

Cirsium *xsudae*: a new interspecific hybrid between rare Alpine thistles

Cirsium xsudae – nový mezidruhový kříženec vzácných alpských pcháčů

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In this study we describe a new nothospecies, *Cirsium xsudae* Michálková et Bureš, a homoploid hybrid between two rare Alpine species, *C. carniolicum* and *C. greimleri*. Hybrid status was confirmed for four morphologically intermediate individuals, found in the Ennstal Alps, Austria (three of them were F1 hybrids and one a backcross with *C. carniolicum*). We used amplified fragment length polymorphism (AFLP) to confirm affiliation to the parental species and exclude the potential contribution of other sympatric species. In addition, we used flow cytometry to confirm the diploid status of this hybrid. The newly assessed genome size of this hybrid is $2C = 1.99 \pm 0.03$ pg, and for *C. carniolicum* $2C = 2.03 \pm 0.04$ pg.

Key words: AFLP, Alps, *Asteraceae*, *Compositae*, *Carduoidae*, *Cynareae*, flow cytometry, genome size, homoploid hybridization, interspecific hybridization, thistle

Introduction

Cirsium Mill. (thistle, *Asteraceae*) is a large genus composed of roughly 400–450 species distributed in the Northern Hemisphere, spanning the subtropical to boreal latitudes (Bureš et al. 2018). Because of its strong tendency to produce interspecific hybrids in nature (Wagenitz 1987, Bureš et al. 2004, 2010, Keil 2006, Stöhr 2006, Segarra-Moragues et al. 2007, Sheidai et al. 2016), this genus has been engaging the attention of botanists since the Linnean era. While some interspecific hybrids are frequently reported, others are extremely rare (Bureš et al. 2004, 2010, Segarra-Moragues et al. 2007). The frequency of interspecific hybridization is conditioned by overlap of distribution ranges and/or flowering periods of parental species, by their phylogenetic (dis)similarity, or ploidy difference (Bureš et al. 2004, 2010).

Within *C. waldsteinii* Rouy, two cytotypes/species were recently recognized: a tetraploid distributed in the Eastern and Southern Carpathians and a diploid distributed in the Eastern Alps and the Dinaric Mountains, which was separated as *C. greimleri* Bureš (Bureš et al. 2018). While the diploid *C. greimleri* hybridizes frequently with other diploid congeners, the Carpathian, tetraploid *C. waldsteinii* hybridizes much less frequently (Bureš et al. 2018).

The binomials for the majority of *C. waldsteinii* hybrids are based on Alpine plants: *C. xscopolii* Khokhlov (= *C. erisithales* × *C. waldsteinii*), *C. xjuratzkae* Reichardt (= *C. heterophyllum* × *C. waldsteinii*), *C. xprzybylskii* Eichenfeld (= *C. oleraceum* ×

C. waldsteinii), *C. ×reichardtii* Juratzka (= *C. palustre* × *C. waldsteinii*), *C. ×stiriicum* Fritsch (= *C. rivulare* × *C. waldsteinii*), *C. ×stroblii* Hayek (= *C. spinosissimum* × *C. waldsteinii*) and *C. ×paradoxum* Hayek (= *C. arvense* × *C. waldsteinii*) (cf. Reichardt 1861, Eichenfeld 1887, Fritsch 1907, Hayek in Halácsy 1907, Khek 1908, 1909, Hayek in Zahlbruckner 1913, respectively). Since all previous records of *C. waldsteinii* from the Alps now refer to *C. greimleri*, any binomials of interspecific hybrids with *C. waldsteinii* should be implicitly ascribed to hybrids with *C. greimleri*, because they are based on plant material collected in the Alps. However, this is not the case for the binomial of the hybrid between tetraploid *C. vulgare* and *C. waldsteinii* (= *C. ×zapalowiczii* Khek), which is based on plants collected by H. Zapalowicz in the Chornohora Mts in the Eastern Carpathians (Khek 1909).

There is one extremely rare hybrid combination that has never been formally described and designated with a binomial, the hybrid between diploid Eastern Alpine *C. carniolicum* Scop. and *C. greimleri*. Despite the overlapping area of distribution, these species hardly co-occur, because of their overall rarity and different ecologies. *Cirsium carniolicum* is considered calciphilous whereas *C. greimleri* is a calcifuge (Janchen 1958, Meusel & Jäger 1992). This hybrid was reported only once from the mountains along the Austrian-Slovenian border: near Wackendorfer Alm south of the village Unterort (Podkraj) in the Eastern Karawanks by Meltzer (1973). Morphological determination of hybrids can, however, be deceiving (Rieseberg & Ellstrand 1993). The overall morphological variation of hybrids is usually broader than that in their parental species because the parental features are combined uniquely in each hybrid individual (Bureš 2004). In *Cirsium*, where most species are sympatric and able to hybridize, the parental species are in principle always uncertain, particularly when more species co-occur. Furthermore, thistles are also known to produce triple hybrids (Wagenitz 1987, Bureš 2004). Since the achenes of *Cirsium* can spread over long distances, the co-occurrence of parents with hybrids is not the rule (unpubl. field observ.), as is also documented for hybrids in other genera, e.g., in *Potamogeton* (Kaplan et al. 2009). For the exact identification of the parental species of a particular hybrid it is therefore necessary to consider not only the co-occurring species but also those growing in the broader surroundings.

Whole genome dominant marker AFLP (amplified fragment length polymorphism; Vos et al. 1995) is an effective molecular technique for examining hybrids and determining their affiliation to parental species (Bonin et al. 2007), as documented in particular case studies such as Kirk et al. (2004), Minder et al. (2007), Segarra-Moragues et al. (2007), Hersch-Green & Cronn (2009), Goldman et al. (2011), Cires et al. (2012) and Szczepaniak et al. (2016). Another efficient method for examining hybrids, which is mostly used when the parental species differ in ploidy level, is flow cytometry (Suda et al. 2007b), as documented in case studies such as Bureš et al. (2003), Morgan-Richards et al. (2004), Suda et al. (2007a), Kúr et al. (2016) and Macková et al. (2017). This method has been successfully used even when detecting homoploid hybridization in cases where the genome sizes of the parental taxa differ (Suda et al. 2007b). Moreover, this method effectively detects possible polyploidy consequences of hybridization (Morgan-Richards et al. 2004).

In this study we examine, using AFLP and flow cytometry, a putative hybrid of *C. carniolicum* and *C. greimleri*, found sympatrically with the putative parental species, and eventually describe it as a new nothospecies.

Materials and methods

Plant material and sampling strategy

The plant samples for this study were collected while sampling material for another, broader study concerning interspecific hybridization among Central-European species of the genus *Cirsium*. At the locality near Kölblwirt in the Ennstal Alps, we fortuitously discovered four individuals whose morphological features suggested their origin from co-occurring *C. carniolicum* and *C. greimleri*. To prove that the hybrids originated from these two species and to exclude affiliation with any other potential parent, a set of samples based on the regional species pool was created. Besides the putative hybrid plants resembling *C. carniolicum* × *C. greimleri*, (i) samples of all *Cirsium* species present at the locality near Kölblwirt, *C. carniolicum*, *C. greimleri* (which was present as only one clone at the locality) and *C. erisithales*, were included in the sample set (Electronic Appendix 1). To complete AFLP profiles of the above-mentioned species, (ii) the sample set was enhanced with samples from other localities. Additionally (iii) samples of all other *Cirsium* species occurring in the Austrian Easternmost Alps: *C. acaulon*, *C. arvense*, *C. eriophorum*, *C. heterophyllum*, *C. oleraceum*, *C. palustre*, *C. pannonicum*, *C. rivulare*, *C. spinosissimum* and *C. vulgare*, were included in the sample set. The nomenclature and taxonomical treatment follow Flora Europaea (Werner 1976), apart from *C. heterophyllum* (L.) Hill. and *C. acaulon* (L.) Scop.

To avoid clones, we sampled plants that were at least 10 m apart. From every sampled individual we collected few pieces of young, undamaged leaves for subsequent AFLP and flow cytometry analyses. Samples for AFLP were placed in plastic bags and stored in a deep freezer at -80°C until analyzed. Samples for flow cytometry were placed in plastic bags with few droplets of water and analyzed 3–4 days later. After the leaf samples were collected, the shoot was kept as a herbarium specimen (preserved in BRNU – acronyms of herbaria follow Thiers 2018). The measurements of achene, corolla and pappus (Electronic Appendices 5–7) and their statistical analyses follow Bureš et al. (2018).

Molecular data processing

Using AFLP, we analyzed all four putative *C. carniolicum* × *C. greimleri* hybrid individuals and 18–24 samples of each species (see Electronic Appendix 2). Genomic DNA was extracted from deep frozen leaves using 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 AFLPtm plant mapping kit (Applied Biosystems). Genomic DNA (300 ng) was double-digested at 37°C for 4 h with EcoRI and MseI and ligated to EcoRI and MseI adaptors in the same reaction. The preselective amplification was done with EcoRI+A and MseI+C primers in a GenePro (Bioer) thermocycler. The following four selective primer pair combinations were chosen: 6-FAM-EcoRI+ACT/MseI+CTC, VIC-EcoRI+AGG/MseI+CAG, NED-EcoRI+AGC/MseI+CAT, PET-EcoRI+ACA/MseI+CTA. The products of selective amplification were mixed with GS500 LIZ size standard and Hi-DiTM Formamide (Applied Biosystems) for fragment analysis on an ABI 3500 Genetic Analyzer (Life Technologies). The error rate estimation (according to Bonin et al. 2004) was based on 23 replicate samples. Sizing and scoring of the raw data was performed using GeneMarker 2.4.0 (SoftGenetics). A panel

of scorable peaks (loci) for each primer combination was created manually. Fragments from 60 to 560 bp were scored. Scoring of samples was done automatically and then checked and corrected manually. The final file was then exported as a binary matrix.

To identify the parental species of putative hybrid individuals, we performed a two-step Bayesian analysis using STRUCTURE 2.3.4 software (Pritchard et al. 2000). Within the first step, we analyzed the morphologically “pure” samples, without prior membership information, in order to verify whether they were also genetically “pure”. We ran STRUCTURE for $K = 13$ (since we had 13 species) with 10 separate runs, each of 100,000 iterations and 100,000 burn-in, and used an admixture model with independent allele frequencies. The ploidy level was set as “2” – diploid – since all species (except for tetraploid *C. vulgare*) are diploid. Individuals with a q value lower than 0.900 in their species cluster were considered genetically eroded.

Within the second step, we applied the USEPOPINFO model implemented in STRUCTURE. The genetically “pure” individuals (confirmed in the previous step) were set to be of “known” origin and divided into 13 populations according to their species. The putative hybrids and genetically eroded individuals were set to be of “unknown” origin in order to be clustered by the program. The STRUCTURE was run for $K = 13$ with 10 separate runs, each of 100,000 iterations and 100,000 burn-in, and we used an admixture model with independent allele frequencies and diploid ploidy level. The STRUCTURE diagram was generated using R package pophelper (Francis 2017).

To complement the Bayesian approach with distance-based analyses, relationships between the hybrids, their putative parental species (*C. carniolicum* and *C. greimleri*) and co-occurring *C. erisithales* were also visualized as a NeighborNet network using SplitsTree software 4.14.4 (Huson & Bryant 2006) and PCoA diagram using Canoco5 (ter Braak & Šmilauer 1998) based on Jaccard distances.

Flow cytometry

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 standard *Bellis perennis* ($2C = 3089.89$ Mbp, 39.54 % GC, Veselý et al. 2012), whose genome size and genomic GC contents were derived from comparisons with the completely sequenced *Oryza sativa* subsp. *japonica* ‘Nipponbare’ (International Rice Genome Sequencing Project 2005). For the genome size (Electronic Appendix 3) or ploidy level estimations, propidium iodide was used as a fluorochrome, and for the genomic GC content (Electronic Appendix 4) estimation, the samples were co-processed with DAPI (4',6-diamidino-2-phenylindole) fluorochrome. For improving the signal/background ratio, the original OTTO I solution (Otto 1990) was mixed 1: 1 with 0.1 M hydrochloric acid and two drops of Tween. This “acid” buffer was prepared to reduce the influence of secondary metabolites on the measurement. The genomic GC content was calculated using an ATGCFlow spreadsheet prepared by P. Šmarda: <http://www.sci.muni.cz/botany/systemgr/download/Festuca/ATGCFlow.xls> (Šmarda et al. 2008). In parental species, each sample was analyzed once, in hybrids, three times on different days.

Results

Genetic proof of hybrid status and parental affiliation of hybrid: AFLP analyses

There were 563 AFLP loci scored in 283 individuals from all 13 species. The highest number of fragments per individual was found in *C. vulgare* (mean = 131.7), which was the only tetraploid. In diploid species, the mean number of fragments was evenly distributed from 95.7 to 122.9 (Electronic Appendix 2). The overall error rate was 0.4 % for compared AFLP-replicates.

Within the first step of the STRUCTURE analysis, it was discovered that the only *C. greimleri* individual (GREI-A52a) found at the locality near Kölblwirt is likely to be genetically eroded, because its q value for the *C. greimleri* cluster was only 0.837, while its q value for *C. erisithales* was 0.151. Since this individual is the only representative of the species *C. greimleri* from this locality and is therefore likely to be either the parent or a close relative of the parent of the four putative *C. carniolicum* × *C. greimleri* hybrids we decided to keep this “impure” individual in further analyses. Nevertheless, the hybrid status of this individual was taken into account and it was set as of “unknown” origin during the second step of the analysis.

Remaining individuals clustered exactly according to their species membership, and the q values ranged from 0.943 to 0.998 (results not shown), indicating that the level of “genomic” admixture in morphologically “pure” samples is negligible and no hybrids are within the dataset.

During the second step of the STRUCTURE analysis, the four putative hybrids were split between the two parental species, *C. carniolicum* and *C. greimleri* (Fig. 1). While for Hyb-A52a, Hyb-A52b and Hyb-A52c, the q value ratio of *C. carniolicum* and *C. greimleri* was approximately 1:1 suggesting their F1 hybrid status, the q value ratio was increased in favour of *C. carniolicum* in Hyb-A52d, suggesting that this individual could be a BC1 hybrid originating from F1 via backcrossing with *C. carniolicum*. Within the only *C. greimleri* individual from the locality near Kölblwirt (GREI-A52a) there was

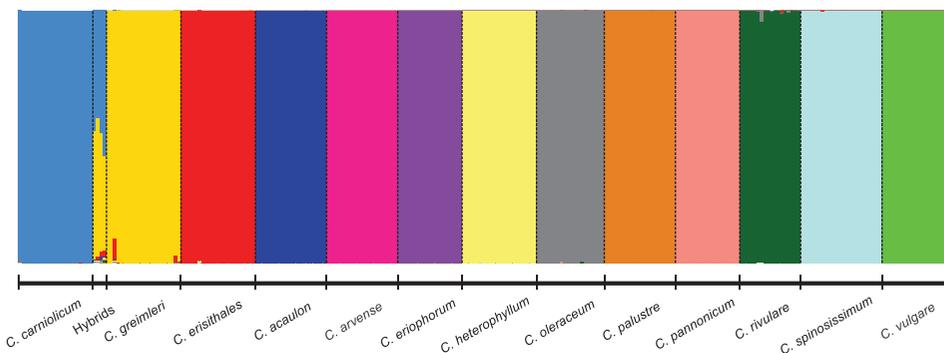


Fig. 1. – Affiliations of hybrids collected near Kölblwirt to 13 species occurring in the Easternmost Austrian Alps estimated using STRUCTURE. The hybrids are clustered between *Cirsium carniolicum* and *C. greimleri* with a negligible admixture of *C. erisithales* and other species. Results of the STRUCTURE analysis are visualized as a bar plot showing Bayesian assignment probabilities for 13 clusters derived from AFLP profiles of all 13 species and hybrids.

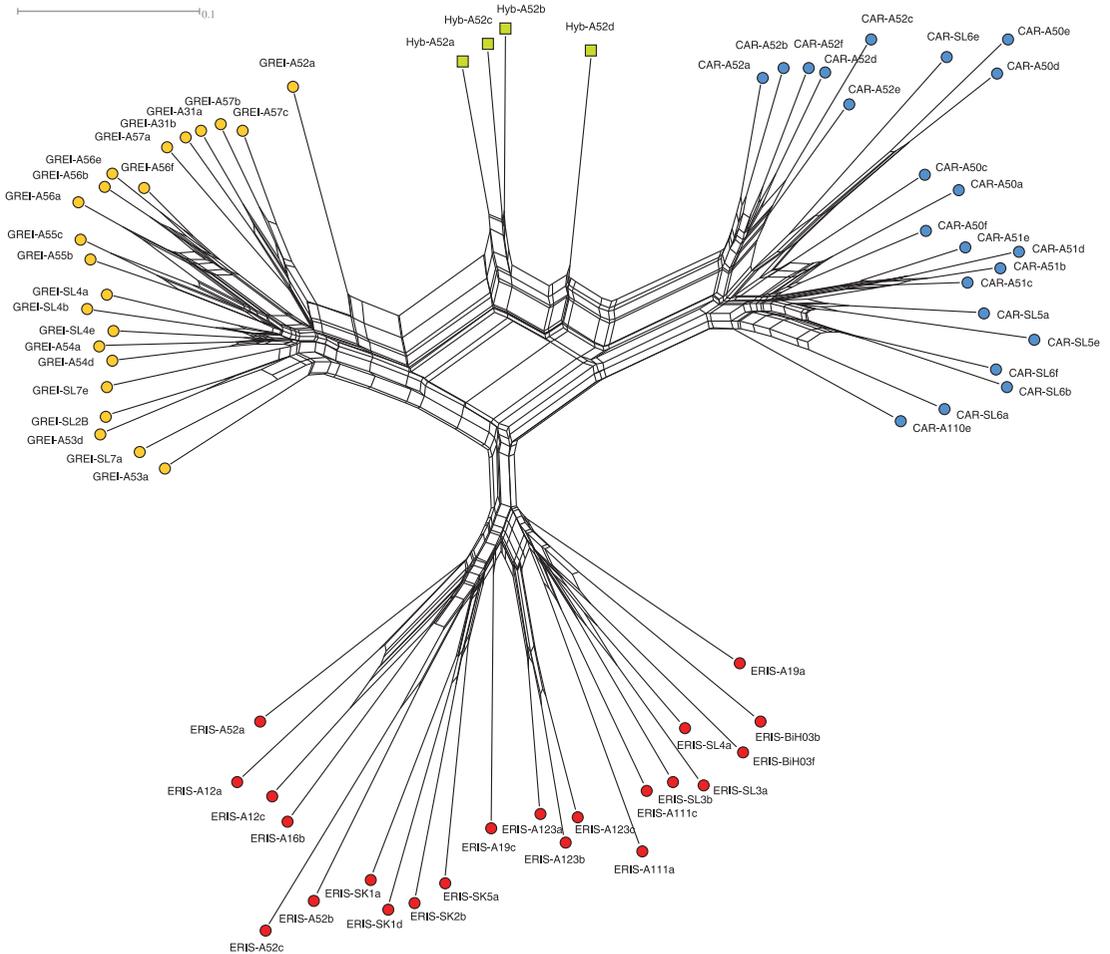


Fig. 2. – Genetic relationship among hybrids collected near Kölblwirt (Hyb, green squares) and representatives of the potential parents, *Cirsium carnioolicum* (CAR, blue circles), *C. erisithales* (ERIS, red circles) and *C. greimleri* (GREI, yellow circles), visualized using NeighborNet based on Jaccard distances derived from AFLP profiles. Numbers on labels refer to respective localities (Electronic Appendix 1).

an admixture of *C. erisithales* in accordance with the first step of the STRUCTURE analysis.

The NeighborNet (Fig. 2) and PCoA diagram (Electronic Appendix 8) depicting relationships between the hybrids, their parental species *C. carnioolicum* and *C. greimleri* and co-occurring species *C. erisithales*, provided a fine resolution of all the taxa examined. The hybrid individuals were clustered intermediately between the parental species. It is also evident that the hybrids are most affiliated with the samples of *C. carnioolicum* from Kölblwirt and the single sample of *C. greimleri* from the same locality (A52). The individual Hyb-A52d was shifted towards *C. carnioolicum*, as it was in the STRUCTURE analysis. Neither the NeighborNet, nor the PCoA diagram showed any tendency of the hybrids towards *C. erisithales*.

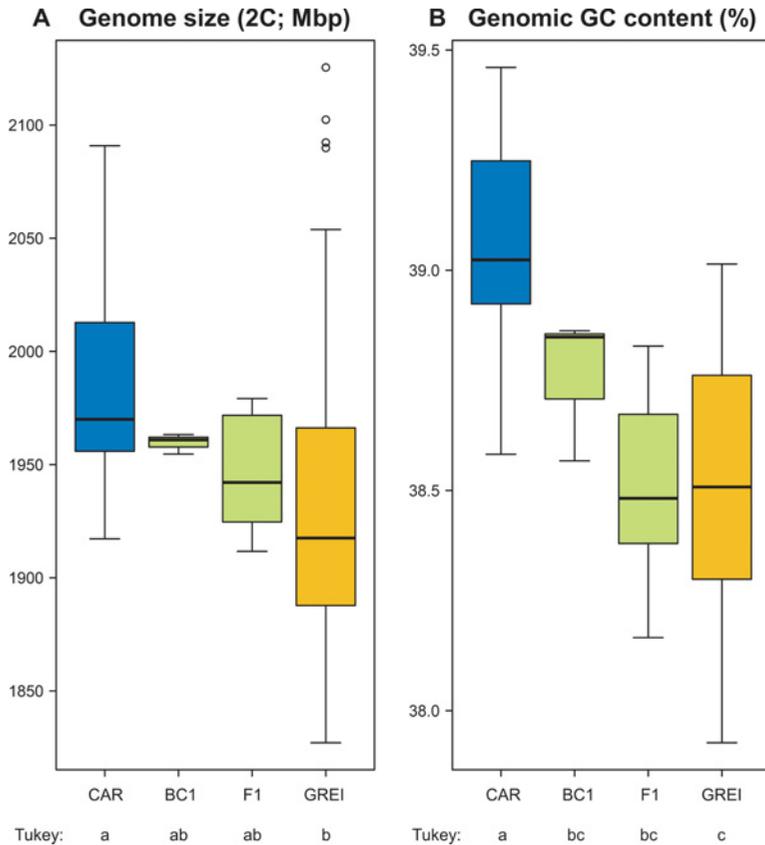


Fig. 3. – Variability in genome size (A), and genomic GC content (B) detected in *Cirsium ×sudaе* (F1, green), backcross *C. ×sudaе* × *C. carniolicum* (BC1, green), and parental species *C. carniolicum* (blue) and *C. greimleri* (yellow). Boxplots show median (thick line), interquartile range (boxes), non-outlier range (whiskers) and outliers (empty circles). Boxplots marked with the same letter do not differ significantly at $P > 0.05$ (Tukey HSD test).

Genome size, ploidy level and genomic GC content in hybrid and parental species

The variation in the amount of somatic nuclear DNA in the F1 hybrids (Hyb-A52a, Hyb-A52b and Hyb-A52c) was intermediate between the parental species (see Fig. 3A and Electronic Appendix 3). Mean was $2C = 1.99 \pm$ standard deviation 0.03 pg for the F1 hybrid, 2.03 ± 0.04 pg for *C. carniolicum* and 1.97 ± 0.06 pg for *C. greimleri*. Although the difference in $2C$ of the two parental species was rather small it was statistically significant (Tukey HSD test; Fig. 3A). The hybrids, on the other hand, could not be considered significantly different from either parental species (Fig. 3A). The genome size of the BC1 hybrid was $2C = 2.00 \pm 0.01$ pg.

The detected genomic GC content was $38.52 \pm 0.22\%$ for the F1 hybrids and $38.76 \pm 0.17\%$ for the BC1 hybrids; $39.02 \pm 0.29\%$ for *C. carniolicum* and $38.51 \pm 0.27\%$ for *C. greimleri* (Fig. 3B; Electronic Appendix 4). As for genome size, the difference

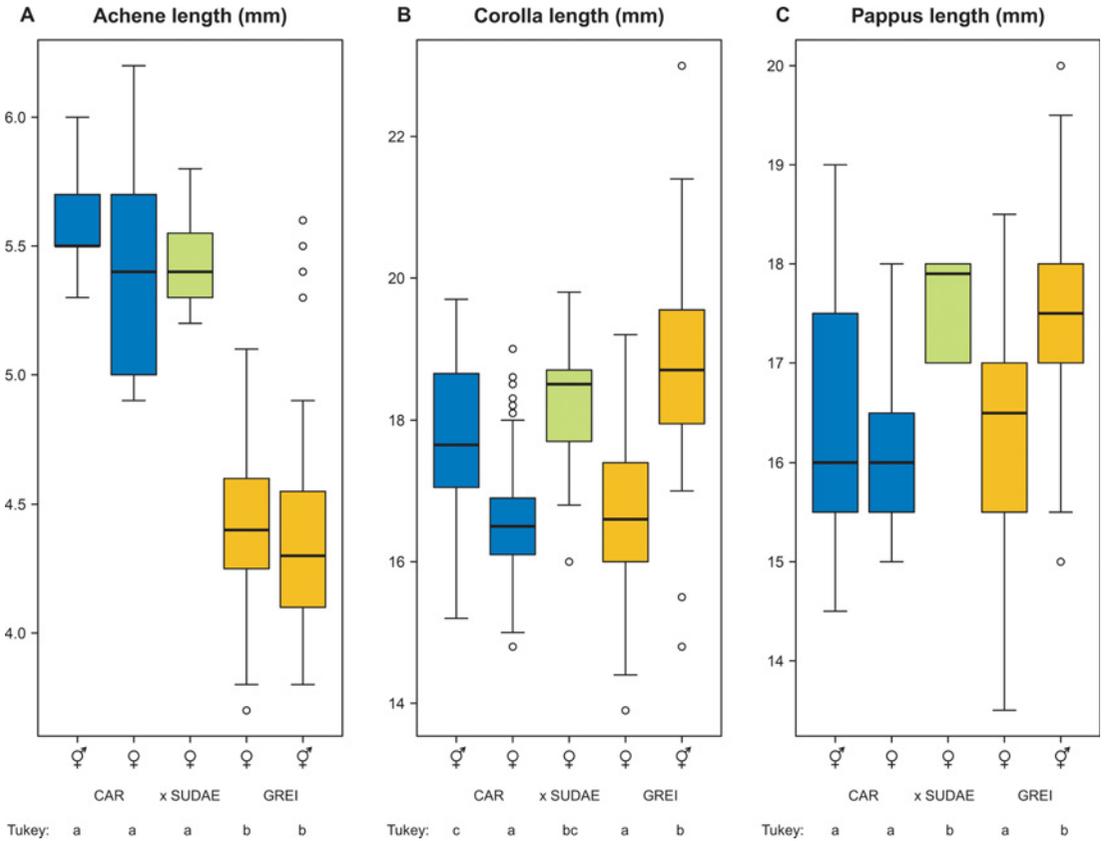


Fig. 4. – Variability in length of achenes (A), corollas (B) and pappi (C) of *Cirsium x sudae* (green) compared with that of parental species *C. carnioolicum* (blue) and *C. greimleri* (yellow). Boxplots show median (thick line), interquartile range (boxes), non-outlier range (whiskers) and outliers (empty circles). Boxplots marked with the same letter do not differ significantly at $P > 0.05$ (Tukey HSD test).

between parental species is negligible but significant (Tukey HSD test, Electronic Appendix 4), moreover, both hybrids F1 and BC1 differ significantly from *C. carnioolicum* (Tukey HSD test, Electronic Appendix 4).

Morphology of hybrid plants

All four hybrid plants were females. That is, their anther tubes were not fully developed and did not contain any pollen. Two of them, individuals Hyb-A52b and Hyb-A52d, had fully developed ripe achenes.

The plant sample Hyb-A52d differed from the other three in having deeply lobed leaves and longer (6–10 mm) spines on lateral lobes of upper leaves. Because in the molecular analyses the sample Hyb-A52d was identified as BC1 to *C. carnioolicum*, the following morphological characteristics are based on the three F1 hybrid plants (Hyb-A52a, Hyb-A52b and Hyb-A52c).

Table 1. – Most important characters differentiating *Cirsium xsudae* from the parental species *C. carnioolicum* and *C. greimleri*; ¹ = anther tubes; ² since only female hybrids were found, no developed synanthelia were recorded in hybrids, but in hermaphrodites their colour could be intermediate; ³ (minimum–) 0.05percentile–0.95percentile (–maximum); ⁴ = involucre bracts (of mature terminal capitulum).

	<i>Cirsium carnioolicum</i>	Hybrid (<i>C. xsudae</i>)	<i>C. greimleri</i>
Flowers			
Corolla colour	Creamy yellowish	Pinkish white	Ruby red to brownish purple
Synanthelia ¹ colour (of hermaphrodite plants)	Pinkish–white; after pollination whitish–brownish	?	Violet–brownish purple; after pollination whitish
Stem			
Colour / indumentum below capitulum	Green / densely hirsute	Greenish white / sparsely arachnoid or woolly	White / densely arachnoid
Uppermost leaves / bracts			
Shape and position	Continual transition between ciliate phyllaries and many (10–20) uppermost marginally spinose leaves/bracts surrounding capitulum	5–10 uppermost marginally spinose leaves/bracts distinctly larger than entire phyllaries	1–3 uppermost shortly spinose leaves/bracts distinctly larger than entire phyllaries
Colour of terminal spines of lateral lobes	Yellowish, later dark brown	Yellowish, later dark brown	Whitish to brownish purple
Length ³ of terminal spines on lateral lobes	(2.3–) 2.7–10.1 (–10.5) mm	(1.2–) 1.8–7.9 (–10) mm	(0.7–) 0.9–2.7 (–3.7) mm
Indumentum	Sparsely setose or hirtelous on both sides	Sparsely setose or hirtelous above / sparsely arachnoid beneath	Subglabrous or sparsely arachnoid above / densely arachnoid beneath
Outer phyllaries ⁴			
Length	Longer than 3/4 of involucre length	Longer than 1/3 and shorter than 2/3 of involucre length	Outer shorter than 1/4 of involucre length
Arrangement in involucre	Spreading, ± erect	Spreading, ± erect	A pressed, only with slightly outstanding apices
Shape	Narrowly lanceolate to linear	Narrowly lanceolate	Lanceolate to narrowly lanceolate
Apex	With apparent (up to 3 mm long) yellowish terminal spine	With apparent (up to 2 mm long) yellowish terminal spine	With slightly patent ruby red apex
Indumentum / margin	Shortly (up to 0.6 mm) densely hirsute on both sides in upper 3/4–2/3 with curved lateral short spines	Shortly (up to 0.3 mm) ciliate in upper 2/3	Entirely glabrous or very shortly ciliate (whitish cilia 0.1–0.2 mm long) in upper 2/3
Colour	Green – glaucescent	Green – purplish or brownish green	Purplish-brown or purplish-black

The most apparent morphological features of putative hybrids were (i) pinkish white flowers (Fig. 5B and E), (ii) sparsely arachnoid or woolly stems below the capitulum (Figs 5B, E, 6H), (iii) greenish or purplish–brownish green narrowly lanceolate phyllaries, whose outer representatives were longer than 1/3 but shorter than 2/3 of involucre length (Table 1), and (iv) 1.2–10.0 mm long terminal spines on lateral lobes of upper leaves (Fig. 5B and E, Table 1). Features (i)–(iv) were all intermediate compared with parental species (Figs 5A, C, D, and F; 6G; and I, Table 1). In other features, putative hybrid plants resembled one of the parental species: in (v) spreading, more-or-less erect, and apically spinose phyllaries (Fig. 5B), in (vi) sparsely setose or hirtelous indumentum on upper surface of leaves (Fig. 6B), and in (vii) 5.2–5.8 mm long achenes (Fig. 4A; Electronic Appendix 5), they resembled *C. carniolicum* (Figs 4B, 5A, 6A, respectively, Table 1); while in (viii) glabrous phyllaries (Fig. 5B and E) and in (ix) sparsely arachnoid indumentum on abaxial surface of upper leaves (Fig. 6E), they resembled *C. greimleri* (Figs 5C and F, 6F, respectively, Table 1). Finally, in some features, such as (x) corolla length (Fig. 4B; Electronic Appendix 6) or (xi) pappus length (Fig. 4C; Electronic Appendix 7), they were larger than both putative parental species. For the synopsis of differences between hybrid and parental species, see Table 1.

Description of the hybrid

Based on morphological intermediacy and genetic analyses we can claim that three of the individuals sampled (Hyb-A52a, Hyb-A52b and Hyb-A52c) are F1 hybrids of co-occurring *C. carniolicum* and *C. greimleri*, and based on these three plants we describe a new nothospecies, whose formal description is as follows:

***Cirsium xsudae* Michálková et Bureš, nothosp. nova**

= *C. carniolicum* Scop., Fl. Carniol., ed. 2., 2: 128, 1772 × *C. greimleri* Bureš, Preslia 90: 105–134, 2018

D i a g n o s i s: The hybrid differs from both parental species in its pinkish white corollas, sparsely arachnoid or woolly indumentum on stem below capitulum, in (1.2–) 1.8–7.9 (–10.0) mm long spines on lateral lobes of upper leaves, in purplish or brownish green phyllaries, in length of outer phyllaries that are longer than 1/3 and shorter than 2/3 of involucre length; from *C. carniolicum* it differs in sparse arachnoid indumentum on abaxial surface of upper leaves and in phyllaries glabrous on both sides with only a shortly ciliate margin; from *C. greimleri* it differs in sparsely setose or hirtelous adaxial surface of upper leaves and in narrowly lanceolate, spreading, more-or-less erect phyllaries with terminal spines (Figs 5B, E, H; 6B, E, and H).

Type: Austria: Ennstal 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; altitude 1560 m a.s.l.; coll. P. Bureš & J. Šmerda, 30 July 2016 (holotype: BRNU; isotypes: PR; WU).

D e s c r i p t i o n: Perennial gynodioecious plant 0.9–1.4 m tall. Stem erect, on the top rather nodding, unwinged, shallowly ribbed, sparsely arachnoid or woolly below the capitula. Leaves shallowly-roughly double serrate, softly herbaceous, fresh green and sub glabrous, sparsely setose or hirtelous (by multicellular hairs) above, greenish and sparsely arachnoid beneath, softly spinose at the margins; leaf spines of upper leaves (1.2–) 1.8–7.9 (–10.0) mm long; basal cauline leaves from petiolate with broadly ovate



Fig. 5. – Habit of *Cirsium ×sudae* (B, E, H) compared with that of the parental species *C. carnioolicum* (A, D, G) and *C. greimleri* (C, F, I). Terminal flower head (capitulum, A–C; scale 1 cm); terminal cluster of flower heads (D–F; scale 1 cm); whole plant (G–I; scale 10 cm).

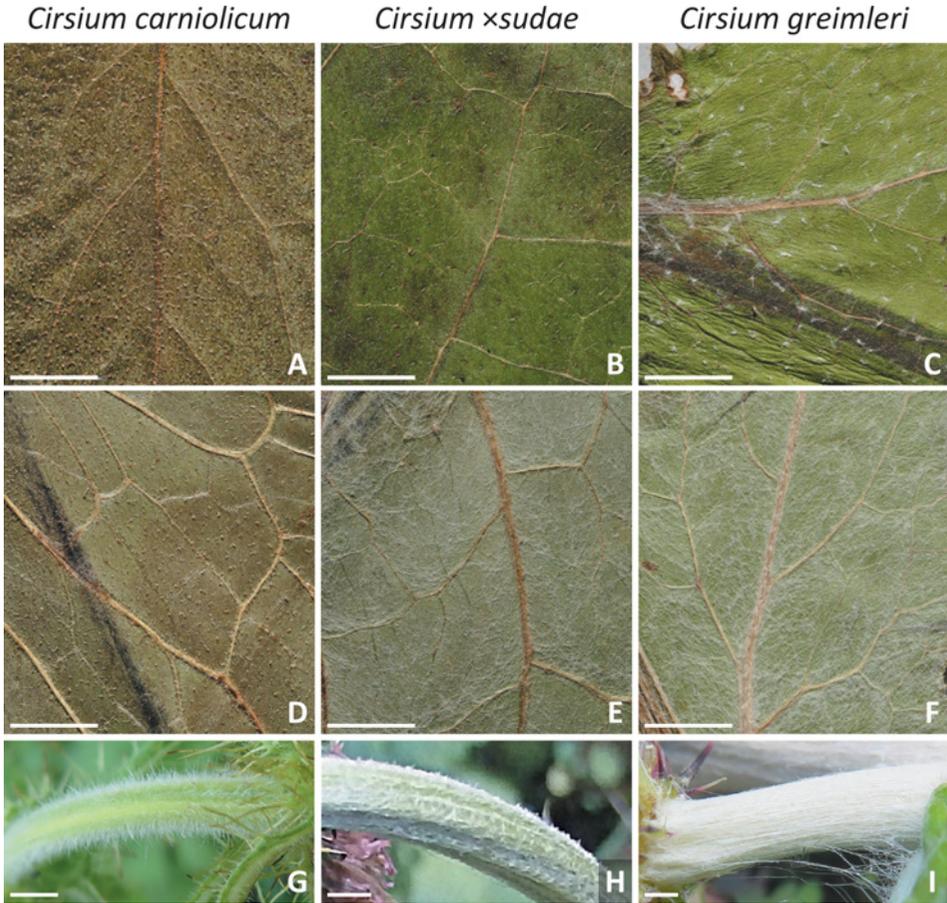


Fig. 6. – Indumentum on stem and leaves of *Cirsium x sudae* (B, E, H) compared to that of the parental species *C. carniolicum* (A, D, G) and *C. greimleri* (C, F, I). Upper (adaxial) leaf surface (A–C); lower (adaxial) leaf surface (D–F); stem below terminal flower head (G–I); all scales 5 mm.

blades to amplexicaul broadly fiddle-shaped or lyrate, blades almost entire, irregularly dentate, serrate or shallowly lobed; median cauline leaves from broadly fiddle-shaped or lyrate to ovate, amplexicaul, roughly double serrate or lobed; upper cauline leaves from lance-ovate to narrowly lanceolate, semi-amplexicaul, shallowly lobed bracts. Capitula nodding, solitary or corymbosely terminally clustered and shortly pedunculate, rarely also solitary on elongate lateral peduncles/branches subtended by upper cauline leaves below the terminal cluster. Peduncles sparsely arachnoid or woolly (= green colour of stem is partly visible). Involucres (of mature terminal capitulum) ovoid, with apices of phyllaries strongly outstanding. Phyllaries (of mature terminal capitulum), narrowly lanceolate, outer and median with short (up to 2 mm) terminal spine, glabrous the margins of which bear short (up to 0.2 mm) cilia, purplish-brownish green. Flowers hermaphroditic or functionally female (with rudimentary synantheria without developed pollen); corolla pinkish white, lobed to 3/10–6/10 of its length, (16.0–) 16.9–19.7 (–19.8) mm long in

females; stigma pinkish shortly bi-lobed, straight or undulate (twisted) in females; pappus plumose, whitish or stramineous, 17–18 mm long in females. Achenes oblong, asymmetric, compressed, creamy ochre, 5–6 mm long in females, with 0.2–0.3 mm long umbo and ca. 0.1 mm high apical ring. Flowering in July.

Distribution: Southeastern Austria in the Eastern Karawanks, in the Kamnik–Savinja Alps (Steiner Alpen, Kamniško-Savinjske Alpe) and in the Ennstal Alps. It might also be present in Slovenia where both parental species occur.

Etymology: The specific epithet is derived from the surname of Prof. RNDr. Jan Suda, Ph.D. (*16. IV. 1974 – †9. III. 2017), an excellent Czech botanist at Charles University, Prague, and at the Institute of Botany, Czech Academy of Sciences, Průhonice.

Discussion

Genetic analyses

Although AFLP is currently considered to be rather outdated, it still provides a reliable way of detecting parental species as shown by the current study. The additive presence of nuclear genomic profiles from both parents was easily detected and confirmed by the analysis of the four individuals recognized, based on their intermediate habitus, as hybrids. In addition, the parental species were well recognized even in a robust dataset containing 13 different species. The applicability of this technique for studying hybridization events was demonstrated recently by Čertner et al. (2015), Hulber et al. (2015), Szczepaniak et al. (2016), Zelener et al. (2016), An et al. (2017), Píšová et al. (2017) and others. In the genus *Cirsium* it was also used for the molecular confirmation of a new hybrid by Segarra-Moragues et al. (2007).

AFLP was also successful in the detection of genetic erosion of a morphologically “pure” individual GREI-A52a by *C. erisithales*. The NeighborNet network showed the strongest affiliation of the hybrids to plants from the same locality (Fig. 2), suggesting they originated from these individuals or their close relatives. The transport of “hybrid” achenes from another site is therefore unlikely. No direct relation of the hybrids to co-occurring *C. erisithales* was revealed by NeighborNet or PCoA analysis (Electronic Appendix 8), which is also consistent with the morphological features of the hybrids.

In the STRUCTURE analysis, a negligible admixture of *C. erisithales* (mean $q = 0.012$) was detected in the hybrid plants (Fig. 1). One could interpret this as an artefact of STRUCTURE computation, nevertheless this analysis detected some admixture of *C. erisithales* in GREI-A52a, the only individual of *C. greimleri* in the locality studied, despite its clear morphological *C. greimleri* identity. This could have two explanations: (i) the only plant of *C. greimleri* at the Kölblwirt locality has been genetically eroded by *C. erisithales* but originated from a “pure” *C. greimleri* individual, which was the parent of *C. ×sudae* or (ii) it is a true parent of the local *C. ×sudae* in whose gametes, from which the alleles of *C. erisithales* were purged by random meiotic chromosomal segregation. Although we consider the latter is more likely. Regarding the frequent hybridization of *C. greimleri* with *C. erisithales* (Bureš et al. 2018), the occasional presence of *C. erisithales* alleles could be expected in populations of *C. greimleri* and weak genetic erosion could become an irreversible feature of this species, at least in some populations. Although triple hybrids can be found in *Cirsium*, their affiliation to the least represented

species is usually larger (10–25%; Michálková in prep.), therefore dominant and even the q values of *C. greimleri* and *C. carniolicum* suggest these are likely to be F1 hybrids rather than triple hybrids.

Flow cytometry

Although both *C. carniolicum* and *C. greimleri* are diploid ($2n = 34$; Czapik 1958, Marcucci & Tornadore 1997, Bureš et al. 2018), the occasional presence of triploid (or higher) ploidy levels cannot be excluded in *Cirsium* species or hybrids (Bureš et al. 2004). Moreover, the closest relative of *C. greimleri*, i.e., *C. waldsteinii*, is tetraploid (Bureš et al. 2018). Flow cytometry was, therefore, used in our study. However, no ploidy variation was detected, neither in the hybrids nor in the pure species. Based on flow cytometry, the parental affiliation of the hybrids could not be stated. On the other hand, the results do not disprove the hybrid nature of the samples examined and the results confirm their diploid status, i.e., the hybridization is homoploid.

Since *C. xsudae* is diploid, its chromosome number is most probably $2n = 34$, because the most frequent chromosome number within the diploid species in the genus *Cirsium* is $2n = 34$ (Bureš et al. 2004), including the parental species (Dobeš et al. 1996, Marcucci & Tornadore 1997).

The genome size of *C. carniolicum* ($2C = 2.03 \pm 0.04$ pg) estimated here for the first time looks rather small compared to the values for other *Cirsium* taxa, among which $2C$ genome size varies from 2.14–3.60 pg (cf. Bureš et al. 2004). This, however, is due to a change in the estimated genome-size of the standards. Bureš et al. (2004) used $2C = 1916$ Mbp, 1.96 pg for *Solanum lycopersicon* ‘Stupické polní tyčkové rané’ standard based on Doležel et al. (1992). By contrast, the present study used *Bellis perennis*, with a genome size ($2C = 3089.89$ Mbp; Veselý et al. 2013) derived from comparisons with the completely sequenced *Oryza sativa* subsp. *japonica* ‘Nipponbare’ (International Rice Genome Sequencing Project 2005). If *Solanum lycopersicon* ‘Stupické polní tyčkové rané’ standard was also derived from *Oryza sativa* subsp. *japonica* ‘Nipponbare’ (International Rice Genome Sequencing Project 2005), its genome size would be $2C = 1696.81$ Mbp, 1.73 pg (Veselý et al. 2012), not $2C = 1916$ Mbp, 1.96 pg. When these smaller standard genome sizes are considered, the newly detected genome size of *C. carniolicum* does not differ from the $2C$ values estimated for other species of *Cirsium* (reported by Bureš et al. 2004), which after re-calculation varies from 1.90 to 3.19 pg at the diploid level.

Morphology

Morphological recognition of hybrids between most *Cirsium* species is easier than in other frequently hybridizing plant genera because the parental species (despite their genetic similarity) are usually highly morphologically diverse, e.g., in height, robustness, colour of flowers or phyllaries, size and number of capitula, degree of spinosity, indumentum on stems or leaves, leaf shape, or life history (Werner 1976, Wagenitz 1987, Bureš 2004, Bureš et al. 2010). In *C. xsudae*, both parental species share uniquely shaped large, shallowly lobed and softly spinose leaves (Fig. 5G and I), but differ substantially in flower colour (Fig. 5A, C, D, F). The intermediate pinkish flower colour is therefore the most apparent feature of the hybrid *C. xsudae* (Fig. 5B and E). In other features it is either

intermediate (indumentum on stem below capitulum, length of spines on lateral lobes of upper leaves, colour and length of phyllaries – Fig. 5A–F) or a mosaic patchwork related to the features of the parental taxa (indumentum on adaxial or abaxial surface of upper leaves, indumentum and spinosity of phyllaries – Fig. 6A–F, 4A–F).

The plants of *C. ×sudae* from the locality near Kölblwirt have consistently pinkish white flowers (Fig. 5B and E), congruent with Meltzer (1973), who reports reddish flowers. All hybrid plants found near Kölblwirt had aborted synantheria (anther tubes), which could indicate this hybrid is sterile. However, in *Cirsium* where gynodioecious sexual dimorphism is often present (Bureš et al. 2010, 2018), these individuals are actually standard females that co-occur with hermaphrodites in natural populations of hybrids or pure species (Delannay 1979, Bureš et al. 2010). Moreover, “ovular” fertility of the female hybrid plants is supported by the presence of fully developed achenes detected in the individuals Hyb-A52b and Hyb-A52d. Gynodioecy is usually associated with differences in the size of generative features of females and hermaphrodites (Delannay 1979), which is also the case in *Cirsium*, where females usually have smaller corollas and pappi (Fig. 4B and C; Bureš et al. 2018). The floral characters of the hybrid females of *C. ×sudae* collected near Kölblwirt were longer than those of the females of both parental species (Fig. 4B and C; Electronic Appendices 6 and 7), which could be a consequence of the vigorous effect of heterozygosity acting in hybrid plants.

Distribution and ecology

Although most species of *Cirsium* are heliophilous, there are some species that occupy shady habitats. This is the case of both of the parental species, *C. carniolicum* and *C. greimleri*. It is therefore not surprising that *C. ×sudae* was found in an open, park-like forest.

The presence of the four individuals of *C. ×sudae* near Kölblwirt could be considered rather peculiar because there was only one clone (three shoots) of *C. greimleri* at this locality. However, based on our field observations, it is not rare for only one of the parental species to prevail at localities where hybrids of *Cirsium* occur (unpubl. pers. observ.). Disequilibrium in pollen availability can facilitate hybrid events at such localities compared to those where both parents dominate (Arnold et al. 1993). Although, both of the parental species, *C. carniolicum* and *C. greimleri*, are promiscuous and regularly hybridize with other diploid congeners (Wagenitz 1987, Bureš et al. 2018), *C. ×sudae* is one of the rarest interspecific hybrids in the genus *Cirsium*. This is due to the rarity of both parental species and small overlap in their distributions (Fig. 7): *C. carniolicum* is distributed particularly in the Southern and Northern Limestone Alps¹, while *C. greimleri* is distributed in the Central Eastern Alps (Stevanović et al. 1991, Meusel & Jäger 1992, Jogan et al. 2001, Bureš et al. 2018)². In Austria, therefore, both species co-occur in the Southern

¹ *Cirsium carniolicum* occurs also in the Southern Alps near Lake Garda in Italy (Alpi Giudicarie, Monti Lessini) where *C. greimleri* is absent (Meusel & Jäger 1992), similarly as in the Pyrenees in France and Spain, where populations similar to *C. carniolicum* are classified as a separate species *C. rufescens* Ramond ex DC. (or subspecies; Meusel & Jäger 1992).

² *Cirsium greimleri* occurs also in the Dinaric Mountains in Slovenia, Croatia, Bosna and Herzegovina, Montenegro and Serbia (Stevanović et al. 1991, Meusel & Jäger 1992, Bureš et al. 2018), where *C. carniolicum* is absent (Meusel & Jäger 1992).

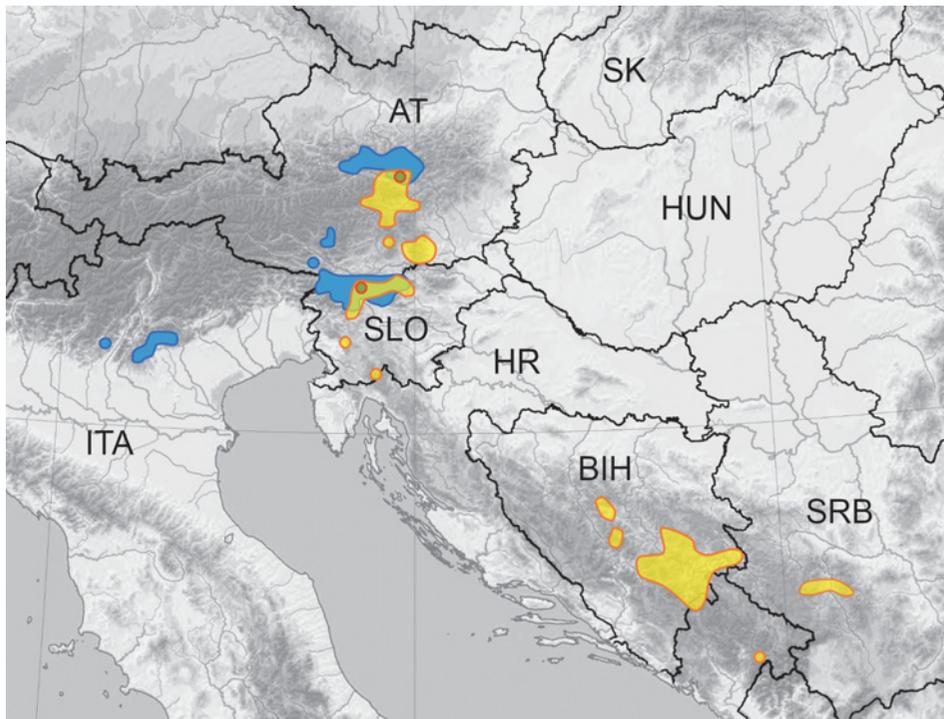


Fig. 7. – Distribution of the parental species *C. carniolicum* (blue) and *C. greimleri* (yellow) and position of the localities where their hybrid *C. x sudae* was recorded by Meltzer (1973) and in this study (green circles).

Limestone Alps, in the Karawanks, where the hybrid has already been reported by Meltzer (1973). Our locality is the first finding of this hybrid in the Northern Limestone Alps, where both species co-occur probably only in the Ennstal Alps.

Parental species are also considered to be ecological vicariants: *C. carniolicum* is calciphilous, whereas *C. greimleri* is calcifuge (Janchen 1958, Meltzer 1973, Wagenitz 1987, Meusel & Jäger 1992). Although we have never found *C. carniolicum* outside limestone areas and have recorded the largest populations of *C. greimleri* on acidic substrates in the Lavanttal Alps (in the subgroups Seetal Alps and Koralpe), we have also recorded *C. greimleri* at limestone areas, particularly in the Karawanks, the Stein Alps and the Julian Alps in Austria and Slovenia. Therefore, we do not consider these species to be substrate/ecological vicariants.

See www.preslia.cz for Electronic Appendices 1–8.

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Souhrn

Na základě morfologické, cytometrické a genetické (AFLP = polymorfismus délky amplifikovaných fragmentů) analýzy jsme identifikovali a popsali hybrida *Cirsium* × *sudae* (*C. carniolicum* × *C. greimleri*). Tento hybrid, který dosud neměl dvouslovné jméno, byl předtím nalezen jen jednou v pásmu jižních vápencových Alp při rakousko-slovenské hranici v Karavankách, kde se oba velmi vzácné rodičovské druhy dostávají ojedinelé do kontaktu. Jedinou další takovou oblastí jsou patrně Ennstalské Alpy, ležící v pásnu severních vápencových Alp. Právě zde poblíž osady Kölblwirt jsme našli čtyři jedince vykazující intermediární habitus. Podrobnější rozbor morfologických znaků ani analýza velikosti genomu pomocí průtokové cytometrie hybridní status těchto jedinců nevyvrátily. U často hybridizujících pcháčů je nicméně vždy namístě určitá opatrnost, protože „hybridní“ nážky mohou být, např. díky chmýru, transportovány i poměrně daleko od místa svého vzniku. Abychom bezpečně identifikovali původ hybridních rostlin, rozhodli jsme se pomocí AFLP analyzovat nejen oba potenciální rodičovské druhy a s nimi spolu na lokalitě rostoucí *C. erisithales*, ale i všechny další druhy pcháčů, rostoucí v rakouské části východních Alp: *C. acaulon*, *C. arvense*, *C. eriophorum*, *C. heterophyllum*, *C. oleraceum*, *C. palustre*, *C. pannonicum*, *C. rivulare*, *C. spinosissimum* a *C. vulgare*. Analýza STRUCTURE předpokládá původ hybridů potvrдила a vizualizace pomocí sítě NeighborNet navíc ukázala, že jsou nejpřibuznější právě s jedinci *C. carniolicum* a *C. greimleri* pocházejícími z lokality u Kölblwirt, takže vznikly zde a ne transportem „hybridních“ nážek odjinud. V jediné rostlině (klonu) *C. greimleri* na této lokalitě byla pomocí STRUCTURE odhalena mírná introgrese *C. erisithales*. U hybridů byla však příslušnost k *C. erisithales* prakticky zanedbatelná. Tato skutečnost má dvě alternativní vysvětlení: buď (i) byly gamety rodičovského mírně geneticky erodovaného *C. greimleri* náhodnou meiotickou segregací homeologních chromozomů „vyčištěny“ od alel *C. erisithales*, nebo (ii) jsou jak hybrid, tak i v rámci lokality jediné, mírně geneticky erodované, *C. greimleri* potomky téhož jedince – čistého *C. greimleri* – který na lokalitě vyhybnul. Geneticky jsou tedy jedinci *C. x sudae* kříženci mezi *C. carniolicum* a *C. greimleri* a ne trojnásobní hybridy. Nejnápadnějšími znaky tohoto nového hybrida jsou velké, mělce laločnaté listy s měkkými ostny a široce vejčitou čepelí u nejspodnějších z nich (jako u obou rodičů) a také jeho růžově bílé květy (intermediární mezi oběma rodiči). Přechodnými jsou ve srovnání s rodiči také řídce pavučinaté až vlnité odění stonku pod terminálním úbořem hybrida a délka ostnů laterálních laloků jeho horních listů (1.2–10.0 mm). Řídce štetinatým až chlupatým oděním svrchní strany listů a terminálními ostny zákrovních listenů je však hybrid bližší *C. carniolicum*, řídce pavučinatou spodní stranou listů a lysými zákrovními listeny naopak *C. greimleri*. Délkou koruny nebo chmýru je hybrid robustnější než oba rodiče, což může svědčit o heterózním efektu.

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