

## MORPHOLOGICAL AND PLOIDY LEVEL VARIATION OF *CENTAUREA PHRYGIA* AGG. (ASTERACEAE) IN THE CZECH REPUBLIC, SLOVAKIA AND UKRAINE

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**Abstract:** *Centaurea phrygia* agg. was studied in part of Central Europe (Czech Republic, Slovakia and Ukraine) to evaluate its karyological and morphological variation. Three ploidy levels were found: diploid (*C. pseudophrygia*, *C. stenolepis* and several populations of *C. phrygia* s.str.), tetraploid (*C. oxylepis* and several populations of *C. phrygia* s.str.), and triploid (very rare, only single individuals). Only one taxon, *C. phrygia* s.str., comprises populations of two ploidy levels, but they seem to be geographically separated. Multivariate morphometric analysis confirmed the separation of the four taxa; the length and the width of appendages of middle involucre bracts, visibility of appendages of inner involucre bracts, the length and the width of an involucre, and the length/width ratio of middle cauline leaves are the most important discriminant characters. Several populations intermediate between two species (either *C. pseudophrygia* and *C. stenolepis* or *C. oxylepis* and *C. phrygia* s.str.) were found. Their possible origin from introgressive hybridization is discussed considering their morphology (intermediate between putative parental species), chromosome numbers (always the same as in putative parents), and distribution (both spatially and ecologically more or less intermediate between distribution ranges of putative parents). A determination key for the four studied species, including intermediates, is also presented.

**Keywords:** Chromosome number, Hybridization, Introgression, Multivariate morphometric analysis, Polyploidy

### INTRODUCTION

The genus *Centaurea* L. comprises between 400 and 700 species, depending on taxonomic concepts (GREUTER et al. 2001). They are distributed mainly in Eurasia (HELLWIG 2004), with 211 species and many infraspecific taxa reported from Europe in Flora Europaea (DOSTÁL 1976). Because of extreme morphological variation, both the delimitation of the genus and its infrageneric classification have remained largely unresolved. Recent molecular studies have provided first insights into phylogenetic relationships, revealing some major patterns and proposing informal monophyletic groups in the genus (SUSANNA et al. 1995, WAGENITZ & HELLWIG 1996, GARCIA-JACAS et al. 2000, 2001).

Complicated and unsettled taxonomy led to the inconsistent and confusing use of some names that differ among authors. In this paper the name *Centaurea* L. is henceforth used as proposed by GREUTER et al. (2001). The name *Centaurea* sect. *Jacea* (MILL.) DC. is applied to the group of closely related species corresponding to the *Centaurea* subgen. *Jacea* (MILL.) HAYEK as delimited in Flora Europaea (DOSTÁL 1976), to the genus *Jacea* MILL. in the sense of Dostál's other works (e.g. DOSTÁL 1973, 1989), or to the "*Jacea-Lepteranthus* subgenus"

as defined by WAGENITZ & HELLWIG (1996) and HELLWIG (2004). However, this delimitation of the section *Jacea* should not be confused with the “*Centaurea jacea* group” by GARCIA-JACAS et al. (2000), in fact corresponding to the whole genus *Centaurea* in the sense of Greuter’s concept (GREUTER 2001).

Based on the recent phylogenetic studies, the section *Jacea* is recognized as a phylogenetically primitive group within the genus *Centaurea* (WAGENITZ & HELLWIG 1996, GARCIA-JACAS et al. 2000). All species of the section are perennial herbs of a mesophilous character. Typical morphological features of the section are undivided leaves (sometimes lower leaves lobate to pinnatifid, but with fewer and relatively wider lobes in comparison to other groups of *Centaurea*), middle-sized capitula (involucre ca. 1–2 cm in diameter), involucre bracts bearing a scarious appendage usually not decurrent on a bract and never spiny, purple florets, outer florets usually strongly radiating, and pappus of achenes short or absent (DOSTÁL 1976). The section *Jacea* is characterized by the base chromosome number  $x=11$  (HELLWIG 2004), and includes diploid ( $2n=22$ ) and tetraploid ( $2n=44$ ) taxa (DOSTÁL 1976). The majority of species of the section inhabit mountains of the Mediterranean and SW Asia, some species reaching Central Europe and rarely as far as Scandinavia and northwestern Russia (HELLWIG 2004).

*Centaurea phrygia* agg., classified within the section *Jacea*, is morphologically characterized by the shape of appendages of involucre bracts, which are ovate to linear with a pectinate-fimbriate margin attenuated into a filiform, laterally fimbriate acumen; terminal fimbriae are longer than lateral and the whole appendage is recurved in the upper part. The pappus is always present and is usually 1 mm long, whereas it is absent or is much shorter in other species of the section. The stem is usually branched and thus bears several to many capitula, which is the most obvious difference from the *C. uniflora* group, which has similar appendages but it regularly has a single capitulum on the top of an unbranched stem (DOSTÁL 1976, 1989).

From Central Europe (including Germany, Czech Republic, Slovakia, Poland, Austria, northern Hungary, and western Ukraine) up to seven taxa have been reported within *Centaurea phrygia* agg. at a specific or subspecific rank. DOSTÁL (1976) in *Flora Europaea* treated the group as consisting of three species, *Centaurea phrygia* L., *C. stenolepis* A. KERN., and *C. indurata* JANKA. Within *C. phrygia* he recognized nine subspecies, four of them occurring in Central Europe: subsp. *phrygia*, subsp. *melanocalathia* (BORBÁS) DOSTÁL, subsp. *nigriceps* (DOBROČZ.) DOSTÁL, and subsp. *pseudophrygia* (C.A. MEY.) GUGLER. Within *C. stenolepis* he reported three subspecies, only one of them reaching the Central European region (subsp. *stenolepis*). Other authors preferred a narrower species concept and raised all these taxa to the specific level (WAGENITZ 1987, ADLER et al. 1994, ROTHMALER 1994, OCHSMANN 1998, and also older detailed monographs by HAYEK 1901 and WAGNER 1910; none of these works, however, dealt with all the taxa mentioned).

*Centaurea pseudophrygia* C.A. MEY. and *C. stenolepis* are diploid taxa (DOSTÁL 1976, WAGENITZ 1987). Only the diploid chromosome number was published for *C. phrygia* by DOSTÁL (1976) and WAGENITZ (1987); however, both ploidy levels (diploid and tetraploid) were later reported for this taxon by DOSTÁL (1989). *Centaurea melanocalathia* BORBÁS is

tetraploid (DOSTÁL 1989). There are no chromosome counts available for *C. nigriceps* DOBROČZ.

The distribution ranges of particular taxa seem to be distinct from each other, overlapping only in relatively small areas (MEUSEL & JÄGER 1992), but there are some contradictory records based probably on wrong determinations, e.g. occurrence of *C. phrygia* s.str. in the southern part of the Czech Republic reported by DOSTÁL (1989), or several localities of *C. stenolepis* recorded for Poland (MEUSEL & JÄGER 1992). The distribution of some taxa of *C. phrygia* agg. is insufficiently known, as e.g. *C. nigriceps* was reported only from the Ukrainian Carpathians (DOSTÁL 1976), *C. melanocalathia* from the Carpathians (Slovakia and Romania, DOSTÁL 1976, 1989, MEUSEL & JÄGER 1992), and *C. indurata* from Slovakia, Hungary, Romania, and Bulgaria (DOSTÁL 1976).

Extensive hybridization occurs in the section *Jacea*. Numerous hybrids were described, and in addition, for many taxa originally described as non-hybrid ones, hybrid origin has been shown. In the majority of the known hybrids, *C. jacea* L. was involved as one of the parents. Its hybrids can be easily distinguished, because this species clearly differs from the other taxa in the shape of appendages of involucre bracts. Hybrids between *C. jacea* and all the taxa of *C. phrygia* agg. included in the present study are known. The extent of hybridization between particular taxa depends on the ploidy level; taxa of the same ploidy level can cross easily and their hybrids are fertile and capable of backcrossing, whereas taxa differing in ploidy levels hybridize only rarely and their hybrids are almost sterile (GARDOU 1972, HARDY et al. 2001, ŠTĚPÁNEK & KOUTECKÝ 2005).

*Centaurea oxylepis* (WIMM. et GRAB.) HAYEK and *C. macroptilon* BORBÁS are two other taxa occurring in Central Europe, which are apparently close to *C. phrygia* agg. They are usually included in *C. jacea* agg. (WAGENITZ 1987, ADLER et al. 1994, ROTHMALER 1994) or treated as subspecies of *C. macroptilon* (DOSTÁL 1976, 1989). Morphologically they differ from taxa of *C. phrygia* agg. by having narrower leaves and in the shape of appendages of involucre bracts, which are triangular and not attenuated into a filiform acumen. However, both taxa also share some morphological features with *C. phrygia* agg., particularly the regular fimbriation of appendages of involucre bracts and the overall shape of the appendages, which are recurved (DOSTÁL 1976).

Major taxonomic problems in *C. phrygia* agg. originate from (i) unclear morphological differentiation of individual taxa; (ii) unclear relationships between morphological variation and chromosome numbers; and (iii) contradictory distribution data for some taxa.

The main diagnostic features reported for the individual taxa in *C. phrygia* agg. include size and shape of the involucre and details of the shape of appendages of middle and inner involucre bracts. Due to the lack of clear differences in the qualitative characters, few quantitative ones (length of the appendage, number of lateral fimbriae, etc.) are used (DOSTÁL 1976, 1989). Plant identification is often obscured by high intra-population variation, occurrence of intermediate morphotypes, and hybridization. Usually none of these problems are considered in determination keys, which report only small variation of the main diagnostic characters, resulting in a rather high number of misidentifications. Re-evaluation of the morphological variation within taxa and populations is therefore necessary, and it is one of the main aims of the present study.

The present study aims to evaluate variation of *C. phrygia* agg. in Central Europe from morphological and karyological points of view. Two main questions were addressed: (1) What is the karyological variation both within the taxa and populations? (2) What are the morphological differences between the Central European taxa and what is the variation within the taxa?

## MATERIAL AND METHODS

### Field sampling

Sixty-five population samples were collected in the Czech Republic (42), Slovakia (17), and Ukraine (6) during 2001–2003 (see Appendix). Populations tentatively determined as *C. phrygia*, *C. stenolepis*, *C. pseudophrygia*, and *C. oxylepis* were included. Only populations not affected by hybridization with *C. jacea* were included, because including such hybrids into morphometric analyses would greatly distort the results. Hybrids with *C. jacea* can be easily identified, because they have wider and shorter appendages of involucre bracts and the lateral fimbriae of the appendages irregularly fused into groups. At each locality, 25–30 mature plants were sampled randomly, except for two populations where 13 and 17 plants were collected, respectively (populations denoted as DOM and KRH, see Appendix). *Centaurea* plants sometimes grow in lax clusters, each cluster corresponding to one genet. Therefore, only one stem from a cluster was sampled. Morphological characters concerning branching of the stem, shape of leaves, and a few characters on the involucre were measured immediately on fresh plants. Plants were then dried and used to measure other morphological characters. At some localities living plants were collected for subsequent DNA ploidy level estimation using flow cytometry. Living plants were cultivated in the experimental garden of the Department of Botany, České Budějovice, Czech Republic (48°58' N, 14°27' E, 390 m a.s.l.). Voucher specimens are kept in the CBFS herbarium.

### Chromosome counting

Chromosome numbers were counted in root apices of seedlings that were germinated from the achenes collected *in situ*. A rapid squash method was used. The roots were pre-treated with a saturated water solution of p-dichlorobenzene for 3 hours at room temperature, fixed in 3:1 ethanol and acetic acid mixture for 24 hours at 4°C, and stored in 70% ethanol. Maceration lasted ca. 1–1.5 min in the ethanol and hydrochloric acid mixture (1:1), and finally, lacto-propionic orcein was used for staining. At least three samples (achenes originating from different individuals) were analyzed for the population, and at least two mitoses per plant were studied (see the Appendix for the number of analyzed plants).

### Flow cytometry

DNA ploidy levels were estimated using a Partec PA II flow cytometer following the procedure as described by OTTO (1990). A karyologically confirmed diploid plant (planted from a seedling that survived root tip removal) was used as an internal standard. Approximately 0.5 cm<sup>2</sup> of a fresh leaf of a studied plant and of the standard was chopped with a new razor blade in 1 ml ice-cold Otto I buffer (0.1M citric acid, 0.5% Tween 20). The

suspension was filtered through a 42 µm nylon mesh. After at least 5 min (at room temperature with occasional shaking) 1 ml of a staining solution containing Otto II buffer (0.4 M Na<sub>2</sub>HPO<sub>4</sub>), β-mercaptoethanol (2 µg/ml), and fluorochrome DAPI (4 µg/ml) was added. The samples were measured after 1–2 min of staining. The cytometer was adjusted so that the fluorescence of G<sub>0</sub>/G<sub>1</sub> nuclei of the diploid standard was localized on channel 200. Fluorescence of 5000 nuclei was recorded and the coefficient of variance (CV) of an analyzed plant varied from 1.6 to 3.0 (measurements with higher CV values were repeated).

### Morphometric analysis

Twenty-five characters were measured or scored on plants collected in the field (12 quantitative characters, 5 ratios, and 8 binary characters; see Table 1). The character set was chosen to include all characters used in determination keys and floras to distinguish the studied taxa. Several other characters not previously used in literature were added based on field observations. Several characters were recorded on achenes. Because the achenes were not available for all the studied individuals and populations, all the analyses were computed on two datasets, with the achene characters either included (i.e., all characters were analyzed on a reduced number of individuals) or not (i.e., all individuals/populations were analyzed).

The data were analyzed using the following software packages: Statistica 6 (STATSOFT 2001), CANOCO for Windows 4.5 (TER BRAAK & ŠMILAUER 2002), and R 1.8 (R DEVELOPMENT CORE TEAM 2003). Basic statistical measures (mean, median, maximum and minimum values, quartiles, 5 and 95 percentiles, and the standard deviation) were computed for each taxon and population. The normality of distribution was tested for each character using Shapiro-Wilk statistics. Values of characters SF, SFT, LLW, MW, and MF (see Table 1) were log-transformed to improve their distribution. Average values for each population were calculated because in some analyses the populations were used as operational taxonomic units (OTUs). For achene characters that were not available for all individuals, an average value for population was computed only if there were data for at least five individuals. Prior to cluster analysis, the data were standardized to zero mean and unit standard deviation.

Intra-population variation was studied because populations were used as OTUs in some analyses. Principal component analysis (PCA) for each population was run to detect outliers (atypical individuals that could distort mean values).

At first, Pearson and Spearman correlation coefficients were calculated for pairs of characters for each taxon and for the whole data set to study relationships between the characters. Cluster analysis (EVERITT 1986) was employed to provide an overall view of the structure present among the populations that were used as OTUs. Various clustering algorithms (average linkage method = unweighted pair-group average method, complete linkage method, Ward's method) using Euclidean distance as a dissimilarity measure were performed. PCA (PODANI 2000) based on a correlation matrix using both populations and individuals as OTUs was also run to obtain further complementary information on the structure of the studied group. Canonical discriminant analysis and classificatory discriminant analysis (KLECKA 1980) using both populations and individual as OTUs were then used to test the groups defined on the basis of the cluster analysis and the PCA. The discriminant power was determined using crossvalidation. As some characters deviated from

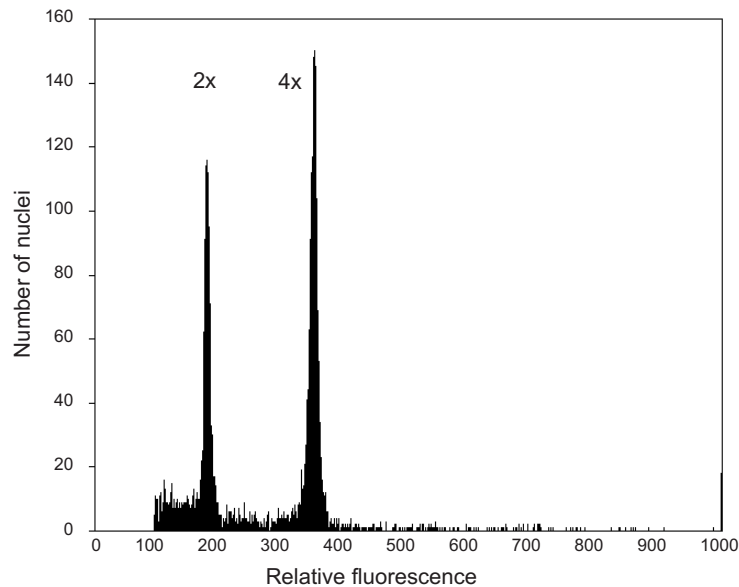


Fig. 1. Flow cytometric profile of diploid *C. stenolepis* (population denoted as PREL, used as internal standard) and a tetraploid intermediate between *C. oxylepis* and *C. phrygia* (population KOZL). Nuclei of both plants were isolated, stained with DAPI, and analyzed simultaneously.

normal distribution, Monte Carlo permutation tests were used in discriminant analysis instead of parametric significance tests.

### Distribution of taxa

The distribution of individual taxa was estimated for the Czech Republic based on the revision of ca. 2000 herbarium sheets from major Czech public herbaria (BRNM, BRNU, CB, CHOM, GM, HR, LIM, LIT, MJ, MP, OLM, OSM, PL, PR, PRC, ROZ, SOKO, ZMT). Data from literature could not be used because of a high number of probable misidentifications. Distribution maps were prepared using DMAP software (A. Morton, ined.).

## RESULTS

### Chromosome numbers and DNA ploidy levels

At least three individuals from each population were studied for chromosome numbers. At some localities (BABU, BOL, BURK, DEM, DUB, KASH, KOZL, KRH, KRIP, LAZ, MIL, MIO, NEJ, OLE1, RTE, PREL, PROS, RUS, STGH, SYB, VAL3, VER1, see Appendix) the DNA ploidy levels of more individuals were also estimated using flow cytometry (see Appendix, Fig. 1). One more population that was not included in the morphometric studies was analyzed to cover the presumed isolated occurrence of the tetraploid cytotype of *C. phrygia* s.str. Both diploid ( $2n=22$ ) and tetraploid ( $2n=44$ ) levels were identified among the studied populations, but no populations with the co-occurrence of these two ploidy levels were found (see Appendix for results).

Table 1. List of the studied morphological characters. Those with asterisks were measured or scored but not included in the morphometric analyses (for details see the Results section).

Character Description	
Quantitative characters:	
SF	height of the flowering part of the stem (i.e., height from the lowest flowering branch to the terminal capitulum) (cm)
*ST	total stem height (i.e., plant height) (cm)
*LL	lamina length of the middle stem leaf (cm)
*LW	lamina width of the middle stem leaf (cm)
CL	involucre height of the terminal capitulum (cm) (see Fig. 8)
CW	involucre width of the terminal capitulum (cm) (see Fig. 8)
ML	length of the longest appendages of middle involucre bracts of the terminal capitulum (mm)
MW	maximal width of the (central) undivided part of the longest appendages of the middle involucre bracts of the terminal capitulum (mm)
MF	number of lateral teeth ("fimbriae") on one side of the longest appendages of the middle involucre bracts of the terminal capitulum
AL	achene length (average value of 5 achenes) (mm)
AW	achene width (average value of 5 achenes) (mm)
AP	pappus length (average value of 5 achenes) (mm)
Ratios:	
*SFT	height of the branched part of the stem / total height of the stem (SF / ST)
LLW	length / width of the lamina of the middle stem leaf (LL / LW)
CLW	height / width of the involucre of the terminal capitulum (CL / CW)
*MLW	length / maximal width of the longest appendages of middle involucre bracts of the terminal capitulum (ML/MW)
*ALW	length / width of achene (AL / AW)
Binary characters:	
LM	leaf margin shape (0 – entire to dentate; 1 – distinctly lobed)
LBA	attenuated base of the upper stem leaves (0 – absent; 1 – present)
LBS	semi-amplexicaul base of the upper stem leaves (0 – absent; 1 – present)
LS	continual decrease of size of the stem leaves (from the lower part of the stem upwards) (0 – absent; 1 – present)
*CS	shape of the involucre of the terminal capitulum (0 – globose; 1 – cylindrical or ovoid, distinctly higher than wide)
CG	grouping of capitula at the end of branches (0 – absent, i.e., capitula single; 1 – present, i.e., capitula in groups of two or more)
IS	shape of the appendages of the innermost involucre bracts of the terminal capitulum (0 – orbicular or ovate; 1 – elongate to elliptical)
IV	visibility of appendages of the innermost involucre bracts of the terminal capitulum (0 – visible, exceeding the appendages of the middle involucre bracts from the side view; 1 – not visible, covered by the appendages of the middle involucre bracts from the side view)

The ploidy level generally agreed with the tentative determination of plants based on their morphology. Both ploidy levels were found among populations determined as *C. phrygia* s.str., which accords with published data. *Centaurea stenolepis* was found to be exclusively diploid, *C. oxylepis* tetraploid. A huge majority of plants assigned to *C. pseudophrygia* were diploid, but one tetraploid population was also found (population KRH from the West Carpathians, see Appendix).

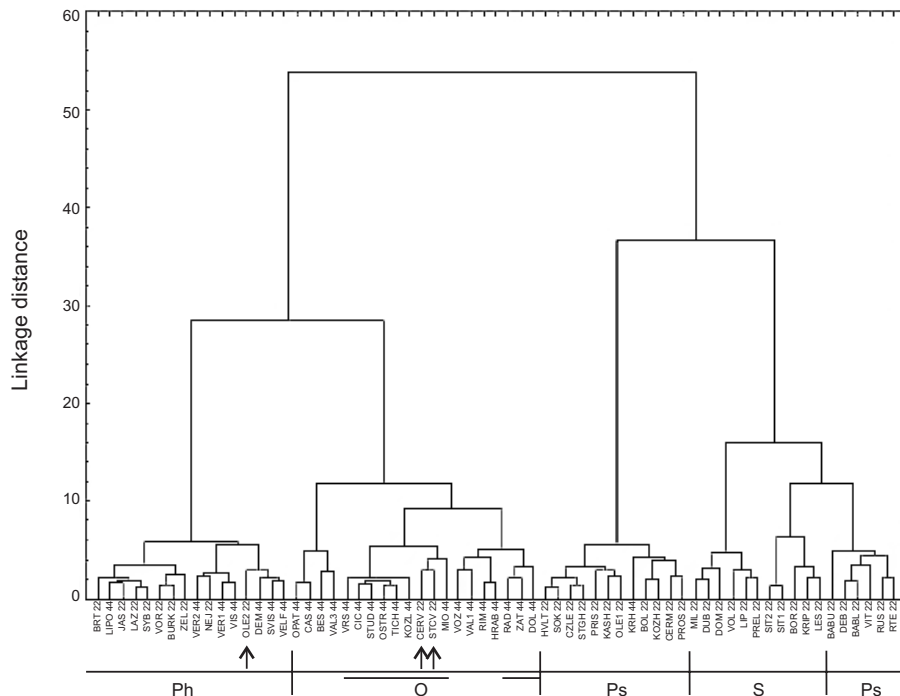


Fig. 2. Cluster analysis of 65 populations of *Centaurea phrygia* agg. (Ward's method) based on 15 characters. Characters on achenes were omitted because they were not available for all populations. Population abbreviations are according to Appendix and include indications of chromosome numbers. The lines and letters indicate the tentative determination of plants (Ph – *Centaurea phrygia* s.str.; Ps – *C. pseudophrygia*; O – *C. oxylepis*, the double line indicates populations intermediate between *C. oxylepis* and *C. phrygia*; S – *C. stenolepis*). “Misplaced” populations are highlighted by arrows.

In three populations (BRT, CZLE, KASH) single individuals with triploid chromosome numbers were found, but otherwise these populations were diploid. The occurrence of the triploid plants is of particular interest, as they were not found in the detailed study of ploidy levels of populations of other species of *Centaurea* sect. *Jacea*: *C. nigra* and the *C. jacea* complex (HARDY et al. 2000).

### Morphometric analysis

Descriptive statistics of all the studied characters were computed. The results were used to evaluate the variation of individual populations and characters (see below). Most characters more or less deviated from the normal distribution, which is required by discriminant analyses. However, these methods are to a large extent robust to the violation of this assumption except for significance tests (LEPŠ & ŠMILAUER 2003).

Intra-population variation detected using PCA was quite low. Nevertheless, some populations contained several outlying individuals. Most of these populations were morphologically intermediate between two of the studied taxa and the outliers usually were closer to the one of these taxa than to the rest of the population. It is hypothesized that these



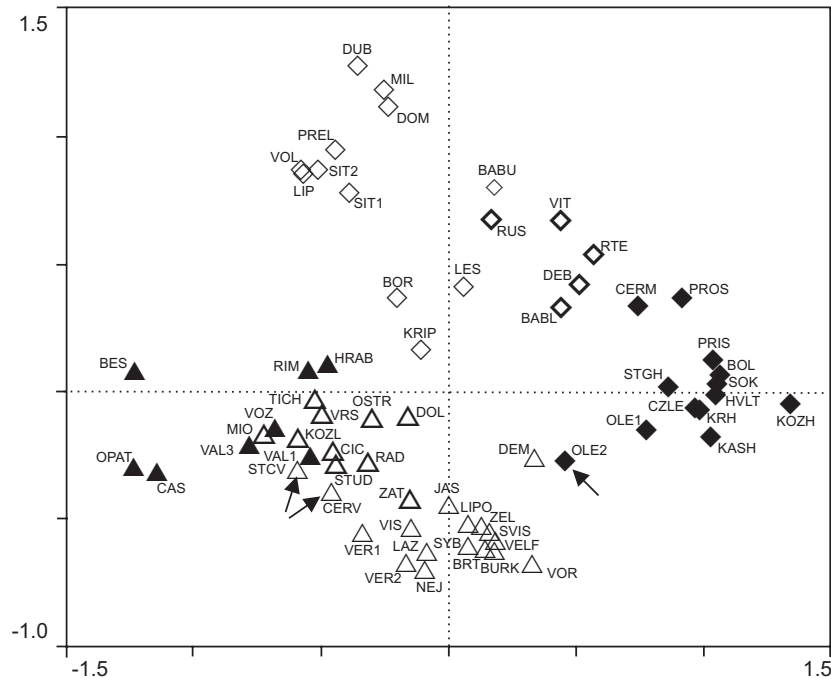


Fig. 3. Principal component analysis of 65 populations of *C. phrygia* agg. based on 15 characters. First (horizontal) and second (vertical) ordination axes explain 37.2% and 26.4% of total variation, respectively. Characters on achenes were omitted because they were not available for all populations. Determination of plants based on cluster analysis (Fig. 2) is indicated by different symbols: *C. pseudophrygia* (full diamonds), *C. stenolepis* (empty diamonds), populations intermediate between them (empty thick-lined diamonds), *C. phrygia* (empty triangles), *C. oxylepis* (full triangles) and populations intermediate between them (empty thick-lined triangles). Populations “misplaced” in cluster analysis (Fig. 2) are indicated by arrows.

populations could be of hybrid origin and the occurrence of individuals “atypical” in this way may be their characteristic feature. Thus, no outliers were omitted from the statistical analyses, except for the canonical discriminant analyses, which were run without intermediate populations (see below).

Only slight differences were observed between Spearman and Pearson correlation coefficients. No highly correlated characters were found (value exceeding 0.9). Nevertheless, some selection of correlated characters expressing similar morphological features was done. Character SF and the ratio SFT both describe branching of the stem. Character SF shows lower variation within populations. The value of character SFT, which is a ratio SF / ST, is highly influenced by the total stem length (ST), which is of little taxonomic value because it depends on many environmental factors (shading, mowing of the meadow, etc.). Therefore, only character SF was taken into analysis and characters ST and SFT were omitted. Similarly, from characters describing the shape of middle stem leaves (the leaf length LL, the leaf width LW, and their ratio LLW) only the last one was used. Two characters described the shape of the involucre, the ratio CLW and the binary character (CS). Because they refer to the same feature, only the former was used. The value of ratio MLW depends strongly on MW and to

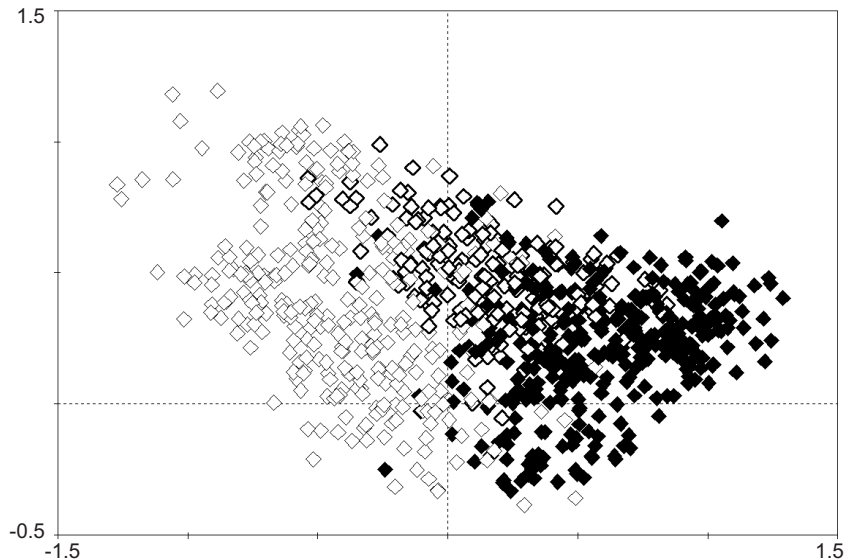


Fig. 4. Principal component analysis of individuals of *C. pseudophrygia* (typical populations – full diamonds; intermediate to *C. stenolepis* – empty thick-lined diamonds) and *C. stenolepis* (empty diamonds). Characters on achenes were omitted because they were not available for all populations. First (horizontal) and second (vertical) ordination axes explain 30.6% and 19.9% of total variation, respectively.

a smaller extent on ML, whereas correlation of the original characters is relatively low. Thus, the ratio MLW was omitted. A similar decision was made with characters AL, AW, and their ratio ALW (see Table 1).

Results of the cluster analysis of 65 populations are presented in Fig. 2. The main clusters agree with ploidy levels and tentative determination of plants with a few exceptions. In a cluster that is assigned to *C. phrygia*, both ploidy levels are found intermingled in congruence with literature. One population determined as *C. pseudophrygia* was also placed into this cluster (population OLE2). Other populations determined as *C. pseudophrygia* formed a distinct cluster composed of two subclusters. The larger subcluster represents morphologically typical populations, while the smaller one is composed of populations that possess some features of *C. stenolepis*. A cluster of *C. oxylepis* comprises tetraploid populations only, except for two “misplaced” diploid populations of *C. phrygia* s.str. (populations CERV and STCV). Apart from typical *C. oxylepis*, the cluster also includes populations that are clearly close to *C. oxylepis* but resemble *C. phrygia* s.str. in some features. These intermediate populations cluster within two separate subclusters, whereas typical populations form another two subclusters. Populations of *C. stenolepis* were placed in two clusters, reflecting their ecological differentiation: the right one represents populations from typical localities (thermophilous oak forests and semixerophilous meadows in the warmest parts of the study area), the left one contains populations from mesophilous meadows of relatively higher altitudes of the Carpathians. Similar results were obtained using other clustering algorithms and/or when characters of achenes were considered, which provide further support for the pattern presented.

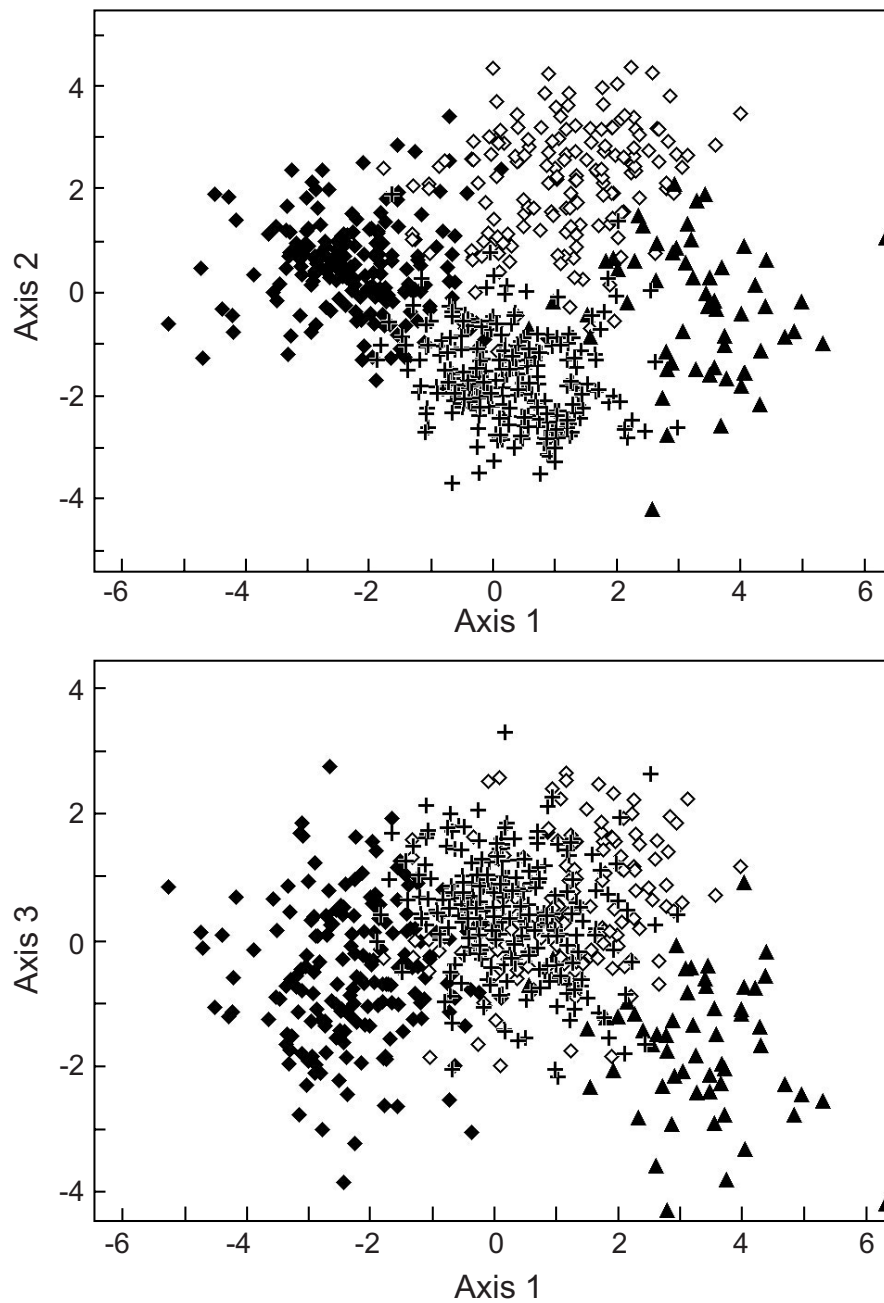


Fig. 5. Canonical discriminant analysis of 634 individuals of *C. phrygia* agg. based on 18 characters. Individuals from intermediate populations were excluded, from other populations only individuals for which all characters (including achenes) were available were included. Individual taxa are marked with different symbols: *Centaurea phrygia* s.str. (empty diamonds), *C. pseudophrygia* (full diamonds), *C. stenolepis* (crosses), and *C. oxylepis* (triangles). First and second, and first and third canonical axes, respectively, are displayed.

Table 2. Contribution of individual characters to the first three canonical axes (Axis 1, Axis 2, and Axis 3). 634 individuals divided into four groups (*C. phrygia*, *C. pseudophrygia*, *C. stenolepis*, and *C. oxylepis*) on the basis of cluster analysis and PCA were analyzed and all the studied characters were included. Absolute canonical correlations exceeding 0.3 are printed in bold.

Character	Axis 1	Axis 2	Axis 3
SF	-0.063	-0.101	-0.218
LLW	0.243	0.033	<b>-0.417</b>
LM	0.140	-0.258	-0.047
LBA	0.247	0.166	-0.033
LBS	-0.295	0.038	<b>-0.337</b>
LS	0.222	<b>0.313</b>	0.283
CL	<b>-0.395</b>	-0.118	0.093
CW	<b>-0.585</b>	<b>-0.569</b>	0.009
CLW	<b>0.393</b>	<b>0.593</b>	0.092
CG	0.002	0.211	-0.134
ML	<b>-0.464</b>	0.273	-0.192
MW	0.072	<b>-0.723</b>	-0.228
MF	<b>-0.348</b>	-0.107	<b>-0.451</b>
IS	-0.069	0.226	0.061
IV	<b>-0.344</b>	0.271	<b>-0.341</b>
AL	<b>-0.353</b>	-0.133	0.145
AW	-0.060	-0.103	-0.101
AP	<b>-0.498</b>	0.016	<b>0.341</b>

Results of PCA of populations are displayed in Fig. 3. They are highly congruent with cluster analysis and tentative determinations of the studied plants. Particular species form more-less distinct groups. Populations that were “misplaced” in cluster analysis occur in intermediate positions between the groups, and that explains their positions in cluster analysis (their “misclassification” reflects rather the clustering algorithm than differentiation of these populations from other populations of the species). In the case of two “misplaced” populations of *C. phrygia* s.str., chromosome numbers should also be considered; both populations are diploid, whereas there are only tetraploid populations within the rest of the *C. oxylepis* cluster.

The intermediate position of “atypical” populations is also clearly visible. In the *C. pseudophrygia*-*C. stenolepis* pair, the intermediate populations do not seem to be closer to the latter species, as indicated by cluster analysis. However, this analysis might not portray the internal structure of the primary groups (here *C. phrygia* + *C. oxylepis*, and *C. pseudophrygia* + *C. stenolepis*) well because the ordination vectors could only be a compromise between somewhat different variation within primary clusters. Therefore both the groups were analyzed separately in the next step. Analyses using both populations and individuals as OTUs were carried out. Results of the analysis of individuals of *C. pseudophrygia* and *C. stenolepis* are shown in Fig. 4. The atypical populations occupy an intermediate position between *C. pseudophrygia* and *C. stenolepis*. In contrary to results at the population level, their variation overlaps with both species (which alone overlap to a small extent) and there are virtually no gaps. Analysis of *C. phrygia* and *C. oxylepis* yielded very similar results (figure not shown), except for a much stronger overlap between the intermediate populations and

Table 3. Results of classificatory discriminant analyses of individuals of *C. phrygia* (Ph), *C. pseudophrygia* (Ps), *C. stenolepis* (S), and *C. oxylepis* (O). Intermediate populations were excluded from the analysis. Two analyses were performed, one including all individuals with achene characters omitted (n=1433, 15 characters), the other only including individuals with all characters available (i.e., including achenes, n=634, 18 characters). Their results are separated by a slash; number of individuals classified into the respective class and percentage of correct classifications are presented.

actual	Group membership predicted				Percent correct
	Ph	Ps	S	O	
Ph	455 / 228	22 / 9	8 / 6	23 / 5	89.57 / 91.94
Ps	29 / 13	335 / 156	12 / 4	0 / 0	89.10 / 90.17
S	15 / 8	18 / 9	276 / 138	2 / 1	88.75 / 88.46
O	17 / 3	1 / 0	15 / 1	205 / 53	86.13 / 92.08
Total					88.70 / 90.69

*C. oxylepis* than between the intermediate populations and *C. phrygia*, in accord with the cluster analysis and the PCA.

In general, there is good coincidence between tentative determination of the studied populations and the results of cluster analyses and PCA at population level. Four taxa can be distinguished, but in contrast to tentative determination of plants several populations were marked as intermediate, combining characters of two different taxa. Resulting classification was studied at the level of individuals using canonical discriminant analysis to reveal which characters differentiate the taxa and using classificatory discriminant analysis to test the discriminant power of the characters. Intermediate populations, which cannot be assigned to any taxon with certainty, were excluded from discriminant analyses.

Figure 5 presents a diagram of canonical discriminant analysis, which enables the best separation of a priori defined groups. Individuals were used as OTUs and all the characters (including achenes) were used. The species form distinct clusters but there is some overlap. Canonical correlation coefficients are 0.85 for the first axis, 0.83 for the second, and 0.56 for the third. Squares of these coefficients, which can be interpreted as the portion of variation of canonical discriminant function explained by the division of objects into groups, are 72.8%, 68.6%, and 30.9%, respectively. The contributions of individual characters to canonical axes are presented in Table 2. Characters most correlated with the first axis, which separates *C. pseudophrygia*, the pair of *C. stenolepis* + *C. phrygia*, and *C. oxylepis*, are (in decreasing order) CW, AP, ML, CL, CLW. The second axis separates *C. phrygia* from *C. stenolepis*, with characters MW, CLW, CW, and LS being the most important. The third axis contributes to separation of *C. oxylepis* (and partly *C. pseudophrygia*) from *C. phrygia* + *C. stenolepis* and characters most correlated with it are MF, LLW, IV, AP, and LBS. If characters on achenes are not considered, the directions of the canonical axes are similar and the most important characters are ML, IV, MW, CL, LLW, CW for the first axis, CW, MW, CLW, and LS for the second axis, and LLW, MF, LBA, and IV for the third axis. Stepwise analysis (forward selection) was performed, too. It has shown that most characters contribute significantly to classification function. In the analysis not considering achenes, all characters were revealed as significant. If achenes are considered, characters SF, AW, and CL do not significantly

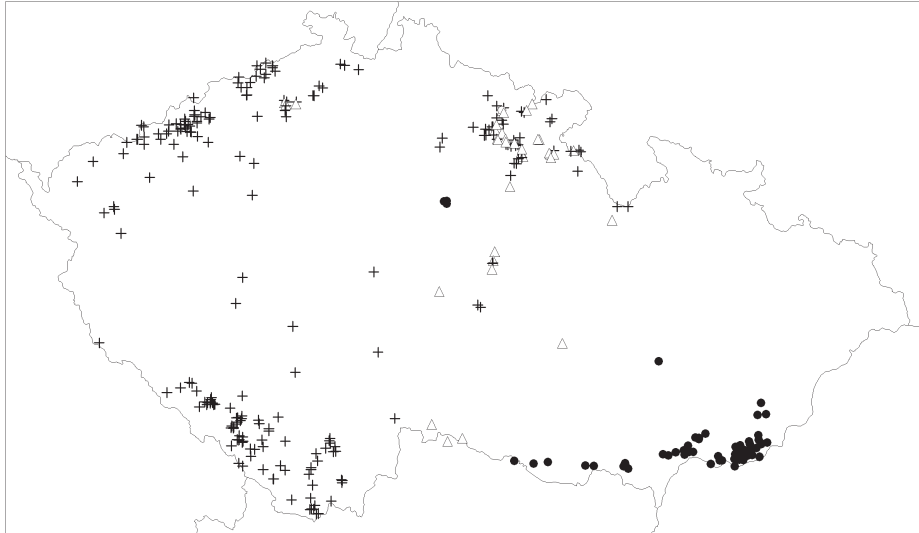


Fig. 6. Distribution of *Centaurea pseudophrygia* (crosses), *C. stenolepis* (circles), and intermediate plants (triangles) in the Czech Republic, based on the revision of herbarium specimens.

contribute to discriminant functions. If Bonferroni correction is applied, the effect of three other variables is not significant at the 5% level (LBA, CLW, IS). The non-significance of these characters in stepwise analysis is partly due to correlation with other characters; however, these variables are not completely useless. For practical determination, several correlated characters can be useful, because it is not always possible to measure all characters on each studied individual.

In total, 88.7% of individuals are correctly classified in classificatory discriminant analysis when achene characters are not considered. If achenes are included, 90.7% of individuals are correctly classified. This improvement is particularly due to the better resolution of *C. oxylepis* and *C. phrygia* on the basis of achene pappus length (Table 3).

The intermediate character of the intermediate populations can be clearly demonstrated when these populations are classified using a classification function computed from the data set that does not include them (i.e., using classification rules for the “pure” species). Table 4 presents the results showing that more than 60% of individuals in both types of intermediate populations are classified as one of the species, whereas more than 30% are classified as the other species.

## DISCUSSION

### Intermediate populations

Some studied populations are morphologically intermediate, either between *C. phrygia* s.str. and *C. oxylepis* or between *C. pseudophrygia* and *C. stenolepis*. Variation within each particular intermediate population is similar to the variation within typical populations of either species but there is considerable variation among them. Intermediate populations differ

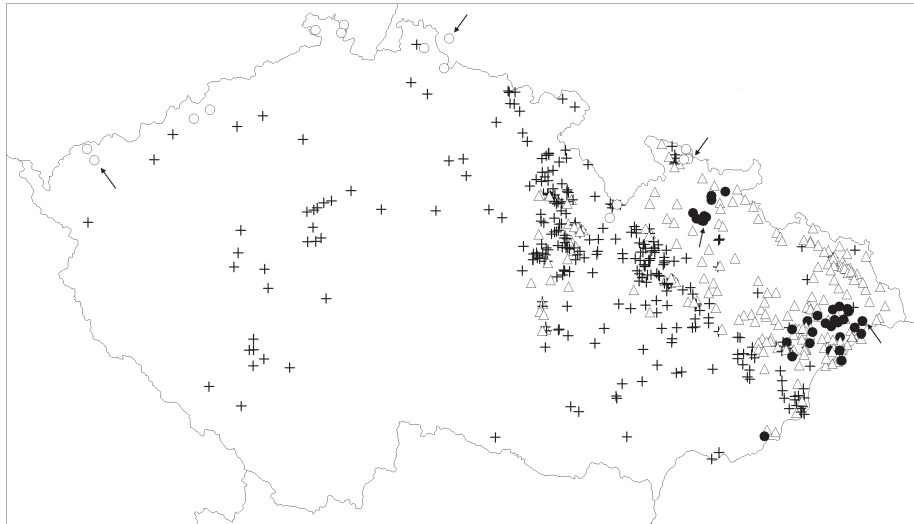


Fig. 7. Distribution of *Centaurea oxylepis* (crosses), *C. phrygia* s.str. (circles), and intermediate plants (triangles) in the Czech Republic, based on the revision of herbarium specimens. For *C. phrygia* probable distribution of two cytotypes is also indicated: white circles – probably diploids, black circles – probably tetraploids. Localities with confirmed chromosome counts are indicated by arrows.

slightly from one another, forming a more or less continuous transition from one species to the other, each of the intermediate populations being one “step”. It is probable that this pattern originated from introgressive hybridization.

Apart from morphological variation, there are other facts that seem to accord with this hypothesis. One is the pattern of ploidy levels. Hybridization experiments have shown that those species within *Centaurea* sect. *Jacea* with the same number of chromosomes cross easily and their offspring is usually fertile. On the contrary, hybridization of species differing in ploidy level is rare and the hybrids are almost sterile (GARDOU 1972, HARDY et al. 2001). Introgressive hybridization is therefore only possible if two species share the same ploidy level and is impossible if they do not. In agreement with the results cited, all the intermediate populations within *C. phrygia* agg. of supposed hybrid origin have putative parents of the same ploidy level. No intermediate population between “parents” differing in the number of chromosomes was found.

Geographic distribution of intermediate populations is also “intermediate”. Detailed data based on the revision of ca. 2000 herbarium sheets are available for the Czech Republic. The distributions of *Centaurea pseudophrygia*, *C. stenolepis*, and plants intermediate between them are depicted in Fig. 6. *Centaurea pseudophrygia* occurs mainly in montane and submontane areas. The species is absent in the lowest (and warmest) parts of the country. *Centaurea stenolepis* is a thermophilous species occurring in warm lowlands (note that some presented localities are only historical, the species declined during the last 100 years). Intermediate plants occur at middle altitudes and also around the middle of the climatic gradient from colder mountains to warmer lowlands. Fig. 7 shows the distributions of *C. oxylepis*, *C. phrygia* s.str., and plants intermediate between these two species. The

Table 4. Classification of intermediate populations. Individuals were used as OTUs. The populations were classified using the classification function computed from data for “pure” species (i.e., intermediate populations excluded). Results of analysis considering / not considering achene characters are presented and number of observation and percent classified into particular species are given. “Ph” stands for *C. phrygia*, “O” for *C. oxylepis*, “Ps” for *C. pseudophrygia* s.str., and “S” for *C. stenolepis*.

Intermediate populations	Predicted group membership			
	Ph	O	Ps	S
Ph - O	36 / 123	72 / 148	2 / 10	2 / 15
Percent	32.1 / 41.6	64.3 / 50.0	1.8 / 3.4	1.8 / 5.1
Ps - S	1 / 0	0 / 0	64 / 113	35 / 67
Percent	1.0 / 0.0	0.0 / 0.0	64.0 / 62.8	35.0 / 37.2

tetraploid cytotype of *C. phrygia* occurs in mountains of northern Moravia. *Centaurea oxylepis* is common in relatively warmer areas (lowlands to submontane regions). Intermediate plants occupy relatively cold middle altitudes both ecologically and spatially “between” putative parents.

However, the problem deserves further investigation. Isozymes are being studied to confirm the working hypothesis of introgressive hybridization (KOUTECKÝ & PLAČKOVÁ, unpubl. data). The first results show that there are almost no qualitative differences among rather closely related species *C. pseudophrygia* and *C. stenolepis*, but there are quantitative differences in frequencies of several alleles that might be useful for solving the studied problem.

### Triploid individuals

Single triploid individuals were found within three diploid populations. They were possibly established by hybridization of diploid and tetraploid plants. Two of these populations (populations CZLE and KASH) belong to *C. pseudophrygia*. The tetraploid parent of the putative hybrid might be *C. jacea*, which occurs in the surroundings. In population CZLE some individuals also seem to have morphological characters of such a hybrid, especially the lateral fimbriae of appendages irregularly fused into groups (see SAARISALO-TAUBERT (1966) for the detailed description and photographs of such plants). The third population, where a triploid individual occurred, was population BRT from the Nízke Tatry Mts., Slovakia. It is the only diploid population of *C. phrygia* s.str. known from the West Carpathians. In the surroundings, the tetraploid cytotype of *C. phrygia* is common and might be the parent of the triploid hybrid. Only small morphological differences exist between the cytotypes and it is therefore almost impossible to identify hybrids using morphological features only.

However, in all cases another possibility of formation of the triploids in diploid populations cannot be ruled out: unreduced gametes. In controlled crossing experiments unreduced gametes occur at very low frequency in *Centaurea* sect. *Jacea* and they can seldom produce offspring (GARDOU 1972, HARDY et al. 2001).



Table 5. Descriptive statistics of selected (diagnostic) characters for particular taxa of *Centaurea phrygia* agg.: Ph – *Centaurea phrygia* s.str. ( $n = 508$ ), Ps – *C. pseudophrygia* ( $n = 359$ ), S – *C. stenolepis* ( $n = 311$ ), O – *C. oxylepis* ( $n = 238$ ). Character IV is binary, therefore only the mean (i.e., proportion of individuals with the character state 1) is displayed. The number of observations was reduced for character AP, because not all individuals had mature achenes. For character explanation see Table 1.

Character	Taxon	Mean	s.d.	Min.	5%	95%	Max.
LLW	Ph	3.3	0.7	2.1	2.4	4.4	6.4
	Ps	3.0	0.6	1.7	2.2	4.2	6.3
	S	3.5	0.8	2.0	2.5	4.9	6.7
	O	5.4	1.8	2.5	3.2	9.0	15.7
CL	Ph	1.7	0.2	1.2	1.4	1.9	2.0
	Ps	1.8	0.1	1.4	1.6	2.0	2.2
	S	1.6	0.1	1.2	1.4	1.8	1.9
	O	1.5	0.1	1.2	1.2	1.7	1.8
CW	Ph	1.6	0.2	1.0	1.3	1.9	2.0
	Ps	1.7	0.2	1.0	1.3	2.0	2.2
	S	1.2	0.2	0.8	0.9	1.5	1.7
	O	1.1	0.2	0.7	0.9	1.5	1.4
CLW	Ph	1.1	0.1	0.9	0.9	1.2	1.5
	Ps	1.1	0.1	0.8	1.0	1.3	1.4
	S	1.4	0.2	0.9	1.1	1.7	2.0
	O	1.3	0.2	0.9	1.1	1.7	2.0
ML	Ph	8.1	1.8	4.5	5.4	11.1	14.1
	Ps	11.7	1.6	7.3	9.2	14.5	17.1
	S	9.4	1.5	6.4	7.2	12.0	13.4
	O	7.3	1.6	3.7	4.9	10.5	11.5
MW	Ph	1.4	0.4	0.3	0.8	2.1	2.8
	Ps	0.9	0.2	0.5	0.6	1.3	1.7
	S	0.6	0.2	0.3	0.4	0.9	2.2
	O	1.4	0.6	0.5	0.7	2.4	3.1
MF	Ph	14.0	1.9	9.0	11.0	17.0	20.0
	Ps	16.4	1.9	9.0	14.0	20.0	24.0
	S	13.0	1.8	8.0	10.0	16.0	18.0
	O	13.6	1.7	9.0	11.0	16.0	18.0
IV	Ph	0.04					
	Ps	0.8					
	S	0.4					
	O	0.04					
AP	Ph	0.8	0.4	0.1	0.5	1.4	1.6
	Ps	1.1	0.2	0.6	0.7	1.5	2.1
	S	0.7	0.2	0.1	0.3	1.1	1.3
	O	0.1	0.2	0.0	0.0	0.5	0.7

### **Cytotypes of *Centaurea phrygia* s.str.**

*Centaurea phrygia* s.str. is the only species studied for which both cytotypes are reported. Nevertheless, there seems to be a distinct geographical pattern of cytotype distribution. Diploids were reported from Russia (PULKINA 1988, LAVRENKO et al. 1990) and Belarus (SEMERENKO 1989). Other literature sources from Central Europe (LÖVE & LÖVE 1961, DOSTÁL 1976, WAGENITZ 1987) reported the diploid cytotype as the only one within *C. phrygia*, but without listing the localities. During my study, diploid populations were found in northern Bohemia near the Czech-German border, in northern Moravia near the Czech-Polish border, in the East Carpathians, and one population in the West Carpathians. MÁJOVSKÝ et al. (1987) reported the tetraploid cytotype from the West Carpathians and DOSTÁL (1989) from former Czechoslovakia without exact localization. During my study it was found at many localities in the West Carpathians and also in adjacent mountain areas (Hrubý Jeseník Mts., part of the East Sudetes). To sum it up, it seems that the cytotypes are geographically differentiated: the diploids occur in lower altitudes of central, northern and north-eastern Europe, and in the East Carpathians, whereas the tetraploids may be confined to the montane altitudes of the West Carpathians and adjacent East Sudetes. Only minor morphological differences exist between the cytotypes (e.g. the appendages of tetraploids are longer than appendages of diploids on average, but the variation ranges strongly overlap). We might consider treating the cytotypes as discrete taxa, which would be homogenous in the chromosome number, similarly to the other taxa of *C. phrygia* agg. However, more data both on morphology and karyology are needed.

### ***Centaurea pseudophrygia* and *C. elatior***

Small morphological differences are reported between plants of *C. pseudophrygia* from eastern and Central Europe (DOSTÁL 1976; some morphological measurements differ between Central European and Russian floras, e.g. CZEREPANOV 1963). Also ecological (continentality and prevailing type of potential vegetation, BOHN et al. 2000–2003) and phytogeographical differences between the areas suggest taxonomical differentiation. Therefore, populations from eastern and Central Europe were sometimes treated as separate taxa (e.g. DOSTÁL 1989, ŠTĚPÁNEK & KOUTECKÝ 2005) and the names *Jacea phrygia* subsp. *elatior* (GAUD.) DOSTÁL and *C. elatior* (GAUD.) HAYEK were then used for those from Central Europe. However, the problem requires further study. No sufficiently detailed study of morphological differences between populations of *C. pseudophrygia* is available for this whole area.

### **Tetraploid *Centaurea pseudophrygia***

One tetraploid population unambiguously assigned to *C. pseudophrygia* (both by a tentative determination and by the results of this study) was found during the present study in the Nízke Tatry Mts., Slovakia (population KRH). It grows on limestone in tall-herb communities at the timberline (ca. 1500 m). Similar plants from the Veľká Fatra Mts. (Slovakia) were seen in herbaria, originating probably from similar plant communities. However, *C. pseudophrygia* is reported to be exclusively diploid. The occurrence of two cytotypes within one taxon is unusual within *C. phrygia* agg., because most taxa are

homogenous as regards chromosome numbers. The other exception is *C. phrygia* s.str., in which, however, taxonomical differentiation of the cytotypes might be justified, as discussed above.

The status of tetraploid *C. pseudophrygia* is therefore uncertain. "Tetraploid *C. pseudophrygia*" possibly represents a different taxon, which was not recognized by previous authors. Other rather poorly known taxa of *C. phrygia* agg. (namely *C. carpatica* PORCIUS from the East Carpathians) should therefore be included in the analysis before the status of tetraploid populations resembling *C. pseudophrygia* is resolved. In the West Carpathians the tetraploid cytotype of *C. phrygia* s.str. is also common. However, it differs considerably from tetraploid *C. pseudophrygia* both morphologically and ecologically (it occurs usually on meadows and pastures at lower altitudes), but it cannot be ruled out that tetraploid *C. pseudophrygia* represents populations of a tetraploid cytotype of *C. phrygia* occurring in atypical ecological conditions and therefore of atypical morphology.

#### **Other taxa of *Centaurea phrygia* agg.**

From six taxa of *C. phrygia* agg. reported from Central Europe, only three were available for analysis (plus *C. oxylepis*). The others are insufficiently known. A single locality is known to the author for *C. indurata*: Veľký vrch hill near the town of Kráľovský Chlmec, SE Slovakia. Determination of plants from this locality is moreover uncertain, because they possess a well-developed pappus, which should be absent according to both the original description of the taxon (JANKA 1858) and descriptions in modern floras (DOSTÁL 1976, 1989). Presence/absence of the pappus is an important character within *Centaurea*, which is believed to be constant within particular species and is often used in determination keys. More data from the whole distribution range (Hungary, Romania, Bulgaria) is therefore necessary to evaluate morphological variation and delimitation of this taxon.

No material (neither in the field nor in herbaria) that could be assigned to *C. melanocalathia* without any doubts has been seen. In the West Carpathians, plants of *C. phrygia* s.str., which have some characters of *C. melanocalathia* as described by DOSTÁL (1976, 1989) occur, but they are single specimens within "normal" populations. The only studied population that contained a high portion of individuals resembling *C. melanocalathia* was the population BRT. However, it had a different ploidy level; population BRT was diploid whereas *C. melanocalathia* is reported to be tetraploid (DOSTÁL 1989). Similarly, some individuals collected in the Ukrainian Carpathians fit the description of *C. nigriceps* to some extent, but no considerable divergence between them and the plants of *C. phrygia* s.str. from other areas exists. To sum it up, for both taxa (*C. melanocalathia* and *C. nigriceps*) a more detailed study and typification of the names is needed. They possibly should not be classified as separate taxa, but their names should be included into the synonymy of *C. phrygia* s.str.

#### **Determination key**

On the basis of morphometric and karyological analyses and from the information gained by field observations, four taxa can be recognized within *Centaurea phrygia* agg. in Central Europe: *Centaurea phrygia* s.str., *C. pseudophrygia*, *C. stenolepis*, and *C. oxylepis* (the last

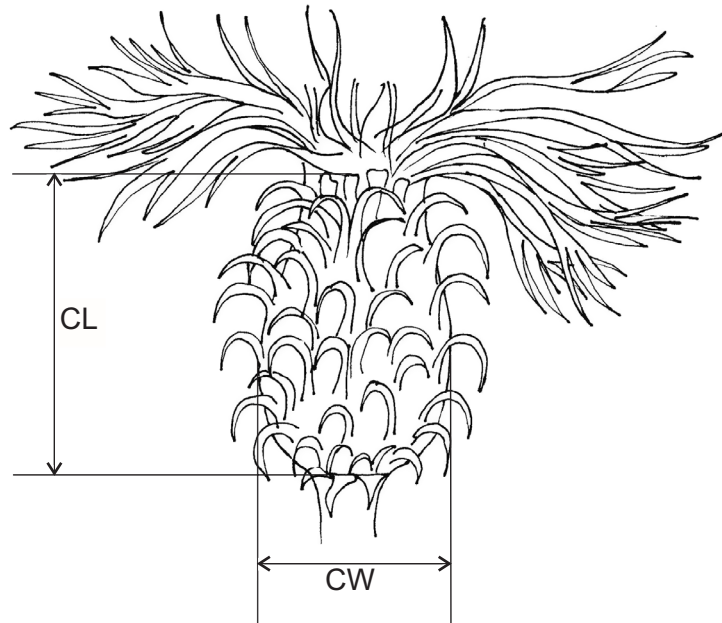


Fig. 8. Measurements on involucre (characters CL and CW).

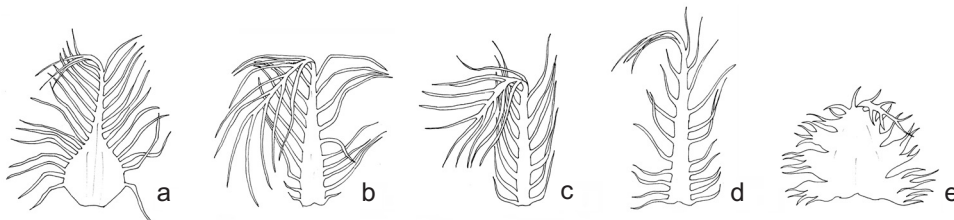


Fig. 9. Shape of appendages of middle involucre bracts: a – *C. phrygia*, b – *C. pseudophrygia*, c – *C. stenolepis*, d – *C. oxylepis*, e – hybrid between *C. jacea* and some taxon from *C. phrygia* agg. (Orig. Eva Koutecká).

taxon is usually not included in this group but was included in this study for its similarity in some principal morphological features). Descriptive characteristics of the main differentiating morphological characters are presented in Table 5. Some studied populations were morphologically intermediate, either between *C. phrygia* s.str. and *C. oxylepis* or between *C. pseudophrygia* and *C. stenolepis*. They possibly originated through introgressive hybridization.

A determination key of *Centaurea phrygia* agg. in Central Europe is presented below. Morphological measurements should be made on leaves of the middle part of a stem and on a terminal capitulum. If branching of a stem is measured, only fully developed individuals with

an undamaged terminal capitulum should be considered. Because of high intra-population variation, more plants from one locality should be collected and average values should be used for determination. “Appendages” are the longest appendages of middle involucre bracts, unless stated differently. Typical shapes of appendages are depicted in Fig. 9.

- 1a. Lateral fimbriae of appendages irregularly fused in groups; pappus absent or short, irregularly developed ..... hybrids with *C. jacea*
- 1b. Appendages regularly fimbriate on the margin, the fimbriae not fused; pappus present or absent ..... 2
- 2a. Leaves linear-lanceolate to lanceolate; involucre ovoid; central undivided part of appendages brown (rarely blackish), narrowly triangular, attenuated into a simple terminal acumen; pappus absent; 2n=44 ..... *C. oxylepis* (typical populations)
- 2b. Leaves lanceolate to ovate; involucre ovoid to globose; central undivided part of appendages blackish, linear to ovate, attenuated into a long laterally fimbriate acumen; pappus present ..... 3
- 3a. Stem branched in the upper 1/3, branches short, forming ±dense corymb of capitula, if the stem branched in lower parts and with longer branches, then several capitula in corymbs at the end of branches; size of cauline leaves usually gradually decreasing towards the top of stem; involucre ovoid or cylindrical; central undivided part of appendages linear, 0.4–0.9 mm wide, distal part of appendages light brown or ochre; 2n=22 ..... *C. stenolepis*
- 3b. Stem branched from the middle, branches long, not forming dense corymb of capitula; size of cauline leaves only slightly decreasing towards the top of stem; involucre ovoid or globose; central undivided part of appendages linear-lanceolate to ovate, 0.5–2.4 mm wide, distal part of appendages brown or blackish ..... 4
- 4a. Appendages 9.2–14.5(–17.5) mm long, forming dense “sheath” around the involucre; central undivided part of appendages 0.5–1.3 mm wide, gradually attenuated into terminal fimbriate acumen; distal part of appendages brown; appendages of inner involucre bracts in 1(–2) row, usually ovate, covered by appendages of the middle involucre bracts from the lateral view on involucre; 2n=22 ... *C. pseudophrygia* (Note: Some populations are intermediate between *C. pseudophrygia* and *C. stenolepis*. They differ from typical *C. pseudophrygia* by having narrower leaves and appendages, and by branching of the stem similar to *C. stenolepis*).
- 4b. Appendages 5.5–11(–14) mm long, forming ± lax “sheath” around the involucre; central undivided part of appendages 0.8–2.4 mm wide, ± abruptly attenuated into terminal fimbriate acumen; distal part of appendages brown or blackish; appendages of inner involucre bracts in several rows, ovate to orbicular, exceeding appendages of the middle involucre bracts from the lateral view on involucre ..... 5
- 5a. Involucre broadly ovoid to globose, 1.4–1.9 × 1.3–1.9 cm; central undivided part of appendages ovate or triangular; pappus 0.5–1.4 mm long; 2n=22 or 44 ..... *C. phrygia* s.str.
- 5b. Involucre ovoid or cylindrical, 1.3–1.7 × 0.9–1.5 cm; central undivided part of appendages narrowly triangular; pappus up to 0.7 mm long; 2n=44 ..... *C. oxylepis* (populations intermediate to *C. phrygia* s.str.)

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## APPENDIX

List of localities of the analyzed populations and corresponding chromosome numbers determined in this study. Localities are arranged according to taxa (determination is based on the results of the present study), and within taxa they are in alphabetical order according to the population abbreviation. Each record is given as follows: population abbreviation – country, region and/or mountain range: nearest town/village, description of the locality, altitude, geographic coordinates (WGS84); chromosome number (number of plants studied using chromosome counting/flow cytometry).

### *Centaurea pseudophrygia*

- BOL – Czech Republic, NW Bohemia, Krušné hory Mts.: Boleboř, wet meadow by the road to Jirkov, ca. 2.9 km SSE of the village, 450 m, 50°37'47" N, 13°25'43" E; 2n=22 (3/15).
- CERM – Czech Republic, E Bohemia: Čermná, meadow at N end of the village, 460 m, 50°34'02" N, 15°46'04" E; 2n=22 (3/0).
- CZLE – Czech Republic, S Bohemia, Šumava Mts.: České Žleby, meadows "V Podkově" ca. 4.0 km S of the centre of the former village, 890 m, 48°50'36" N, 13°46'15" E; 2n=22, single individual 2n=33 (8/0).
- HVLT – Czech Republic, S Bohemia, Šumava Mts.: Horní Vltavice, meadow by the path to railway station, ca. 0.9 km E of the church in the village, 870 m, 48°57'24" N, 13°46'15" E; 2n=22 (3/0).
- KASH – Czech Republic, S Bohemia, Šumava Mts.: Kašperské Hory, meadow ca. 0.7 km S of the church in the town, 670 m, 49°08'12" N, 13°33'09" E; 2n=22, single individual 2n=33 (5/5).
- KOZH – Czech Republic, S Bohemia, Šumava Mts.: Kašperské Hory, meadows N of Kozi Hřbet settlement, ca. 2.5 km NW of the church in the town, 750 m, 49°07'45" N, 13°31'44" E; 2n=22 (3/0).
- KRH – Slovakia, Nízke Tatry Mts.: Liptovský Ján, tall-herb vegetation at the timberline on S slope of Krakova hoľa hill, near Javorie saddle, ca. 8.5 km SSW of the village, 1530 m, 48°58'31" N, 19°37'59" E; 2n=44 (5/3).
- OLE1 – Czech Republic, E Bohemia, Orlické hory Mts.: Olešnice v Orlických horách, meadows by the road to Nový Hrádek, ca. 1.6 km SWW of the church in the village, 580 m, 50°22'05" N, 16°17'23" E; 2n=22 (3/12).
- OLE2 – Czech Republic, E Bohemia, Orlické hory Mts.: Olešnice v Orlických horách, meadows by Čihalka chalet, ca. 2.9 km E of the village, 730 m, 50°22'28" N, 16°21'00" E; 2n=22 (6/0).
- PRIS – Czech Republic, S Bohemia: Přislop, an edge of tree alley by the old road ca. 0.4 km SW of the village, 790 m, 48°57'01" N, 14°07'24" E; 2n=22 (3/0).
- PROS – Czech Republic, E Bohemia, Železné hory Mts.: Prosička, meadow by the road ca. 0.4 km NE of the village, 530 m, 49°49'21" N, 15°41'57" E; 2n=22 (3/8).
- SOK – Czech Republic, S Bohemia: Hradiště, meadow by Mlýn u Dubu settlement, ca. 1.7 km NNE of the centre of the village, 590 m, 48°44'54" N, 14°33'12" E; 2n=22 (3/0).
- STGH – Czech Republic, S Bohemia, Šumava Mts.: Volary, meadow by the forest E of Stögrova Huť settlement, 1.9 km NWW of the railway station in the town, 810 m, 48°54'48" N, 13°51'26" E; 2n=22 (3/3).

### Populations intermediate between *C. pseudophrygia* and *C. stenolepis*

- BABL – Czech Republic, NW Bohemia, České středohoří Mts.: Čeřeniště, meadows ca. 1.4 km S of the village, near the S border of "Babinské louky" nature monument, 590 m, 50°35'38" N, 14°07'20" E; 2n=22 (3/0).
- BABU – Czech Republic, E Bohemia: Žernov, an old orchard on the SW slope of Babiččino údolí valley, ca. 1.4 km WSW of the village, 300 m, 50°25'37" N, 06°02'21" E; 2n=22 (3/9).
- DEB – Czech Republic, E Bohemia: Debrné, meadow at the N edge of the village, 380 m, 50°29'55" N, 15°43'30" E; 2n=22 (3/0).
- RTE – Czech Republic, E Bohemia, Železné hory Mts.: Rtenín, meadow ca. 0.5 km E of the village, 490 m, 49°52'38" N, 15°42'58" E; 2n=22 (3/12).
- RUS – Czech Republic, E Bohemia, Železné hory Mts.: Rušínov, meadow in the curve of the road to Modletín ca. 0.5 km E of the center of the village, 580 m, 49°47'30" N, 15°41'30" E; 2n=22 (3/5).
- VIT – Czech Republic, SE Bohemia, Českomoravská vrchovina Mts.: Staré Město pod Landštejnem, NW edge of Vitiněves settlement, ca. 3.5 km NW of the center of the village, 540 m, 49°01'59" N, 15°14'01" E; 2n=22 (3/0).



***Centaurea stenolepis***

- BOR – Slovakia, Slovenský kras Mts.: Bôrka, dry meadows on Bôrčianska planina plateau, ca. 1.7 km NE of the church in the village, 850 m, 48°38'44" N, 20°46'45" E; 2n=22 (3/0).
- DOM – Czech Republic, central Bohemia: Dománovice, W edge of the small oak forest, ca. 1.2 km NNE of the village, 225 m, 50°07'18" N, 15°19'43" E; 2n=22 (3/0).
- DUB – Czech Republic, S Moravia: Ratíškovice, an old clearing in Dúbrava forest by the yellow marked tourist path ca. 2.2 km of the church in the village, 200 m, 48°54'14" N, 17°10'14" E; 2n=22 (3/12).
- KRIP – Slovakia, Slovenský kras Mts.: Hačava, meadows in Krížna Poľana saddle, ca. 1.1 km NNE of the church in the village, 860 m, 48°40'36" N, 20°50'32" E; 2n=22 (3/2).
- LES – Czech Republic, S Moravia, Bílé Karpaty Mts.: Horní Němčí, meadows on the N slope of the Lesná hill, ca. 3.9 km NW of the church in the village, 630 m, 48°54'10" N, 17°39'11" E; 2n=22 (3/0).
- LIP – Czech Republic, S Moravia, Bílé Karpaty Mts.: Korytná, S border of Lipiny forest and the meadow nearby, ca. 1.3 km SWW of the church in the village, 390 m, 48°56'11" N, 17°38'54" E; 2n=22 (3/0).
- MIL – Czech Republic, S Moravia: Mikulov, an old clearing in Milovický les forest, ca. 1.8 km NW of the castle in the town, 290 m, 48°49'44" N, 16°40'26" E; 2n=22 (3/10).
- PREL – Czech Republic, S Moravia, Bílé Karpaty Mts.: Vápenky, meadows "Přední louky" ca. 1.2 km NW of Porážky hill, ca. 2 km NNW of the village, 570 m, 48°53'25" N, 17°36'57" E; 2n=22 (3/2).
- SIT1 – Slovakia, Štiavnické vrchy Mts.: Ilija, ca. 2.5 km SWW of the church in the village, on the N ridge of Mt. Sitno, 860 m, 48°24'44" N, 18°52'15" E; 2n=22 (5/0).
- SIT2 – Slovakia, Štiavnické vrchy Mts.: Počúvadlo, meadows on the foothill of Mt. Sitno, ca. 4.1 km NE of the church in the village, 690 m, 48°23'47" N, 18°51'52" E; 2n=22 (5/0).
- VOL – Czech Republic, S Moravia, Bílé Karpaty Mts.: Kněždub, "Vojšické louky" meadows, ca. 4.8 km SSE of the church in the village, 400 m, 48°51'03" N, 17°26'09" E; 2n=22 (3/0).

***Centaurea phrygia* s.str.**

- BRT – Slovakia, Nízke Tatry Mts.: Liptovský Hrádok, meadows in Brtkovica valley, ca. 3.5 km SSW of the railway station in the town, 1000 m, 49°00'18" N, 19°42'45" E; 2n=22, single individual 2n=33 (7/0).
- BURK – Ukraine, Čivčinske hory Mts.: Burkut, SSE of the town of Verchovyna, meadow N of the village, 920 m, 47°56'47" N, 24°41'30" E; 2n=22 (3/1).
- CERV – Czech Republic, Silesia: Černá Voda, meadow by the Černý potok brook near the "Rokliny" settlement, ca. 1.4 km NE of the church in the village, 300 m, 50°19'17" N, 17°10'07" E; 2n=22 (3/0).
- DEM – Slovakia, Nízke Tatry Mts.: Demänová, Repiská meadows in Demänovská dolina valley, ca. 8 km S of the village, 900 m, 48°59' N, 19°35' E; 2n=44 (3/5).
- JAS – Ukraine, Svidovec Mts.: Jasinja, meadow in the low end of the Svidovec brook valley, ca. 5.5 km SW of the centre of the village, 650 m, 48°13'58" N, 24°18'57" E; 2n=22 (3/0).
- LAZ – Ukraine, Čornohora Mts.: Lazeščina, in the valley of the Lazeščanka brook, ca. 9 km SSE of the centre of the village, 940 m, 48°11'59" N, 24°27'39" E; 2n=22 (3/2).
- LIPO – Slovakia, Liptovská kotlina: Liptovská Porúbka, meadows by the S edge of the village, 680 m, 49°01'23" N, 19°43'24" E; 2n=44 (3/0).
- NEJ – Czech Republic, W Bohemia, Krušné hory Mts.: Nejde, meadow ca. 1.6 km SSE of Javorník hill, ca. 2 km W of the railway station in the town, 640 m, 50°19'02" N, 12°40'17" E; 2n=22 (3/1).
- STCV – Czech Republic, Silesia: Stará Červená Voda, meadow by the E edge of the village, ca. 0.7 km SSW of the church in the village, 310 m, 50°19'27" N, 17°11'58" E; 2n=22 (3/0).
- SVIS – Slovakia, Nízke Tatry Mts.: Malužiná, meadows in Svidovské sedlo saddle, ca. 4.5 km W of the church in the village, 1140 m, 48°58'11" N, 19°42'37" E; 2n=44 (5/0).
- SYB – Ukraine, Čivčinske hory Mts.: Zelene, part Šybene, meadow at the N edge of the village, 840 m, 47°59'48" N, 24°43'05" E; 2n=22 (3/2).
- VELF – Slovakia, Veľká Fatra Mts.: Vyšná Revúca, meadow in Zelená dolina valley, ca. 3.7 km W of the centre of the village, 860 m, 48°54'40" N, 19°06'40" E; 2n=44 (3/0).
- VER1 – Slovakia, Nízke Tatry Mts.: Vernár, meadows ca. 0.7 km NWW of the church in the village, 800 m, 48°55'15" N, 20°15'40" E; 2n=44 (3/3).
- VER2 – Slovakia, Nízke Tatry Mts.: Vernár, meadow by the left-side tributary to the Hnilec river, ca. 4 km SW of the village, 940 m, 48°53'18" N, 20°14'16" E; 2n=44 (3/0).

- VIS – Czech Republic, NE Moravia, Moravskoslezské Beskydy Mts.: Visalaje, upper part of downhill course ca. 200 m of the parking place in the settlement, 770 m, 49°31'02" N, 18°31'43" E; 2n=44 (3/0).
- VOR – Ukraine, Čornohora Mts.: Vorochta, meadow ca. 7 km SSE of the centre of the village, 900 m, 48°12'25" N, 24°35'20" E; 2n=22 (3/0).
- ZEL – Ukraine, Čivčinske hory Mts.: Zelene, meadow in the middle part of the village, 770 m, 48°02'35" N, 24°45'06" E; 2n=22 (3/0).
- Czech Republic, N Moravia, Hrubý Jeseník Mts.: Karlov pod Pradědem, meadows S of the village, 690 m, 50°01'03" N, 17°18'15" E; 2n=44 (3/0) [Population not included in morphometric analyses].

#### Populations intermediate between *C. phrygia* and *C. oxylepis*

- CIC – Slovakia, Strážovské vrchy Mts.: Čičmany, meadows ca. 2 km SW of the church in the village, 700 m, 48°56'44" N, 18°29'35" E; 2n=44 (3/0).
- DLOM – Czech Republic, NE Moravia, Moravskoslezské Beskydy Mts.: Dolní Lomná, meadow on the right bank of the Lomná river, by the confluence with the Mionší brook, 490 m, 49°32'43" N, 18°40'45" E; 2n=44 (3/0).
- KOZL – Czech Republic, NE Moravia: Kozlovice, meadow ca. 1.4 km SW of the church in the village, 390 m, 49°34'52" N, 18°14'48" E; 2n=44 (3/5).
- MIO – Czech Republic, NE Moravia, Moravskoslezské Beskydy Mts.: Horní Lomná, meadow on the top of Velká Polana hill, ca. 1.5 km NEE of the church in the village, 890 m, 49°32'02" N, 18°39'21" E; 2n=44 (3/3).
- OSTR – Czech Republic, NE Moravia: Ostrava, a lawn in the N part of Komenského sady park, in the centre of the town, 210 m, 49°51'13" N, 18°17'12" E; 2n=44 (3/0).
- RAD – Czech Republic, N Moravia: Krasov, steep slope under the road by Radim nature reserve, ca. 2.2 km E of the church in the centre of the village, 440 m, 50°05'43" N, 17°34'40" E; 2n=44 (3/0).
- STUD – Czech Republic, N Moravia: Studénka, ruderal vegetation between a road and a railway, ca. 0.6 km E of the railway station in the town, 225 m, 49°42'34" N, 18°04'26" E; 2n=44 (3/0).
- TICH – Czech Republic, NE Moravia: Tichá, meadow ca. 0.9 km N of the church in the village, 430 m, 49°34'41" N, 18°13'13" E; 2n=44 (3/0).
- VRS – Slovakia, Biele Karpaty Mts.: Vršatecké Podhradie, meadows ca. 1 km E of the village, 49°03'51" N, 18°09'54" E; 2n=44 (3/0).
- ZAT – Czech Republic, N Moravia: Zátor, meadow at the forest edge ca. 1.5 km NW of the church in the village, 480 m, 50°03'36" N, 17°33'57" E; 2n=44 (3/0).

#### *Centaurea oxylepis*

- BEST – Czech Republic, E Bohemia: Běstvína, xerophilous meadow ca. 1 km NNW of the church in the village, 340 m, 49°50'37" N, 15°35'35" E; 2n=44 (3/0).
- CAS – Czech Republic, E Bohemia: Častolovice, meadow on the SE slope of Za Humny hill, by Strýc settlement, ca. 0.9 km NW of the railway station in the village, 300 m, 50°08'05" N, 16°09'55" E; 2n=44 (3/0).
- HRAB – Czech Republic, N Moravia: Hrabyně, an abandoned orchard by the road to Smolkov, ca. 0.8 km NE of the church in the village, 330 m, 49°53'18" N, 18°04'10" E; 2n=44 (3/0).
- OPAT – Slovakia, Strážovské vrchy Mts.: Opatová, xerophilous sidehill in the Opatovská dolina valley at the SE edge of the village, ca. 0.5 km SEE of the church in the village, 250 m, 48°54'32" N, 18°06'19" E; 2n=44 (3/0).
- RIM – Slovakia, Cerová vrchovina Mts.: Rimavská Sobota, edge of the forest ca. 2.4 km SSW of the centre of the town, 220 m, 48°21'49" N, 20°00'15" E; 2n=44 (3/0).
- VAL1 – Czech Republic, N Moravia: Valšov, a grass in the village, 520 m, 49°55'49" N, 17°26'15" E; 2n=44 (3/0).
- VAL3 – Czech Republic, N Moravia: Valšov, meadow by the Moravice river, ca. 0.7 km SSW of the railway station in the village, 510 m, 49°55'16" N, 17°25'51" E; 2n=44 (3/10).
- VOZ – Slovakia, Štiavnické vrchy Mts.: Voznica, meadow in Voznická dolina valley, ca. 2 km SEE of the village, 250 m, 48°27'11" N, 18°43'19" E; 2n=44 (3/0).