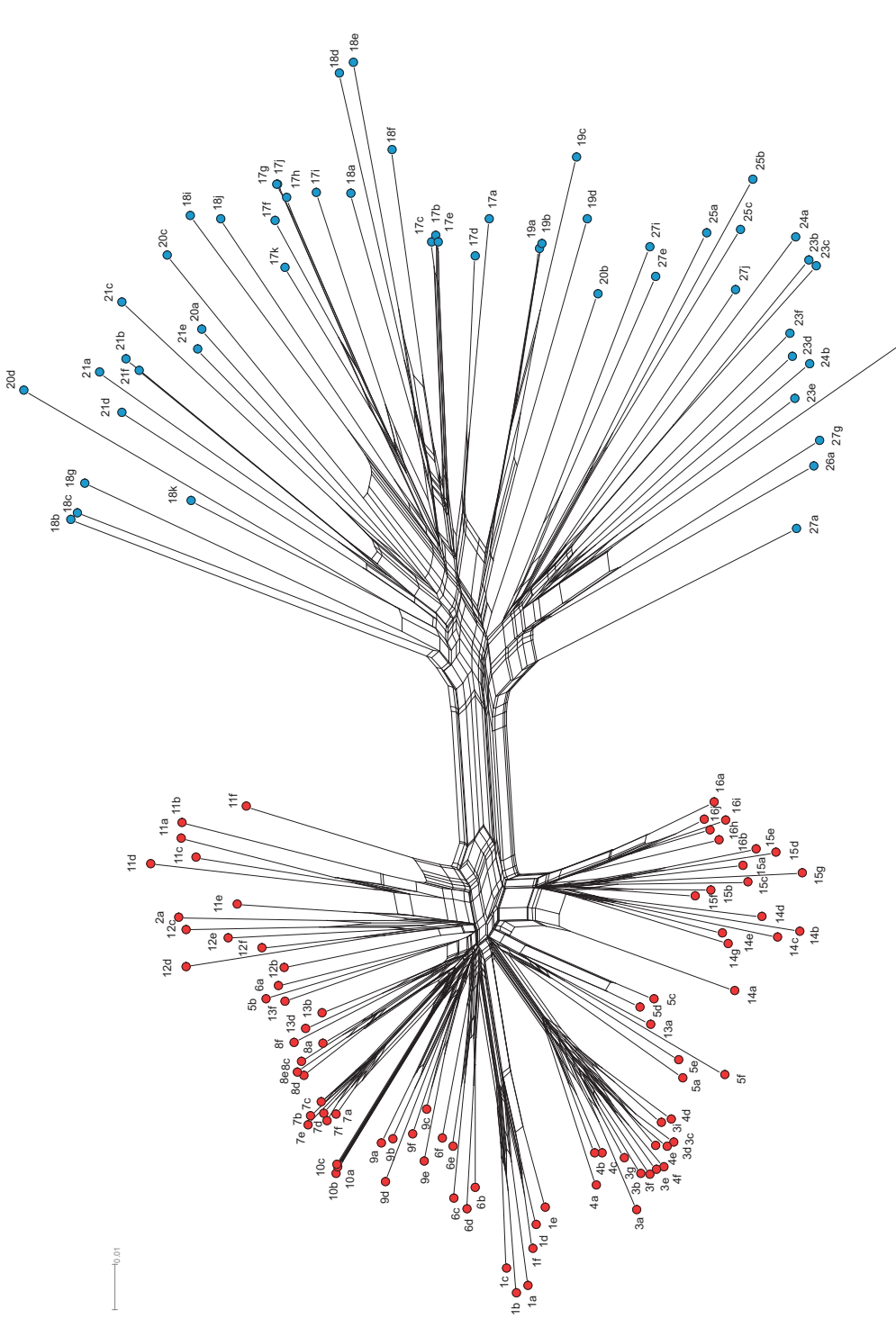

Fig. 3. – Genetic structure and relationship among individuals, populations and cytotypes (recognized as *Cirsium greimleri* and *C. waldsteinii*). NeighborNet based on uncorrected P distances derived from AFLP profiles of Alpine-Dinaric diploid (red circles) and Carpathian tetraploid (blue circles) individuals. The samples of respective populations are marked with the same population number (Appendix 1).

Fig. 4. – Difference in the flower colours. (A) Plants cultivated under uniform conditions in an experimental garden: left – ruby red capitulum of diploid *C. greimleri* (female plant transferred from population 5); right – pinkish-purple capitulum of tetraploid *C. waldsteinii* (female plant transferred from population 17). (B) Diploid *Cirsium greimleri*, hermaphrodite plant in population no. 5. (C) tetraploid *Cirsium waldsteinii*, an hermaphrodite plant from the type locality (population 21). Photo: P. Bureš (A, B), M. Vavrinec (C).

Table 2. – Most important characters that differ in *Cirsium greimleri* and *C. waldsteinii.*

Character	*Cirsium greimleri*	*Cirsium waldsteinii*
Colour of flowers [1]	ruby red – brownish purple (deep crimson)	greyish magenta – purple
Geographic distribution	Eastern Alps and Dinarides	Eastern and Southern Carpathians
Ploidy level (chromosome number)	Diploid (2n = 34)	Tetraploid (2n = 68)
Relative length of lateral lobe of median cauline leaves[2, 3]	(0.0–) 0.1–0.4 (–0.7)	(0.1–) 0.3–0.7 (–0,8)
Somatic nuclear DNA content (2C)[4]	1929.1±60.7 Mbp	3682.3±69.8 Mbp
Stem/stalk indumentum below terminal flower head	adpressed, densely arachnoid = green colour of stem is not visible	adpressed, arachnoid = green colour of stem is slightly visible
Involucre colour	brownish–blackish purple	greenish–brownish purple
Subapical vitta of phyllaries	purplish brown–purplish black	purple–purplish brown
Length of mature achenes[3]	(3.7–) 3.9–5.0 (–5.6) mm	(3.8–) 4.2–5.3 (–5.8) mm
Length of corolla[3]	(14.4–) 15.6–19.9 (–23.0) mm	(14.5–) 15.3–21.9 (–23.1) mm
Length of pappus[3]	(13.5–) 14.5–18.6 (–20.0) mm	(14.0–) 14.3–19.8 (–20.4) mm
Length of guard cell (μm)[3]	(11.6–) 13.8–19.1 (–20.0) μm	(16.6–) 17.7–25.2 (–28.1) μm
Flowering time[6]	end of June – end of July (– beginning of August)	mid of June – mid of July (– end of July)
Basal leaves width[3]	(8.2–) 11.5–23.3 (–26.8) cm	(8.1–) 9.0–26.1 (–28.7) cm
blade shape[3, 7]	(1.0–) 1.2–2.1 (–2.4) × longer than wide	(1.3–) 1.4–2.2 (–2.8) × longer than wide
lateral lobe[3, 8]	0.1–0.4 (–0.5)	0.2–0.9 (–1.0)
Median leaves width[3]	(4.8–) 6.2–18.8 (–20.8) cm	(4.4–) 4.6–15.0 (–16.5) cm
blade shape[3, 7]	(1.2–) 1.3–2.5 (–3.1) × longer than wide	(1.4–) 1.8–3.4 × longer than wide
lateral lobe[3, 8]	(0.0–) 0.1–0.4 (–0.7)	0.3–0.8

[1]For a more exact description of the flower colours, see Electronic Appendix 4, where the colour codes and terms follow the Methuen Handbook of colour (Kornerup & Wanscher 1989); [2]Measured on the largest leaf lobe; see Fig. 7P; [3](minimum–) 0.05 percentile–0.95 percentile (–maximum); [4]Average 2C±standard deviation; [5]Generally, the flowering period is later and longer at high altitudes and at shaded sites compared with that at low altitudes and at open sites. When both species were cultivated together in a fully open state in an experimental garden on the Masaryk University Campus in Brno in the Czech Republic (49°10'44.2"N, 16°34'20.4"E, 268 m s. m.), *C. greimleri* flowered from 29 May to 22 June, and *C. waldsteinii* from 15 May to 6 June in 2017; [6]Measured on abaxial surface in the middle part of median cauline leaves; [7]See Fig. 7N; [8]See Fig. 7P.

Statistically significant differences between the Carpathian tetraploids and Alpine-Dinaric diploids were also recorded in certain leaf parameters, such as the (xii) width of the blade of upper and median cauline leaves (Figs 7, 8; see Electronic Appendices 11–13 for the primary data and statistics), (xiii) shape of the leaf blades (= length/width) of basal leaves (Figs 7, 8; see Electronic Appendices 11–13 for the primary data and statistics), (xiv) position of the broadest parts of the blades of the basal and median cauline leaves (Figs 7, 8; see Electronic Appendices 11–13 for primary data and statistics), and particularly in the (xv) relative length of the lobe of the cauline leaf (Figs 7, 8; Electronic Appendices 11–13 for the primary data and statistics). The stability and degree of these differences were most apparent in the median cauline leaves (Figs 7, 8; Electronic Appendix 12).

During parallel cultivation of the diploid and tetraploid plants in the experimental garden, the tetraploid plants flowered two weeks earlier than the diploid plants (see also footnote below Table 2).

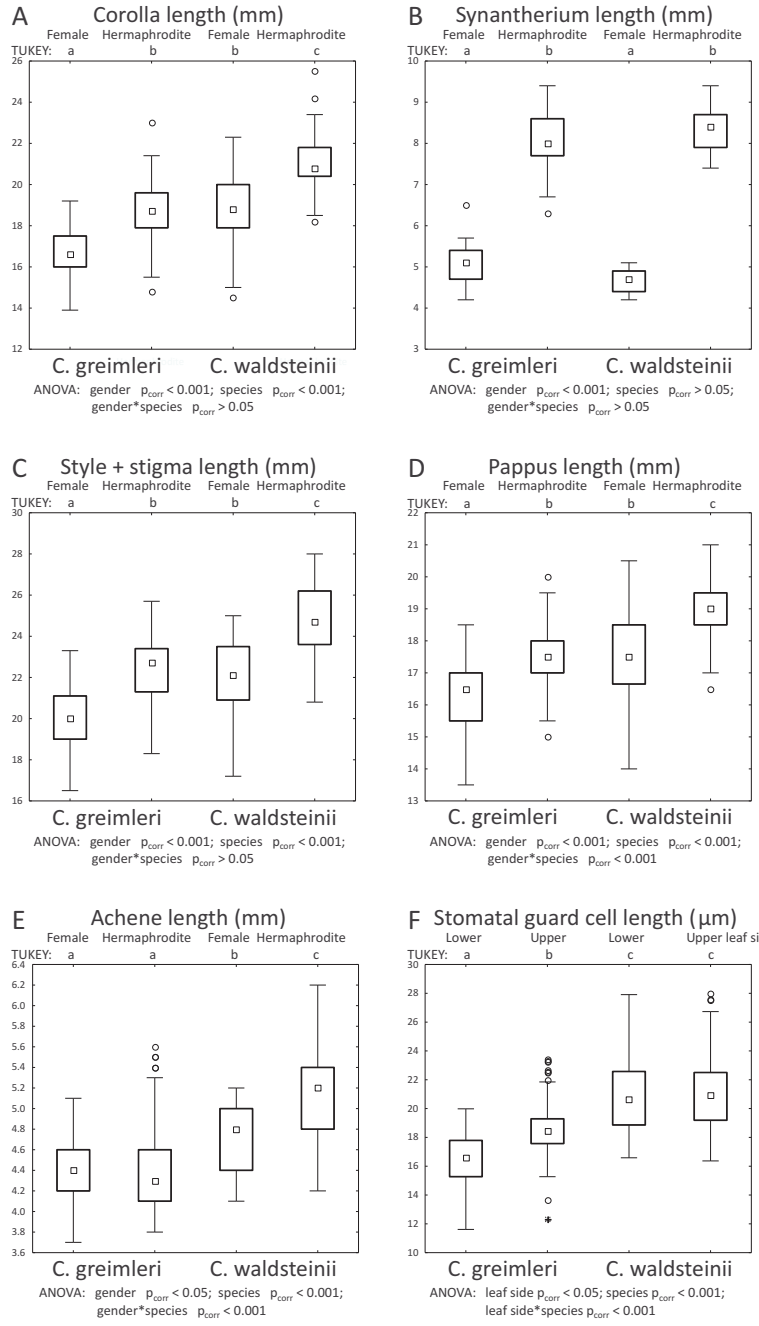

Fig. 5. – Variability in generative features (A–E) and in stomatal guard cells (F) that differ between cytotypes (diploid *Cirsium greimleri* and tetraploid *C. waldsteinii*), sexes and leaf surfaces. Boxplots show median (empty squares), interquartile ranges (boxes), non-outlier range (whiskers), outliers (empty circles). Boxplots marked with the same letter do not differ significantly at $P > 0.05$ (Tukey HSD test). P-values are Bonferroni corrected for multiple comparison.

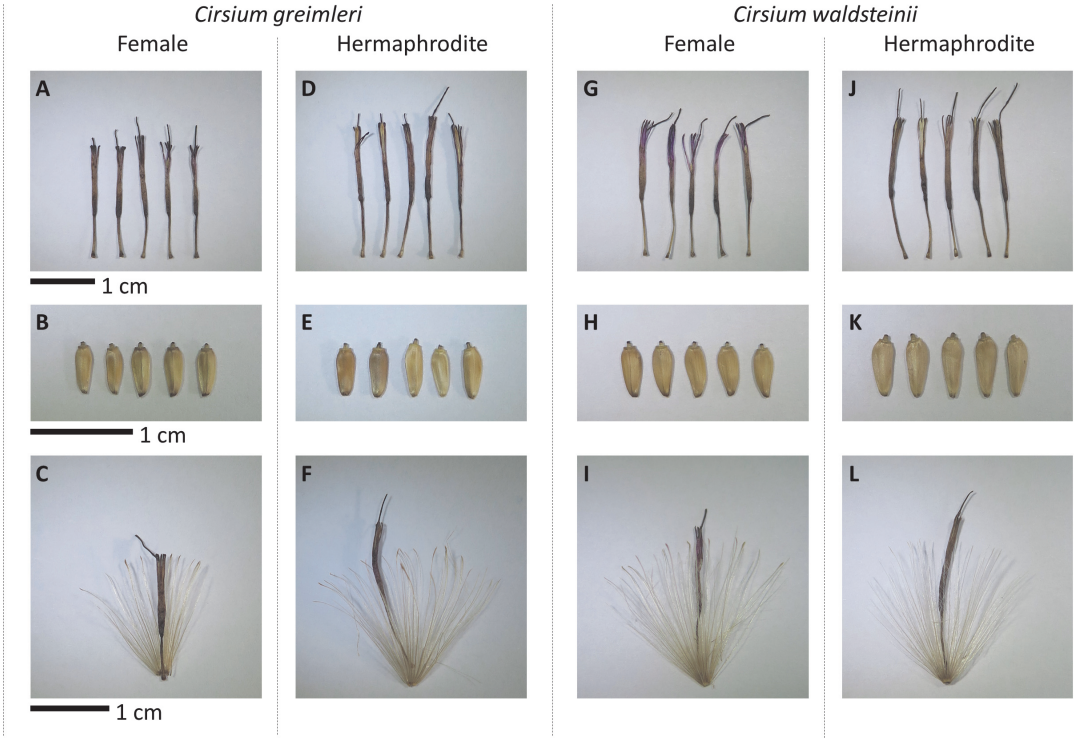

Fig. 6. – Variability in the generative features: corollas (A, D, G, J), achenes (B, E, H, K) and pappi (C, F, I, L) between diploid *Cirsium greimleri* (A–F) and tetraploid *C. waldsteinii* (G–L) and between females (A–C, G–I) and hermaphrodites (D–F, J–L) within the respective species. Photo P. Bureš.

Taxonomic interpretation of the patterns in variability and distribution

Based on the different ploidy levels, the clearly separated geographical distributions, the genetic separation, and the morphological and phenological differences, a new East Alpine and Dinaric species should be distinguished from the *Cirsium waldsteinii* in the south-eastern Carpathians:

Cirsium greimleri Bureš, **sp. nova** (Figs 4A, B; 6A–F; 8A–D, I, J, M, N, Q, R; *Cirsium* sect. *Cirsium*).

D i a g n o s i s: This species differs from *Cirsium waldsteinii* in its ruby red and shorter corollas (14.4–) 15.6–19.9 (–23.0) mm long, shorter pappus (13.5–) 14.5–18.6 (–20.0) mm long, shorter achenes (3.7–) 3.9–5.0 (–5.6) mm long, denser indumentum on the stem below capitula (completely hiding the green colour of the stem), narrower and less deeply lobed median cauline leaves (usually up to 4/10 of half the breadth of a leaf), diploid chromosome number and shorter stomatal guard cells on the abaxial surface of the median cauline leaves (11.6–) 13.8–19.1 (–20.0) μm long.

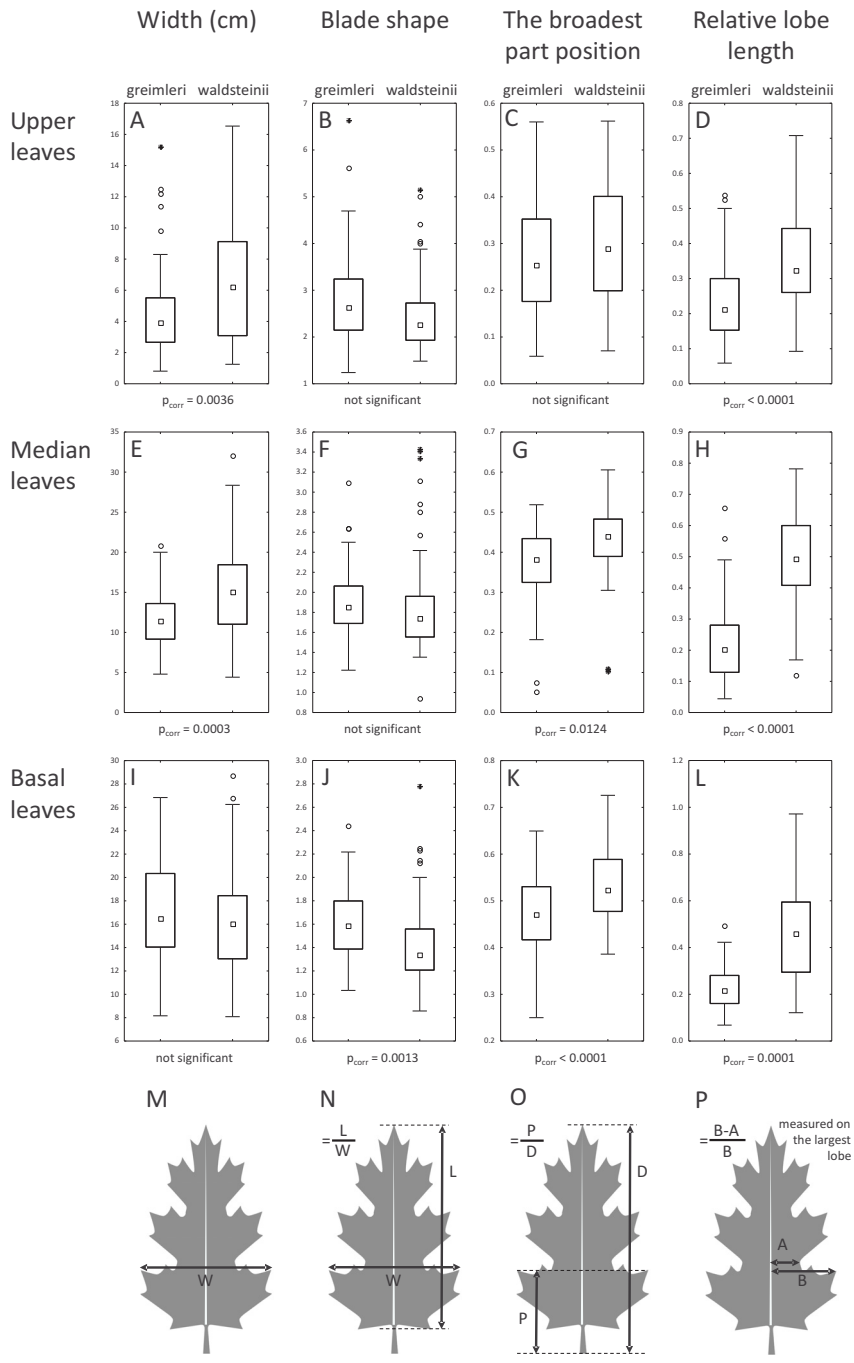

Fig. 7. – Variability in vegetative features (M–P) on the upper (A–D), median (E–H) and basal (I–L) cauline leaves of diploid *Cirsium greimleri* and tetraploid *C. waldsteinii*. Boxplots show median (empty squares), interquartile ranges (boxes), non-outlier range (whiskers), outliers (empty circles) and extremes (asterisks); t-test P-values are Bonferroni corrected for multiple comparisons.

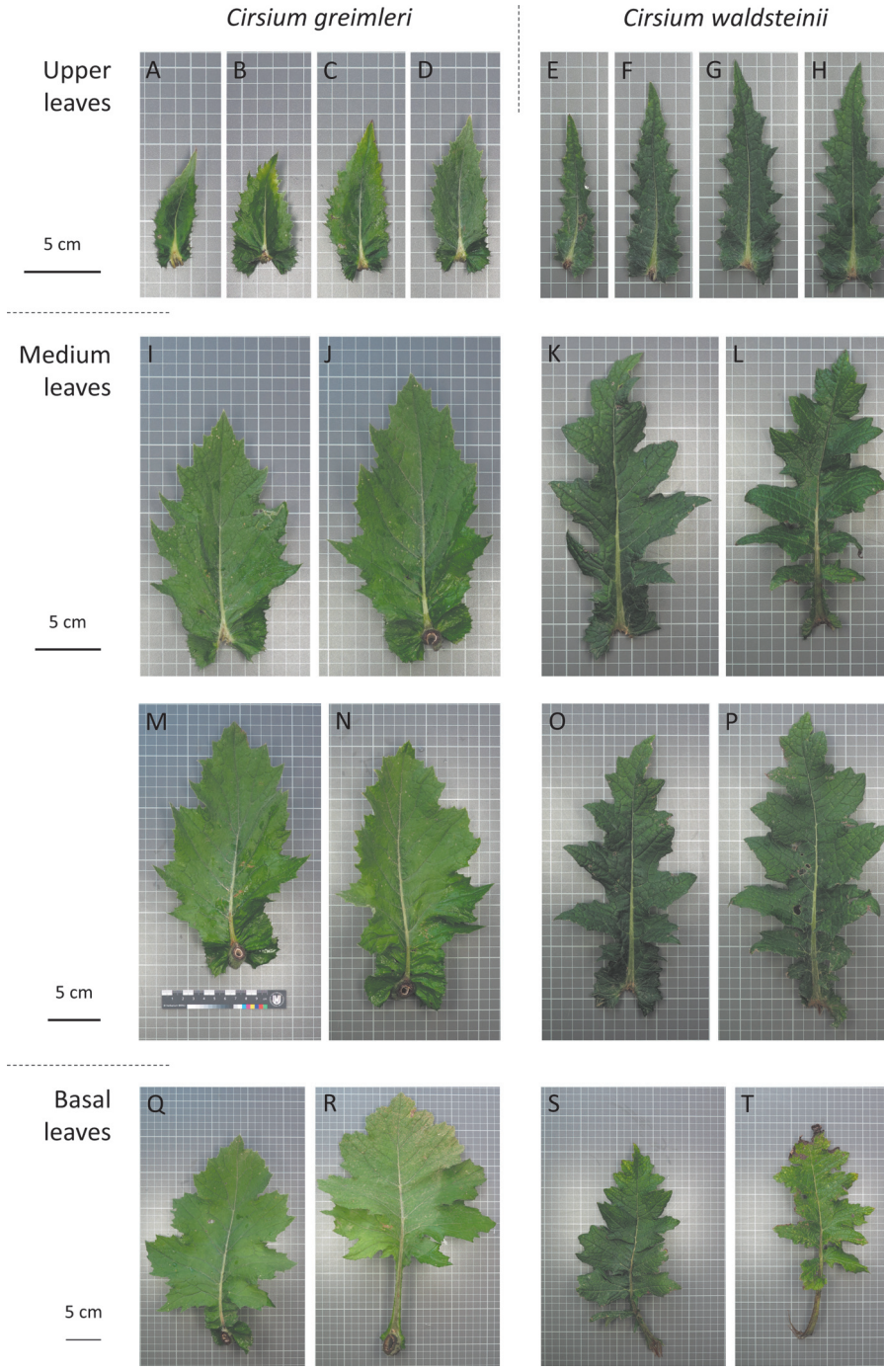

Fig. 8. – Variability in the vegetative features on the upper (A–H), median (I–P) and basal (Q–T) cauline leaves of diploid *Cirsium greimleri* (A–D, I, J, M, N, Q, R) and tetraploid *C. waldsteinii* (E–H, K, L, O, P, S, T). Photo P. Bureš.

T y p e: Austria, Rottenmanner and Wölzer Tauern, Lachtal: along the road Oberer Höhenweg (= road between the village of Oberzeiring and the mountain chalet Klosterneuburger Hütte), 3.5 km NE of the village; 47°16'19.1" N, 14°24'22.5" E; 1739 m s. m.; coll. P. Bureš 15/Sep/2015 (holotype: BRNU 658042; isotypes: PR, WU, W, BP).

D e s c r i p t i o n: Perennial gynodiecious plant (0.7–) 1.1–1.8 (–2.1) m tall. Rhizome oblique, cylindrical. Stem erect, at the top rather nodding, unwinged, shallowly ribbed, green or reddish green and pilose or subglabrous at the base, whitish green and sparsely arachnoid in the middle, white and densely arachnoid in the upper part below the capitula. Leaves shallowly–roughly double serrate to pinnatipartite, softly herbaceous, fresh green and subglabrous or with scattered multicellular hairs above, greenish and arachnoid beneath, shortly and softly spinose at the margins; leaf spines yellowish or brownish-purple particularly on the median and upper leaves, and 0.5–2.0 (–4.5 on upper leaves) mm long; basal cauline leaves from petiolate with broadly ovate blades to amplexicaul broadly fiddle-shaped or lyrate, (1.0–) 1.2–2.1 (–2.4)× longer than wide, with petioles from broadly in the less basal to narrowly winged in the most basal leaves, blades almost entire, irregularly dentate, serrate or shallowly lobed at up to 1/10–4/10 (–6/10) of half the breadth the blade, (15.9–) 20.1–40.1 (–44.1) cm long and (8.2–) 11.5–23.3 (–26.8) cm broad; median cauline leaves from broadly fiddle-shaped or lyrate to ovate, (1.2–) 1.3–2.5 (–3.1)× longer than wide, amplexicaul, roughly double serrate or lobed up 1/10–4/10 (–7/10) of half the breadth of the leaf, (10.4–) 13.6–30.2 (–33.8) cm long, (4.8–) 6.2–18.4 (–20.8) cm broad; upper cauline leaves from lance-ovate to narrowly lanceolate, (1.2–) 1.7–4.2 (–6.6)× longer than wide, semi-amplexicaul, roughly serrate to shallowly lobed at up to 1/10–4/10 (–6/10) of half the breadth of the leaf, (4.2–) 5.4–17.7 (–27.5) cm long, (0.8–) 1.6–9.9 (–15.2) cm broad, subglabrous or sparsely arachnoid above and densely arachnoid beneath; ground leaves of sterile rosettes petiolate with blades broadly ovate, almost entire, irregularly dentate or shallowly sinuately lobed, 20–35 (–45) cm long, 15–35 (–40) cm broad. Capitula nodding, solitary or corymbosely terminally clustered per 1–8 (–12) and shortly pedunculated, rarely also solitary on 1–5 (–10) elongate lateral peduncles/branches subtended by upper cauline leaves below the terminal cluster, (24.2–) 30.1–41.9 (–45.4) mm long, (12.9–) 14.6–21.8 (–23.1) mm broad. Peduncles densely arachnoid (= green colour of stem is fully hidden by dense indumentum). Involucres (of mature terminal capitulum) ovoid, (11.6–) 12.9–21.0 (–21.7) mm long, brownish to blackish-purple with apices of phyllaries protruding to 1/20–1/15 in relation to the involucre diameter. Phyllaries (of mature terminal capitulum) in (5–) 6–7 (–9) rows, lanceolate–narrowly lanceolate, with slightly patent ruby red apices, without terminal spine, glabrous, purplish-brown or purplish-black, 2.8–4.2 mm broad; subapical vitta slightly conspicuous on median phyllaries, entire or very shortly ciliate (whitish cilia 0.1–0.2 mm long), purplish-brown or purplish-black. Flowers hermaphrodite or functionally female (with rudimental synantheria and without developed pollen); corolla ruby red later brownish-purple/deep crimson, lobed to 3/10–6/10, (14.8–) 17.3–21.0 (–23.0) mm long in hermaphrodites, (13.9–) 15.1–18.6 (–19.2) mm in females; narrow corollar tube (5.7–) 6.0–9.0 (–9.5) mm long in hermaphrodites, (5.0–) 5.2–7.0 (–7.5) mm in females; broad campanular corollar tube (4.0–) 4.9–8.0 (–8.8) mm long in hermaphrodites, (3.9–) 4.4–7.3 (–8.2) mm in females; corollar lobes (2.7–) 3.6–7.0 (–9.1) mm long in hermaphrodites, (3.0–) 3.3–6.2 (–7.0) mm in females;

synantherium brownish-purple (in full anthesis) to whitish (when faded), with pollen and clearly protruding from corolla at full anthesis in hermaphrodites, ochre without pollen and never protruding from corolla in females, (6.3–) 6.7–9.3 (–9.4) mm long in hermaphrodites, (4.2–) 4.3–5.7 (–6.5) in females; style whitish (18.3–) 20.0–25.0 (–25.7) mm long in hermaphrodites (incl. stigma), (16.5–) 18.0–22.4 (–23.3) mm in females (incl. stigma); stigma ruby red and then brownish-purple/deep crimson, shortly bi-lobed, straight in hermaphrodites, straight or undulating (twisted) in females; pappus plumose, whitish or stramineous, (15.0–) 16.0–19.0 (–20.0) mm long in hermaphrodites, (13.5–) 14.5–18.0 (–18.5) mm in females. Achenes oblong, asymmetric, compressed, greyish ochre, (3.8–) 3.9–5.4 (–5.6) mm long in hermaphrodites, (3.7–) 4.0–4.9 (–5.1) mm in females, with 0.2–0.5 mm long umbo and ca. 0.1 mm high apex ring in both sexes. Flowering in June–July.

H a b i t a t a n d e c o l o g y: Open high-mountain and subalpine park-like forests/woodlands with *Picea abies*, *Acer pseudoplatanus*, *Sorbus aucuparia* and *Alnus incana*, on the leeward moist slopes of forested valleys and gorges of mountain brooks and along shaded forest roads. Also in open mountain scree forests with *Pinus cembra* and *Larix decidua*, usually on more or less acidic soil but also on calcareous substrates. Often together with subalpine tall forbs, such as *Adenostyles alliariae*, *Cicerbita alpina*, *Gentiana asclepiadea*, *Telekia speciosa* (in Dinarides), *Veratrum lobelianum*, *Prenanthes purpurea*, *Doronicum austriacum*, *Veronica urticifolia*, *Valeriana tripteris*, *Chaerophyllum hirsutum*, *Dryopteris filix-mas*, *Athyrium filix-femina*, *Phyteuma spicatum*, *Senecio nemorensis* agg., *Peucedanum ostruthium*.

D i s t r i b u t i o n a n d h y b r i d i z a t i o n: At altitudes from 800 to 2000 m a.s.l. from southeastern Austria (Styria, Carinthia) to Slovenia, Croatia, Bosnia and Herzegovina, Montenegro and Serbia, i.e. in the mountains of the Eastern Alps: Ennstal Alps, Rottenmanner and Wölzer Tauern, Seckau Tauern, Lavanttal Alps (in the subgroups Seetal Alps, Saualpe, and Koralpe), Karawanks, Stein Alps (= Kamniško-Savinjske Alpe), Pohorje (= Bachergebirge), Julijske Alpe (= Julische Alpen, Alpi Giulie), and in the Dinarides: Notranjski Snežnik and Gorski Kotar near the Slovenian-Croatian boundary, mountains in Bosnia (Srnetica, Cincar, Vranica planina, Bjelašnica, Visočica, Treskavica, Zelengora and Jahorina), Eastern Montenegro (Komovi) and the Western Serbian mountains of Stari Vlah (Golija) and Kopaonik. Most of the populations consist of a few to several tens (or hundreds) of individuals, with the largest populations consisting of thousands of individuals recorded in the Koralpe and Seetaler Alpen. This species often hybridizes with other co-occurring diploid congeners, such as *C. erisithales*, *C. heterophyllum*, *C. palustre*, *C. oleraceum* and *C. carniolicum*.

C o n s e r v a t i o n s t a t u s: Based on its restricted distribution, rare occurrence, small population sizes and frequent interspecific hybridization, this species could be categorized as an endangered species (EN) in the IUCN Red List and the national plant red lists of Austria, Slovenia, Montenegro, Serbia, Bosnia and Herzegovina (and is probably extinct in Croatia).

Etymology: The specific epithet is derived from the surname of Josef Greimler, a botanist from Vienna University, who sampled the material used by Dobeš et al. (1996) to determine the diploid chromosome number (2n = 34) in *C. waldsteinii* for the first time. The chromosomal count detected in Greimler's sample was the first finding that contradicted the previously assumed tetraploid homogeneity of *C. waldsteinii*.

Discussion

Nomenclature and geographical distribution

The south-eastern Carpathian tetraploid species *Cirsium waldsteinii* was recognized for the first time by Waldstein & Kitaibel (1803) under the name *Cnicus pauciflorus* and was accepted as a species occurring in the mountains of the historical territory of Hungary, i.e. in the Carpathians, by the authors in the early decades of the 19th century (Willdenow 1803, Lamarck 1805, Persoon 1807). Later, Sprengel's (1826) combination *Cirsium pauciflorum* (Waldst. et Kit.) Sprengel was recognized by Rouy et al. (1905) as a younger homonym of *Cirsium pauciflorum* Lam. (= *Carduus defloratus* L.), and the illegitimate name was replaced by the new *Cirsium waldsteinii* Rouy Fl. France 9: 84, 1905. A lectotype of *Cnicus pauciflorus* was selected by Kováts (1992: 40). The specimen on sheet XXVIII, No. 123 kept in the collection Herbarium Kitaibelianum and deposited in the Botanical Department of the Hungarian Natural History Museum, Budapest (BP), was labelled by Kitaibel as "Cnicus pauciflorus In pinetis sub Petrosa initio Augusti." The type specimen was collected on 6 August 1796 near the mountain Pietros (= Rom.: Pietrosul Rodnei; 2303 m s. m.), the highest mountain in the Rodna Mts (= Rom.: Munţii Rodnei) in northern Romania in the county of Maramureş, as is reported in Kitaibel's diary referring to the trip to Pietros: "eine *Carduus* mit sehr breiten Blättern, einem mannshohen, 2–3 blüthigen Stamm" among "Pflanzen, die wir bis zum Krumholze fanden" (Gombocz 1945: 123–124). As the more than 200-year-old Kitaibel type specimen is very fragile and consists of only one leaf and one terminal cluster of capitula, its achenes and stomatal cells have not been measured and no molecular samples have been obtained from the type specimen. The tetraploid level was detected in all plants collected at the *locus classicus* (Appendix 1: population 21) using flow cytometry. The tetraploid Carpathian cytotype should therefore be named *Cirsium waldsteinii*, and the separation of the Alpine-Dinaric diploid species under a new name is correct. The oldest report referring to *Cirsium greimleri* is likely that from the Seetal Alps (a subgroup of Lavanttal Alps) by Host (1831: 445) at Bürgersee (recently named Frauenlacke SW of Judenburg), i.e. from a locality close to that of population 5 analysed in this study (Appendix 1).

Leaf shape

Compared with most other species of *Cirsium*, the blade of the median and basal cauline leaves is relatively broad (< 2.5× longer than wide) in *C. greimleri*, and the blades of its leaves in sterile ground rosettes could be even broader (< 2 × longer than wide). The broad leaf blades are shallowly lobed or double serrated in *C. greimleri*, whereas they are often rather deeply lobed in *C. waldsteinii* (Fig. 8). The petioles of the most basal cauline leaves or leaves of sterile ground rosettes are usually longer than 10 cm in both species

(Fig. 8). A similar leaf shape is typical for *C. carniolicum*, a diploid species sympatrically distributed with *C. greimleri* in the Eastern Alps, but different in the yellowish-white flowers, glandular ciliate phyllaries, longer (> 3 mm) spines on upper cauline leaves, underside of leaves subglabrous and stem pilose below the capitula (of which the green colour is clearly visible). In Europe, leaves resembling those of *C. greimleri* or *C. waldsteinii* can also be found in diploid *C. rufescens* (endemic to the Pyrenees) and diploid *C. alpis-lunae* (endemic to Alpe della Luna Mountains, part of the Northern Apennines), both of which differ from *C. greimleri* (and *C. waldsteinii*) in their yellowish-white flowers, green phyllaries and subglabrous leaves, and *C. alpis-lunae* further differs in its stiff and longer (> 5 mm) leaf spines and terminally spiny phyllaries. *Cirsium latifolium*, a diploid species endemic to Madeira, has similar shaped leaves with large basal cauline leaves that are broad, ovate, unlobed, dentate and more than 3 times longer than broad. However, *C. latifolium* differs from *C. greimleri* in having pinkish flowers and less dense indumentum on the stem below the capitula. *Cirsium pseudopersonata* Boiss. et Balansa ex Boiss., which is a diploid Caucasian and Anatolian species, also has broad sessile and almost entire leaves that resembles those of *C. greimleri*, although it differs in having purple flowers, sparse indumentum on the stem below the capitula and outstanding long apices of phyllaries.

Flower colour

Flower colour is a stable taxonomic character in species of *Cirsium* (Wagenitz 1987, Yildiz et al. 2016). In Europe, most species of *Cirsium* (≈80%) have purple, and the remaining ones have white or pale yellow flowers (Werner 1976). In the newly recognized *C. greimleri*, the different and rather unique flower colour is ruby red in fully open flowers and capitula (Fig. 4) or brownish-purple/deep crimson when the flowers are fading and the capitula closing, i.e. having a brush shape. Among European thistles, this colour is almost unknown, although it occurs in the West Asian *C. hypoleucum*, which is distributed in Gruziya and Turkey and rarely in the European part of Turkey near Istanbul (Yildiz et al. 2016). Diploid *C. hypoleucum* resembles *C. greimleri* in its nodding capitula, which are smaller than those of *C. greimleri*. *Cirsium hypoleucum* differs from *C. greimleri* in having narrower involucres, less dense indumentum on the stem below capitula, conspicuous whitish vittae on phyllaries (while those of *C. greimleri* are inconspicuous, brownish/blackish-purple) and longer (> 3 mm) terminal spines on the leaf lobes. The upper and medium cauline leaves of *C. hypoleucum* are variable and sometimes resemble those of *C. waldsteinii* (more than those of *C. greimleri*). The basal leaves of *C. hypoleucum* are oblanceolate and 3–5× longer than broad, with more or less acute lobes and short petioles, which is also the case for the leaves in sterile basal rosettes (see the above discussion of leaf shape). Nevertheless, the phylogenetic relationships of *C. hypoleucum*, *C. greimleri* and *C. waldsteinii* should be thoroughly examined in the future.

Ruby red flower colour is also typical of the garden cultivar 'Atropurpureum' of *C. rivulare*, whose wild populations have standard purple flowers. Occasionally, ruby red flower colours may emerge when the yellow-flowering *C. erisithales* hybridizes with purple flowering thistles (*C. alsophilum*, *C. palustre*, *C. rivulare*, *C. pannonicum*; Bureš & Michálková, field observation) and the hybrids may consequently be mistakenly identified as *C. greimleri*. Such cases may account for the recent reports of *C. greimleri* (as

C. waldsteinii) in the hills of Grmada [46°04'42.1"N, 14°19'35.7"E] and Sveti Lovrenc [46°04'23.0"N, 14°18'00.2"E] near Polhov Gradec (Čarni 2007) and Jalovnik [46°11'14.8"N, 13°48'24.9"E] near Sela nad Podmelcem (Dakskobler et al. 2013), where in July 2016 we only found large populations of ruby red flowering hybrids *C. erisithales* × *C. pannonicum* at the above mentioned geographic coordinates.

Outside Europe, ruby red or brownish-purple flowers are also extremely rare in *Cirsium*; in Asia, they can be found only in *C. borealinipponense* Kitam. et Murata ex Kitam., *C. ficifolium* Fisch. (recently placed in the genus *Synurus* Iljin), *C. hachimantaiense* Kadota, *C. hidakamontanum* Y. Kadota, *C. shimae* Kadota, and in certain varieties of *C. chokaiense* Kitam., and in North America, they are only found in *C. douglasii* DC. and *C. occidentale* (Nutt.) Jepson.

Although the difference in flower colour between *C. greimleri* and *C. waldsteinii* is not large, it is stable and consistent across the distributions of both taxa. Moreover, the colour remains constant when both taxa are cultivated under the same conditions as was tested during the spring of 2017 in an experimental garden (Fig. 4A). Unfortunately, it is almost impossible to use this difference in flower colour to identify herbarium material.

Larger cells, flowers and fruits in tetraploid Cirsium waldsteinii than in diploid C. greimleri

The difference in the length of the stomatal cells in the closely related diploid *C. greimleri* and tetraploid *C. waldsteinii* (1.268 = 20.90 μm/16.48 μm = ratio between the average length of stomatal cells) corresponds well with the difference in nuclear DNA content between these taxa (1.240 = ratio of the cube roots of the average DNA contents). More explicitly, the doubling of the nuclear volume is fully reflected in the doubling of the volume of the stomatal cells. Although the corolla, style + stigma, pappus and achene of the respective genders are also larger in the tetraploid *C. waldsteinii* than in the diploid *C. greimleri* (Fig. 5), their diploid/tetraploid enlargement is rather smaller than that of the stomata in all the cases (from 1.07 fold for the pappus of females to 1.16 fold for achenes of hermaphrodites; see Electronic Appendices 6–8). This finding suggests that the stomatal cells of *C. greimleri* are the smallest possible with respect to the volume of their nuclei (because of the selection for stomatal efficiency; Veselý et al. 2012), and that the doubling of the nucleus volume via polyploidization resulted in the doubling of the stomatal volume in *C. waldsteinii*. Contrastingly, the cells forming the corolla, style, pappus and achene of diploid *C. greimleri* are either larger than what is dictated by their nuclei or the nucleus volume that doubles via polyploidization is compensated by the smaller number of cells constituting these organs in tetraploid *C. waldsteinii* (relative to diploid *C. greimleri*).

Although the underside of the leaves is densely arachnoid, the stomata are well apparent in varnish imprints without the removal/shaving of the indumentum. The adaxial/abaxial difference in size of stomata (Fig. 5F) may be associated with their lower density on the adaxial surface (Fanourakis et al. 2015). Generally, the size of the stomata is associated also with the stage of development of a leaf (its position from the top of stem) or the part of the leaf in which the stomata measured are situated (Dow et al. 2014). For a reliable estimate of the ploidy level of an individual it is necessary to examine the middle part of at least five well-developed (mature) median leaves and at least 50 stomata.

Generative features and gynodioecy

As many other species of *Cirsium* sect. *Cirsium* in Europe, *C. greimleri* and *C. waldsteinii* are gynodioecious (= two sexes: female and hermaphrodite plants occur in their populations). In both species, gender can be easily recognized using the same features as in other gynodioecious thistles (cf. Delannay 1979): in fully flowering hermaphrodites, the synantherium apparently protrudes from the corolla (Electronic Appendix 10), and pollen is pushed out by an elongating style, whereas in females, the synantherium remains hidden in the campanular part of the corolla, and its apex is hardly visible in the incisions between the corolla lobes at any flowering phenophase (Electronic Appendix 10). Moreover, a female synantherium does not contain any pollen. In hermaphrodites, the synantherium has the same colour as the corolla during anthesis, although it subsequently (when the flowers fade) becomes white and moderately shortened (only slightly exceeding the ends of the corollar lobes). The short synantherium of females is whitish and then ochre. In hermaphrodites, the stigma is almost consistently straight, whereas in females, it is usually undulating or twisted, although it may also be straight.

In addition to the synantherium, other floral/generative structures (corolla, style + stigma, pappus and achenes) were larger in the hermaphrodites than the females. Although these findings are congruent with the between-gender difference detected in the Japanese gynodioecious thistle *C. chikushiense* by Kawakubo (1994), the smaller size of female achenes compared with those of hermaphrodites is nevertheless rather surprising. In most gynodioecius species, female seeds or fruits are larger in order to compensate for the genetic disadvantage of females (relative to hermaphrodites, which transfer their genes to the next generation more effectively via ovules and pollen, whereas females do it only via ovules) and thus stabilize gynodioecy (presence of females) in this species (Dufay & Billard 2012). Moreover, the larger female seeds or fruits can be easily explained either by the absence of inbreeding depression in the progeny (in the seed or fruits) of obligatorily outcrossed females compared to those that are at least partly selfing hermaphrodites or by the allocation of resources that hermaphrodites must invest in the development of pollen to the development of seeds or fruits by females (Dufay & Billard 2012). The female advantage or compensation also stabilizes gynodioecy in *Cirsium*; however, in certain species (likely also in *C. greimleri* and *C. waldsteinii*), the smaller achenes produced by females may be counterbalanced by a larger number of achenes, higher germination and lower infestation by achene predators, such as fruit flies (*Tephritidae*) or weevils (*Curculionidae*), compared with the hermaphrodites (Bureš et al. unpublished data).

Because between-gender size differences in floral structures could be even greater within a particular species than between the diploid *C. greimleri* and tetraploid *C. waldsteinii* (Figs 5, 6), the generative characters should therefore be carefully used for determining ploidy level and only used when the gender of analysed plant is clear (as described above).

Interspecific hybridization

During sampling, a considerable number of interspecific hybrids were recorded in populations of *C. greimleri*: (i) *C. erisithales* × *C. greimleri* in the Austrian populations 2, 3, 6 and 8 as well as in the Slovenian populations 11 and 12 (Appendix 1); this hybrid was previously reported from Rottenmanner and Wölzer Tauern (Heimerl 1884, Fritsch 1906,

Khek 1908), Lavanttal Alps (Fritsch 1906, Benz 1922, Leute & Zeitler 1967), Eastern Karawanks (Melzer 1968) and Notranjski Snežnik (Khek 1908, Tommasini ap. Wraber 1998). (ii) *C. carniolicum* × *C. greimleri* was rarely recorded in Austrian population 2 (Appendix 1), and was previously reported once from Karawanks by Melzer (1973). (iii) *C. greimleri* × *C. heterophyllum* occurred scattered in Austrian populations 3 and 5 (Appendix 1) and previously reported from Rottenmanner and Wölzer Tauern (Heimerl 1884, Fritsch 1906), Seckau Tauern (Fritsch 1906) and Lavanttal Alps (Fritsch 1906, Benz 1922). (iv) *C. greimleri* × *C. oleraceum* was rare in population 6 (Appendix 1) in the Lavanttal Alps, where it was previously collected by Eichenfeld (1887), Fritsch (1906) and Benz (1922) as well as at Rottenmanner and Wölzer Tauern by Khek (1905). (v) *C. greimleri* × *C. palustre* was abundant in population 3 and scattered in 5 and 6 (Appendix 1), and was previously reported at Rottenmanner and Wölzer Tauern by Khek (1905), Stein Alps by Juratzka (1859), Seckau Tauern by Fritsch (1906) and the Lavanttal Alps by Fritsch (1905, 1906) and Benz (1922).

In addition to the aforementioned five hybrids there are previous reports of two other hybrids of *C. greimleri*: (vi) *C. greimleri* × *C. rivulare* in the vicinity of Deutschlandsberg east of Koralpe in Austrian Lavanttal Alps by Fritsch (1906) and Benz (1922) and in the Gorski Kotar Mts in Croatia by Tommasini (ap. Fritsch 1906); and (vii) *C. greimleri* × *C. spinosissimum* described as *C.* ×*stroblii* Hayek based on a specimen from Grosser Bösenstein in Rottenmanner and Wölzer Tauern collected by Strobl as *C. carniolicum* × *C. spinosissimum* (Hayek ap. Halácsy 1907).

Although hybridization is common among species of *Cirsium* in nature, particularly in the type section in Europe (Wagenitz 1987, Bureš et al. 2004, 2010, Stöhr 2006, Segarra-Moragues et al. 2007), the "interspecific promiscuity" of *C. greimleri* should be considered among the highest, particularly when the frequency of hybrid plants is related to the small number of "pure" individuals in the populations. The potential risk of recent genetic erosion by other mountain congeners should therefore be carefully considered for this species.

No interspecific hybrids were found in the sampled populations of tetraploid *C. waldsteinii*. In addition, only a few specimens of *C. erisithales* × *C. waldsteinii* from two localities in the Romanian Carpathians (Curmătura Zârnei in Făgăraş Mts and Colibiţa in Calimani Mts) and one specimen of *C. palustre* × *C. waldsteinii* from the Ukrainian Carpathians (valley of Lopuschanka River near Yasinia) were found during the revision of Carpathian material of *C. waldsteinii* in herbaria BP and BRNU. Moreover, reliable literary reports of *C. waldsteinii* hybrids are sporadic: *C. palustre* × *C. waldsteinii* is reported by Nyárády (1964) at Hoverla, which is close to the aforementioned locality at Yasinia; *C. rivulare* × *C. waldsteinii* was supposedly recognized in the Lerchenfeld Transsilvanian collection by Schur (1885), and no specimens of this hybrid were found in BP. Even though the ability of *C. waldsteinii* to hybridize with diploid congeners is dramatically lower than that of *C. greimleri*, the occasional hybrid event suggests that tetraploid *C. waldsteinii* is not fully reproductively isolated from co-occurring diploid congeners, which is similar to the rare hybridization of tetraploid *C. vulgare* (cf. Bureš 2004).

Genetic structure of populations of Cirsium greimleri and C. waldsteinii and their evolution

Compared to diploids, polyploids are supposed to produce higher numbers of AFLP fragments (Meudt & Clarke 2007, Dufresne et al. 2014). This might account for the (i) higher mean number of AFLP fragments (127–139 per individual) in the populations of tetraploid *C. waldsteinii* than in those of diploid *C. greimleri* (96–111 per individual; Electronic Appendix 3), the (ii) higher total number of polymorphic loci (265) detected in the smaller sample-set of tetraploid *C. waldsteinii* (52 individuals) than of the diploid *C. greimleri* (212 polymorphic loci in 86 individuals) and the (iii) ratio between the numbers of private alleles (106: 58), in respective species. Since tetraploid populations contain twice as many copies of each gene (alleles) as the diploid populations of the same size, the effect of genetic drift is smaller in the tetraploid populations, moreover, their genetic homogenization via migration of individuals is stronger even when the migration rate is the same as that among diploid populations, since a tetraploid migrant carries more gene copies than a diploid migrant (Meirmans & Van Tienderen 2013). Lower effect of genetic drift associated with more effective homogenization is therefore likely to be responsible for the smaller genetic divergence among tetraploid *C. waldsteinii* populations and their higher intrapopulation genetic diversities compared to diploid *C. greimleri* (Fig. 3; Table 1; Electronic Appendix 3). In addition, historical processes could have deepened on the effect of genetic drift in Alpine *C. greimleri*. Because the Alps were repeatedly and continuously covered by glaciers during cold periods in the Pleistocene while the Carpathians remained largely free of ice even during the coldest periods, the Carpathian species experienced lower fragmentation of suitable habitats and depauperation of their genetic diversity than the Alpine species (Mráz et al. 2007). Substantially different intensities of gene flow into either species via interspecific hybridization (see above discussion of hybridization) could have also deepened the genetic diversification among populations of *C. greimleri* (Fig. 3, Table 1). The natural ranges of *C. greimleri* and *C. waldsteinii* are consistent with the distribution of many East Alpine or south-eastern Carpathian endemics, respectively, that often share specific glacial refugia, usually with respect to their habitat preferences (Schönswetter et al. 2005, Mráz & Ronikier 2016).

Because AFLP is considered an effective tool for the identification of the parental taxa of allopolyploid species (Winkler et al. 2017), we preliminarily analysed the relationships of *C. waldsteinii* with other European species of *Cirsium*. In these AFLP analyses, the close relationship between *C. greimleri* and *C. waldsteinii* was clearly indicated; however, no other species was identified as the "second parent" of the putatively allotetraploid *C. waldsteinii* (Michálková et al. unpublished). Nevertheless, the higher number of region diagnostic loci (106 private alleles) in tetraploid *C. waldsteinii* compared to the diploid *C. greimleri* (58 private alleles; Electronic Appendix 3) could support the allopolyploid origin of *C. waldsteinii*. The presence of the uncommon ruby red flower colour in the "putatively ancestral" diploid *C. greimleri* and not in the "derived" tetraploid *C. waldsteinii* indicates that the ruby red flower colour could have evolved independently and later than the origin of the polyploid *C. waldsteinii* from the purple-flowering common ancestor of *C. greimleri* and *C. waldsteinii*. In this case, gene flow to the diploid *C. greimleri* from sympatric yellow flowering diploid congeners (*C. erisithales* or *C. carniolicum*) in the Eastern Alps could easily have resulted in the ruby red phenotype (see above discussion of hybridization and flower colour) and could have been fixed later, e. g., via

a bottleneck that occurred during one of the glacial periods (see above discussion of genetic drift). In contrast, the tetraploid nature strongly prevented the gene flow from the diploid yellow-flowering *C. erisithales* to the purple-flowering *C. waldsteinii* in the Carpathians (see above discussion of hybridization). Thorough molecular analyses are needed to explain the mode of polyploid evolution of *C. waldsteinii* or homoploid hybridogenic speciation of *C. greimleri*, which can only be speculated upon at this time. Importantly, these analyses should also include Anatolian and Caucasian species because the Carpathians and Dinarides could be considered stepping-stone areas in historical migrations between Asian and European mountain systems (Schönswetter et al. 2006).

See http://www.preslia.cz for Electronic Appendices 1–14

Acknowledgements

This study was supported by the Czech Science Foundation (project no. P505 14-30313S and the Centre of Excellence PLADIAS, project no. 14-36079G) and Masaryk University (project no. MUNI/A/1301/2016). Fragment analysis of AFLP was arranged by Aleš Knoll using the infrastructure of CEITEC (Central European Institute of Technology) supported by project CEITEC 2020 (LQ1601). We thank Ondřej Knápek and Pavel Veselý for help with sampling; Jana Kocmanová for the cultivation of samples and DNA standards and for the measurement of stomata; and Ondřej Hájek for preparing the map matrix. For help during this study with the herbarium material of *C. waldsteinii* in the Hungarian Natural History Museum (BP), we are obliged to the custodians Lajos Somlyay and Zoltán Barina. For the data relevant to recent populations of *C. greimleri* and *C. waldsteinii* from the European vegetation archive, we are obliged to Milan Chytrý, Milan Valachovič and Ilona Knollová. For valuable information regarding recent occurrences of *C. greimleri*, we are also obliged to Dmitar Lakušič, Faruk Bogunić, Jakub Hloušek, Pavel Novák, Alexander Mrkvicka and Josef Greimler.

Souhrn

Nově popsaný diploidní druh *Cirsium greimleri* (2n = 34; 2C = 1929,1±60,6 Mbp) patří do typové sekce rodu a vyskytuje se vzácně ve východních Alpách a Dinárských horách, zatímco blízce příbuzný tetraploidní vikariantní druh *C. waldsteinii* (2n = 68; 2C = 3682,3±69,8 Mbp) je endemitem jižních a východních Karpat. 169 rostlin z 27 populací pokrývajících reprezentativně areály obou druhů bylo analyzováno cytometricky, geneticky (AFLP) a morfometricky. Všechny tyto analýzy prokázaly jasnou separaci obou taxonů. Druhy se liší také v barvě květů: rubínově červené až hnědavě nachové u *C. greimleri* vs. růžově nachové až nachové u *C. waldsteinii*; tento barevný rozdíl je stabilní i tehdy, pokud jsou oba druhy pěstovány ve stejných podmínkách. Mezidruhový rozdíl ve velikosti průduchů, nažek, korun, čnělek je statisticky signifikantní a v souladu s nárůstem ploidní úrovně. Vzhledem k tomu, že oba druhy jsou gynodioecické (jejich populace obsahují samičí a hermafroditní rostliny), měly by mezidruhové rozdíly ve velikosti generativních znaků být používány k určování pouze tehdy, pokud známe pohlaví determinované rostliny, tj. srovnávat lze jen samice se samicemi, nebo hermafrodity s hermafrodity, protože rozdíly mezi pohlavími uvnitř druhu mohou být větší než rozdíly mezidruhové. Dolní a střední lodyžní listy *C. waldsteinii* mají užší tvar a jsou hlouběji členěné v laloky než listy *C. greimleri*. Mírný posun v době kvetení byl zjištěn, když byly oba druhy pěstovány společně na experimentální zahradě v Brně: *C. waldsteinii* začal kvést o dva týdny dříve než *C. greimleri*. Tento rozdíl zjištěný v nízké nadmořské výšce (268 m n. m.) je však třeba extrapolovat s opatrností na podmínky přirozeného výskytu obou druhů (800–2000 m n. m.). Oba druhy sdílejí ekologické a stanovištní preference k subalpínské řídké lesní vegetaci s podrostem vysokých, často širokolistých bylin. Genetická struktura jejich populací se však podstatně liší. Zatímco druh *C. waldsteinii* akumuloval větší variabilitu a více vzácnějších alel v méně separovaných populacích než *C. greimleri*, u něhož je variabilita uvnitř populací relativně nízká s ohledem na větší mezipopulační rozdíly a také průměrný počet alel na jedince je u tohoto druhu v jeho populacích nižší. Různé rozložení genetické variability je pravděpodobně důsledkem toho, že zvýšení ploidie zpomaluje působení genetického driftu a zároveň zefektivňuje genetickou homogenizaci populací vlivem mezipopulační migrace u *C. waldsteinii*. Nesmíme však zapomínat ani na to, že populace *C. greimleri* prošly v Alpách pravděpodobně dramatičtějším pleistocenním vývojem, než který prodělaly populace *C. waldsteinii* v Karpatech, což mohlo genetickou diferenciaci *C. greimleri* ještě prohloubit. Zatímco tetraploidní *C. waldsteinii* hybridizuje extrémně vzácně se společně se vyskytujícími

diploidními pcháči, *C. greimleri* hybridizuje naopak velmi ochotně. Rozdílná intenzita genového toku z jiných druhů může dále umocňovat genetické rozdíly mezi populacemi *C. greimleri*, ale i zvyšovat riziko jeho vymření následkem genetické koroze.

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Received 13 November 2017
Revision received 23 February 2018
Accepted 23 March 2018

Appendix 1. – List of populations sampled (Alpine-Dinaric *Cirsium greimleri*: 1–16, Carpathian *C. waldsteinii*: 17–27).

1. Austria, Ennstal Alps (subgroup Eisenerz Alps), Eisenerz: forest slopes along the brook, 8.5 SW of the town; 47°29'48.8"N, 14°47'58.4"E; 1093 m s. m.; coll. P. Bureš & J. Šmerda 30/Jul/2016. (FCM/AFLP: 6/6 samples); **2**. Austria, Ennstal Alps (subgroup Eisenerz Alps) Kölblwirt (near Johnsbach): along the path from Kölblwirt up to Hesshütte in Gesäuse between Untere Koderalm and Stadlalm (right of the road to the top), 3 km NE of the village; 47°33'00.0"N, 14°38'38.0"E; 1560 m s. m.; coll. P. Bureš & J. Šmerda 30/Jul/2016. (FCM/AFLP: 1/1 sample); **3**. Austria, Rottenmanner and Wölzer Tauern, Lachtal: along the road Oberer Höhenweg (= road between the village of Oberzeiring and the mountain chalet Klosterneuburger Hütte), 3.5 km NE of the village; 47°16'19.1"N, 14°24'22.5"E; 1739 m s. m.; coll. P. Bureš 15/Sep/2015. (FCM/AFLP: 13/8 samples); **4**. Austria, Rottenmanner and Wölzer Tauern, Möderbrugg: open N facing forest slopes on Steinerkogel Hill, 4.8 km WSW of the village; 47°16'18.9"N, 14°24'59.6"E; 1660 m s. m.; coll. P. Bureš 26/Jul/2016. (FCM/AFLP: 6/6 samples); **5**. Austria, Lavanttal Alps (subgroup Seetal Alps), Judenburg: in open S facing forested slope near the chalet Winterleitenhütte, 10.7 km SW of the town; 47°05'41.7"N, 14°34'07.5"E; 1796 m s. m.; coll. P. Bureš 26/Jul/2016. (FCM/AFLP: 6/6 samples); **6**. Austria, Lavanttal Alps (subgroup Koralpe), Rieding, wet slopes along the road Koralpenhöhenstrasse, 4 km ESE of the village; 46°48'30.6"N, 14°56'34.3"E; 1546 m s. m.; coll. P. Bureš 24/Jul/2016. (FCM/AFLP: 6/6 samples); **7**. Austria, Pohorje Mts, Sankt Oswald ob Eibiswald: along the forest road on S facing slope, 3.5 k W of the village; 46°42'37.0"N, 15°05'49.0"E; 973 m s. m.; coll. P. Bureš 24/Jul/2016. (FCM/AFLP: 6/6 samples); **8**. Austria, Stein Alps, Unterort (Podkraj): Alpine meadow on ski slope 3.5 km SSSW of the village; 46°30'38.5"N, 14°46'07.9"E; 1848 m s. m.; coll. P. Bureš & J. Šmerda 28/Jul/2017. (FCM/AFLP: 6/5 samples); **9**. Austria, Stein Alps, Vellach (Bela): along the forest road 2.1 km SSW of the village; 46°25'09.6"N, 14°33'06.7"E; 883 m s. m.; coll. P. Bureš & J. Šmerda 29/Jul/2017. (FCM/AFLP: 6/6 samples); **10**. Austria, Stein Alps, Ebriach (Obirsko): wet rocky slopes on Trögener Klamm, 4.5 km SW of the village; 46°26'26.1"N, 14°29'06.3"E; 852 m s. m.; coll. P. Bureš 25/Jul/2016. (FCM/AFLP: 3/3 samples); **11**. Slovenia, Julian Alps (subgroup Cerkljansko hribovje), Podbrdo: in the forest along the road E of the Massif Porezen, 2.7 km SSE of the village; 46°11'34.6"N, 13°59'21.8"E; 1444 m s. m.; coll. P. Bureš & E. Michálková 16/Jul/2016. (FCM/AFLP: 10/6 samples); **12**. Slovenia, Notranjski Snežnik Mts, Sviščaki: wet forest 4.1 km E of the village; 45°34'24.8"N, 14°27'24.0"E; 1340 m, s. m.; coll. P. Bureš & E. Michálková 12/Jul/2016. (FCP/AFLP: 6/5 samples); **13**. Austria, Stein Alps, Podvolovljek: along the forest road in Velika Planina, 2.9 km WNW of the village; 46°18'35.7"N, 14°39'23.4"E; 1408 m s. m.; coll. P. Bureš & E. Michálková 14/Jul/2016. (FCM/AFLP: 6/4 samples); **14**. Bosnia and Herzegovina, Vranica planina Mts, Fojnica: along the forest roads in the surroundings of the tourist rest place with fountain, 11.4 km WNW of the town; 43°59'29.3"N, 17°45'37.1"E; 1500 m s. m.; coll. P Bureš & M. Vavrinec 14/Jul/2017. (FCM/AFLP 9/6 samples); **15**. Bosnia and Herzegovina, Bjelašnica Mts (subgroup Igman), Hadžići: along the forest road near the chalet Sarajevski Begluk, 6.6 km SSE of the town; 43°46'18.4"N, 18°14'45.9"E; 1209 m s. m.; coll. P. Bureš & M. Vavrinec 13/Jul/2017. (FCM/AFLP 6/7 samples); **16**. Serbia, Kopaonik Mts, Brzece: wet forest slopes along the road to Kopaonik, 3.5 km NW of the village; 43°19'00.6"N, 20°50'53.3"E; 1550 m s. m.; coll. P. Bureš & M. Vavrinec 17/Jul/2017. (FCM/AFLP 10/5 samples); **17**. Slovakia, Bukovské vrchy Mts, Runina: mountain meadows near Ďurkovec Hill, 3.3 km N of the village; 49°06'03.1"N, 22°25'11.9"E; 1095 m s. m.; coll. O. Knápek & M. Vavrinec 11/Jul/2016. (FCM/AFLP: 11/11 samples); **18**. Slovakia, Bukovské vrchy Mts, Runina: mountain meadows between the hills Rabia skala, Jarabá skala and Čelo, 4.5 km NE of the village; 49°06'11.0"N, 22°26'47.9"E; 1145 m s. m.; coll. O. Knápek & M. Vavrinec 13/Jul/2016. (FCM/AFLP: 11/10 samples); **19**. Ukraine, Ukrainian Carpathians (subgroup Gorgany), Krasnyi: along the road to Lake Sinĕvir, 0.5 km W of the village; 48°36'52.6"N, 23°41'45.4"E; 942 m s. m.; coll. P. Veselý & M. Vavrinec 20/Jul/2016. (FCM/AFLP: 4/4 samples); **20**. Ukraine, Ukrainian Carpathians (subgroup Chornohora), Zavoyelya: mountain meadow on ski slope near chalet Zarosliak, 6.5 km SW of the village; 48°09'54.9"N, 24°32'13.7"E; 1293 m s. m.; coll. O. Knápek & M. Vavrinec 20/Jun/2017. (FCM/AFLP: 5/4 samples); **21**. Romania, Rodna Mts, Borşa: wet mountain meadow along tourist road to the Pietros Mt., 3.9 km S of the town; 47°37'09.2"N, 24°38'58.9"E; 1166 m s. m.; coll. O. Knápek & M. Vavrinec 21/Jun/2017. (FCM/AFLP: 6/6 samples); **22**. Romania, Ceahlău Massiv, Ceahlău: forest margin along the tourist road from the chalet Cabana Fântânele to the rocks Cuşma Dorobanţului, 5.5 km S of the village; 46°59'33.3"N, 25°57'06.5"E; 1355 m s. m.; coll. Ester Michálková & Martin Vavrinec 5/Jun/2017. (FCM: 4 samples); **23**. Romania, Bucegi Mts, Sinaia: along the road on the E shore of Bolboci Lake, 8 km W of the town; 45°20'29.0"N, 25°26'04.3"E; 1455 m s. m.; coll. P Veselý. & M. Vavrinec 22/Jul/2016. (FCM/AFLP: 6/5 samples); **24**. Romania, Bucegi Mts, Sinaia: along the road on the W shore of Bolboci Lake, 9.5 km W of the town; 45°20'42.5"N, 25°25'12.3"E; 1450 m s. m.; coll. P Veselý. & M. Vavrinec 22/Jul/2016. (FCM/AFLP: 2/2 samples); **25**. Romania, Făgăraş Mts, Cârţişoara: along the road

Transfagarasan, 8.5 km SSE of the village; 45°38'57.1"N, 24°36'33.7"E; 1388 m s. m.; coll. P. Veselý & M. Vavrinec 23/Jul/2016. (FCM/AFLP: 3/3 samples); **26**. Romania, Făgăraş Mts, Cârţişoara: along the road Transfagarasan, 15 km S of the village; 45°33'43.2"N, 24°36'31.5"E; 1274 m s. m.; coll. P. Veselý & M. Vavrinec 23/Jul/2016. (FCM/AFLP: 1/1 sample); **27**. Romania, Lotru Mts, Voineasa: wet forest slopes along the road to lake Vidra, 8.5 km WNW of the village; 45°25'55.3"N, 23°51'01.4"E; 1174 m s. m.; coll. P. Bureš & M. Vavrinec 19/Jul/2017. (FCM/AFLP 10/6 samples).