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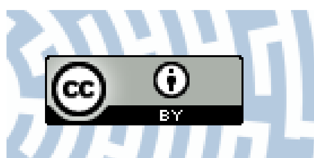

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Cytogenetic studies of three European species of *Centaurea* L. (Asteraceae)

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Cytogenetic analysis of several populations of *Centaurea jacea* (2n = 4x = 44), *C. oxylepis* (2n = 4x = 44) and *C. phrygia* (2n = 2x = 22) was performed using flow cytometry, differential chromosome staining and FISH. In all species *Arabidopsis*-type telomeric repeats hybridized only to the terminal part of chromosomes. In *C. phrygia* three pairs and in *C. oxylepis* six pairs of chromosomes revealed the hybridization signals of 45S rDNA. *Centaurea jacea* showed polymorphism in the 45S rDNA loci number, five or six pairs of sites were observed. 5S rDNA loci were located in two pairs of chromosomes in *C. phrygia*. In *C. jacea* and *C. oxylepis* the number and position of 5S rDNA loci were the same: three pairs located interstitially and one terminally. The genome size of the diploid *C. phrygia* was established as 2.14 pg/2C. The genomes of tetraploid species were nearly two times larger and genome size polymorphism was observed among *C. jacea* populations.

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The genus *Centaurea* L. is one of the largest genera in Asteraceae in the traditional understanding of Flora Europea (Dostal 1976). Depending on taxonomical revision it comprises 400 or even 700 species, predominantly distributed in the Old Word (Wagenitz and Hellwig 1996; Greuter et al. 2001). *Centaure*a species have been studied at the morphological and molecular phylogenetic levels (Wagenitz and Hellwig 1996; Garcia-Jacas et al. 2001). The genome size of *Centaurea* species ranges from 2C = 1.67 to 4.30 pg (Bancheva and Greilhuber 2006). The karyological data for these taxa are scarce and mainly focus on a determination of chromosome number (Romaschenko et al. 2004). Karyotype studies have only been done on a few species (Gardou 1972; Siljak-Yakovlev and Yakovlev 1981). The genus *Centaurea* has several basic chromosome numbers, ranging from x = 7 to x = 16. Considering these basic numbers, the genus seems to comprise mostly diploid and tetraploid species (Gardou 1972; Garcia-Jacas et al. 1996; Romaschenko et al. 2004; Siljak-Yakovlev et al. 2005; Bancheva and Greilhuber 2006).

In northwestern Europe the extremely polymorphic subgenus *Jacea* (Miller) Hayek. has attracted particular scientific interest. Depending on the taxonomic attempts, this taxon is treated as a single species which forms an extremely polymorphic polyploidy complex (Briguet 1931) or is subdivided into several species (Dostal 1976). Better understanding of the subgenus *Jacea* was brought about by the work of Gardou (1972). According to her observation the complex consists of morphologically well-defined units at the diploid levels (2n = 22), with distinct ecogeographical distributions, linked by intermediate tetraploids (2n = 44). Members of the complex freely hybridize, at least within the same ploidy level and usually yield viable and fertile offspring (Saarisalo-Taubert 1966; Gardou 1972). Based on these results Gardou (1972) suggested that the complex could be regarded as a single biological species (*C. jacea* complex). However she admitted that for practical reasons three taxa could be retained: *C. nigra* L., *C. jacea* L. and *C. pratensis* Thuill. This taxonomic treatment was later supported by biosystematic studies, which indicated that a low level of genetic diversity exists within the complex (Sommer 1990; Hardy et al. 2000).

In Poland, after Dostal (1976) taxonomical treatment, subgenus *Jacea* is represented by five species: *C. jacea* L., *C. oxylepis* Hayek (Wimm. et Grab.), *C. phrygia* L., *C. pseudophrygia* C.A. Mey. and *C. pannonica* (Heuff.) Hayek (Mirek et al. 2002). Among them, *C. jacea* has the widest distribution range and it is common both in lowlands and mountains. In this part of Europe *C. jacea* relatively easily crosshybridizes with *C. oxylepis* Hayek, which occurs mainly in the mountains region in southwestern part of Poland (Madalski and Ciaciura 1972). *Centaurea jacea* can also hybridize with *C. phrygia* although offspring of these crosses are observed more rarely (Nowak unpubl.). To our knowledge, no biosystematic data on Polish

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knapweeds (*Centaurea* subgenus *Jacea* (Mill.) Hayek) are available. Based on populations samples in other parts of Europe, two different ploidy levels have been reported for *C. jacea* and *C. phrygia* (2n = 2x = 22 and 2n = 4x = 44) and for *C. oxylepis* only tetraploid plant has been observed (Gardou 1972; Dostal 1976; Hardy et al. 2000; Stepanek and Koutecky 2005).

Centaurea chromosomes are small and the karyotypes consist of mostly metacentric and submetacentric chromosomes, which makes karyotype analysis very difficult. Fluorochrome banding as well as fluorescence *in situ* hybridization (FISH), have recently been used for cytotaxonomical studies within different plant groups (Weiss-Schneeweiss et al. 2008). Fluorochrome banding CMA_3 (chromomycin A3) and DAPI (4',6'-diamidino-2-phenylindole) applied to chromosome analysis has uncovered additional chromosome markers for some groups of species (e.g. Rutaceae-Aurantioideae, Guerra et al. 2000). More informative markers are often provided by FISH with repetitive or single copy sequences as probes. For example rRNA genes have been widely used as chromosome markers for karyotyping and for studying the evolutionary relationships within many genera (Hasterok et al. 2001; Weiss-Schneeweiss et al. 2008). Another highly conserved sequence is the telomeric repeat present in the physical ends of the chromosomes of nearly all plant species. Telomeric DNA in the majority of plant species analyzed thus far consists of *Arabidopsis*-type telomeric repeats (TTTAGGG) (Fuchs et al. 1995). To date, there is no data regarding the characteristics of telomeres in *Centaurea*.

The knowledge about the genome and karyotype structure is very important for better understanding the evolution of the subgenus *Jacea.* The aim of this study was to investigate the diversity at genome size and karyotype level among various *C. jacea* populations using flow cytometry, FISH and chromosome banding. We also applied these methods to comparative cytogenetic analysis of *C. jacea* and two other close related member of subgenus *Jacea*: *C. prygia* and *C. oxylepis*.

MATERIAL AND METHODS

Plant material

Three *Centaurea* species were studied: *C. jacea*, *C. oxylepis* and *C. phrygia*. *Centaurea oxylepis* and *C. phrygia* were represented by two populations and *C. jacea* by eight populations (Table 1). The study area is located mainly in the southern part of Poland but one population was collected in northwestern part of Poland. The species were determined on the basis of morphological features (Madalski and Ciaciura 1972).

Chromosome preparation, staining methods and karyotype morphology analyses

Seeds were germinated on moist filter paper in petri dishes. Whole seedlings (approximately 2 cm long) were pretreated with 2 mM 8-hydroxyquinoline for 4 h and fixed in 3:1 ethanol/acetic acid. Fixed roots were washed in a 0.01 M citric acid–sodium citric buffer (pH 4.8) and digested in a mixture of 20% pectinase (Sigma P0690) and 2% cellulose (Onozuka R-10 Serva) for 1 h at 37°C. A single roottip was washed in cold distilled water and transferred into a drop of 45% acetic acid on microscope slide and squashed. The coverslips were removed after freezing and slides were air-dried.

Fluorescent staining with two fluorochromes: CMA_3 (chromomycin A3) and DAPI (4',6'-diamidino-2-phenylindole) were performed as described Hajdera et al.

Table 1. *Geographic origin of the investigated Centaurea populations.*

No.	Taxon	Locality (altitude)
1	*C. phrygia*	Western Bieszczady Mountains, between Smerek and Wetlina villages (800 m)
2	*C. phrygia*	Western Bieszczady Mountains, The Wyżniańska Pass (855 m)
3	*C. oxylepis*	Eastern Sudetes, Złote Mountains, near Lądek-Zdrój (400 m)
4	*C. oxylepis*	Western Carpathians, Żywiec Beskid, Rajcza village (700 m)
5	*C. jacea*	Great Poland Lake Districts, Słubice (27 m)
6	*C. jacea*	The Western Carpathians, Żywiec Beskid, Ujsoły village (500 m)
7	*C. jacea*	Sub-Tatra furrow, Zakopane town, Antałówka settlement (900 m)
8	*C. jacea*	Pieniny Mountains, The Kaletówka Glade (560 m)
9	*C. jacea*	Krosno-Jasło Basin, near Krosno (250 m)
10	*C. jacea*	Eastern Beskids, Sanok-Turka Mountain, Chrewt village (375 m)
11	*C. jacea*	Eastern Beskids, Sanok-Turka Mountains, Michniowiec village (530 m)
12	*C. jacea*	Eastern Beskids, San Valley, Procisne village (550 m)

(2003). The transcriptional activity of 45S rRNA gene loci was determined using silver staining. Slides were incubated in borate buffer (pH 9.2), air-dried and several drops of freshly prepared 50% (w/v) $AgNO_3$ (Merck) in re-distilled water were applied. Slides were covered with a nylon mesh (Nylbot) and incubated in a moisture chamber for 1 h at 42°C, washed in re-distilled water, air dried and mounted in DPX (a mixture of distyrene, a plasticizer and xylene; Fluka). Fluorescence staining and silver staining were done for at least ten individuals from every population.

The chromosome number was calculated for 100 individuals from each population. Karyotype morphology analyses were done using well-spread metaphase plates (at least five for every species) stained with DAPI (4',6-diamidino-2-phenylindole) and Analysis software. The chromosome nomenclature followed LEVAN et al. (1964). The karyotype asymmetry was estimated using two numerical parameters: the intrachromosomal asymmetry index (A_1) and the interchromosomal asymmetry index (A_2) (ROMERO ZARCO 1986). The intrachromosomal asymmetry index (A_1) was calculated using the equation:

$$A_1 = 1 - \frac{\sum_{i=1}^{n} \frac{q_i}{p_i}}{n}$$

Where q_i is the mean length for short, and p_i for long arms in every homologous chromosome pair; n is the number of homologous chromosome pairs. The A_2 (the interchromosomal asymmetry index) is the ratio between the standard deviation (s_{CL}) and the mean chromosome length (x_{CL}):

$$A_2 = \frac{s_{CL}}{x_{CL}}$$

The fluorescence in situ hybridization

The 2.3 kb sub-clone of the 25S rDNA coding region of *Arabidopsis thaliana* (UNFRIED and GRUENDLER 1990), labelled with digoxigenine-11-dUTP by nick translation, was used for detection of 45S rRNA gene loci. To detect 5S rDNA loci a 410 bp clone isolated from *Triticum aestivum* (GERLACH and DYER 1980) was amplified and labelled with rhodamine-4-dUTP by PCR using universal M13 "forward" (5'-CAG GGT TTT CCC AGT CAC GA-3') and "reverse" (5'-CGG ATA ACA ATT TCA CAC BAGG A-3') sequencing primers. The thermal cycling program was as follow: 94°C × 1 min, 35 cycles of 94°C × 40 s, 55°C × 40 s, 72°C × 1 min and 1 cycle of 72°C × 5 min. As a telomeric probe clone HT100.3 containing approximately 30 copies of *Arabidopsis*-type telomeric repeats was used (HAJDERA et al. 2003). The clone HT100.3 was labeled as described for 5S rDNA probe.

FISH was carried out following the method of SCHWARZACHER and HESLOP-HARRISON (2000) with modifications. In short, the hybridization mixture consisting of 100 ng of labelled DNA probe, 50% formamide, 10% dextran sulphate, 0.1% SDS (sodium dodecyl sulphate) and 10 μg of sheared salmon sperm DNA was denatured for 10 min at 85°C, then chilled on ice and applied to the chromosome preparation. The slides and hybridization mixture were denatured together at 72°C for 5 min in an in situ Thermal Cycler (Hybaid) and then allowed to hybridize for 48 h in a humid chamber at 37°C. Stringent washes (20% formamide in 0.1 × SSC at 42°C) were followed by immunodetection of digoxigenin-labelled DNA probe using FITC-conjugated primary anti-digoxigenin antibodies. The preparations were mounted in Vectashield containing 2 μg ml^{-1} DAPI. Images were acquired with a Hamamatsu CCD camera attached to an Olympus Provis epifluorescence microscope, then processed uniformly and superimposed using Micrografx Picture Publisher software. For every used DNA probe at least ten individuals from each population were analysed.

Flow cytometry

Genome size was determined using flow cytometry. Samples of nuclei for the measurements were obtained from plants growing in pots in a greenhouse with 16 h days and 8 h nights at 20°C. Young leaves were chopped with a razor blade in a nuclei extraction buffer (DOLEZEL and GOHDE 1995), filtered through a 30 μm nylon sieve and stained with propidium iodide using a Partec high-resolution DNA kit according to the manufacturer's instructions. For *C. jacea* and *C. oxylepis* nuclei isolated from leaves of *Zea mays* L. line CE 777 (2C DNA = 5.43 pg, LYSAK and DOLEZEL 1998) were used as an internal standard. *Brassica oleracea* L. (2C DNA = 1.3 pg, Siwinska unpubl.) was used as an internal standard for *C. phrygia*. Genome size for at least ten plants were measured for each population with DAKO Galaxy flow cytometry and 10 000 nuclei were measured for every plant. Differences in DNA content between populations were tested using ANOVA and Kruskal–Wallis test. This allowed detection of populations with significantly different DNA contents.

RESULTS AND DISCUSSION

Chromosome number, size and morphology

Somatic chromosomes of three *Centaurea* species were analyzed. One of these, *C. phrygia*, is a diploid with the chromosome number 2n = 2x = 22 and two others *C. oxylepis* and *C. jacea* are tetraploids with 2n = 4x = 44 chromosomes (Table 2). In this study eight *C. jacea* populations coming from different elevations ranging

Table 2. *Somatic chromosome number, ploidy level, ranges of chromosome length, total karyotype length (TKL), asymmetric index (A1, A2) of* Romero Zarco *(1986), and symmetry classes (SC) of* Stebbins *(1971) for Centaurea species.*

Taxon	2n	Ploidy level	Chromosome length range (µm)	TKL (µm)	A1	A2	SC
C. jacea	44	4x	1.93 – 3.04	108.61	0.26	0.12	2A
C. oxylepis	44	4x	2.00 – 3.28	114.42	0,29	0,13	2A
C. phrygia	22	2x	2.03– 3.26	57.60	0.25	0.11	2A

from 27 to 900 m (Table 1) were analyzed and all were pure tetraploid; no diploid *C. jacea* plant was observed (unlike in western Europe where diploid and tetraploid cytotypes were described, Gardou 1972; Hardy et al. 2000). Diploid cytotypes of *C. jacea* were reported earlier only from western Europe and the tetraploid *C. jacea* was seen to have a wider ecogeographical distribution than diploids (Hardy et al. 2000). Chromosome numbers for *C. phrygia* and *C. oxylepis* were the same as was described earlier (Dostal 1976; Stepanek and Koutecky 2005).

The chromosomes of all analyzed *Centaurea* species were small. *Centaurea phrygia* and *C. oxylepis* have a similar mean chromosome length (2.62 µm and 2.60 µm respectively) whereas *C. jacea* has a little smaller mean chromosome length (2.47 µm) (Table 2). The *C. phrygia* karyotype was composed mainly of metacentric chromosomes; only one pair was described as submetacentric (10m + 1sm). Tetraploids have the same karyotype formula composed of twelve metacentric and ten submetacentric chromosome pairs (12m + 10sm). The karyotypes showed low intra- and interchromosome asymmetry and a gradual decrease in chromosome size was observed within the complements. The variation of A_1 and A_2 asymmetry indices between the analyzed species was very small; however, the tetraploid species, especially *C. oxylepis*, had a higher A_1 and A_2 value than the diploid. Under Stebbins' classification of karyotypes, the taxa fall into the 2A category (Table 2). In karyotypes of the 2A category the ratio between the largest and smallest chromosome is lower than 2. The proportion of chromosomes with arm ratio (length of long arm /length of short arm of a chromosome) lower than 2 is between 0.99–0.55.

Earlier karyotype morphology analyses were done only for diploid *C. jacea*. Gardou (1972) indicated that the karyotype formula for the diploid is 5m + 6sm (five metacentric and six submetacentric chromosome pairs). Polyploid *C. jacea* is thought to be an autotetraploid (Hardy et al. 2000). However, the karyotype formula obtained for the tetraploid *C. jacea* analyzed in this study is not simply a duplication of the diploid karyotype described by Gardou (1972). Instead of the expected 10m + 12sm (ten metacentric and twelve submetacentric chromosome pairs), the tetraploid revealed 12m + 10sm (twelve metacentric and ten submetacentric chromosome pairs). Polyploid genomes usually undergo chromosome rearrangements that cause genome diploidization (Wendel 2000). It is possible that the observed differences in the diploid and tetraploid *C. jacea* karyotype formula reflect the tetraploid *C. jacea* genome rearrangement after polyploidization. However, the results were obtained using different methods of the slide preparation and staining so we cannot excluded some technical reasons for the observed differences.

Distribution of telomeric repeats

The *Arabidopsis*-type telomeric repeat comprising the $(TTTAGGG)_n$ sequence is known to hybridise with chromosomes of numerous angiosperm species (Fuchs et al. 1995). However recent studies of the chromosome ends in *Aloe* and *Othocallis* have revealed the presence of vertebrata type telomeric repeats (TTAGGG) (Weiss and Schferthan 2002; Weiss-Schneeweiss et al. 2004). In analyzed *Centaurea* karyotypes the *Arabidopsis*-type telomeric probe efficiently hybridized to the ends of all chromosomes (Fig. 1a–c) but the size of the signals varied. This can indicate a different number of repeats at the telomere of each arm, but the differences were too faint to be used as a landmark for chromosome identification. Similar variation was observed in *Lupinus angustifolius* chromosomes (Hajdera et al. 2003).

rRNA genes distribution and activity

The fact that *Centaurea* species have small and morphological undifferentiated chromosomes makes a karyotype morphology analyses very difficult. As indicated for other genera, the application of various staining techniques and FISH are usually very useful in cytogenetic analysis and allows an exact determination of homologous chromosomes (Hasterok et al. 2001). FISH with 5S and 25S rDNA probes allowed the number and location of 45S and 5S rRNA gene loci to be determined in three *Centaurea* species. Double FISH with rDNA probes to metaphase chromosomes showed that these two types of rRNA genes were located in different chromosomes. Among the analyzed *C. jacea* populations, the number and location of 5S

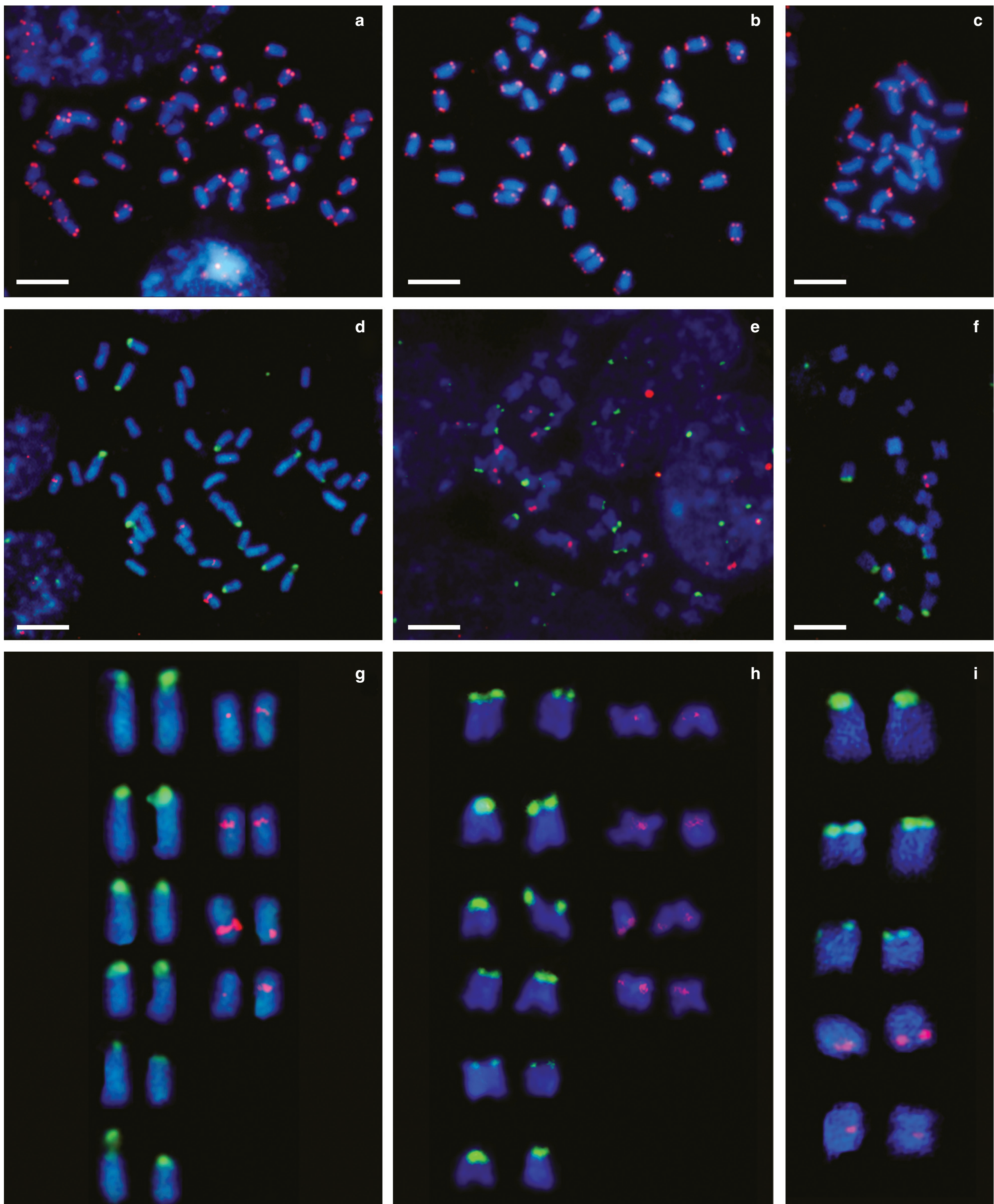

Fig. 1a–i. Somatic metaphase chromosomes of (**a**, **d**, **g**) *C. jacea* (population 5); (**b**, **e**, **h**) *C. oxylepis* and (**c**, **f**, **i**) *C. phrygia*; (**a**–**c**) localization of plant telomeric $(TTTAGGG)_n$ repeats by fluorescent hybridization; (**d**–**f**) localization of 45S rRNA gene loci (green) and 5S rDNA loci (red) by FISH; (**g**–**h**) partial karyotype showing 45S rDNA and 5S rDNA carrying chromosomes. Bar = 10 µm.

rDNA loci were constant. All of them showed four pairs of 5S rDNA loci, one located in the terminal and three in the interstitial part of the chromosome arms (Table 3, Fig. 1d, 1g). Polymorphism occurs in a number 45S rDNA loci. Most of the studied populations showed six pairs of 45S rDNA loci but five pairs were also observed in some populations (Table 3, Fig. 1d, 1g). All of 45S rDNA loci found in the *C. jacea* genome are located terminally in chromosomes. In the genotypes with six pairs of 45S rDNA loci, five were bigger and one was smaller. In genotypes with five pairs of loci, all of them were of similar size. As indicated by silver staining, most often in genotypes with six pairs of 45S rDNA loci, four pairs were transcriptionally active, with the exception of one population that had five pairs of NOR chromosomes (Table 3, Fig. 2a). Among the populations with five pairs of 45S rDNA loci most often all of them were transcriptionally active, and only in one case four pairs of NOR chromosomes were present (Table 3).

The second analyzed tetraploid, *C. oxylepis*, exhibited the same number and location of rDNA sites as the most often observed *C. jacea* genotype (Table 3, Fig. 1e, 1h). After FISH, 5S rDNA hybridization signals were observed in four pairs of chromosomes. Three of these were located proximally and one terminally in the chromosomes. Genes of 45S rRNA were located in the terminal part of six pairs of chromosomes. The loci differ in size – five pairs were major and one pair was minor. After silver staining ten chromosomes with positive bands were observed, which indicated that in the *C. oxylepis* genome five pairs of 45S rDNA loci were transcriptionally active and one pair was inactive (Table 3, Fig. 2b).

The FISH with rDNA to the diploid *C. phrygia* chromosomes tagged 8 of 22 chromosomes (Table 3, Fig. 1f, 1i). Two pairs of chromosomes carried the 5S rRNA gene locus. In one of them the 5S rDNA locus was located terminally and in the second it was located intercalary near the centromere. A hybridization signal of 45S rDNA was observed in the terminal part of three chromosome pairs and they were detected with different intensities, suggesting a different copy number of basic repeats. Three pairs of 45S rDNA loci were major and one was minor. After silver staining four chromosomes exhibited positive AgNOR bands indicating that only two pairs of 45S rRNA gene loci were transcriptionally active. (Fig. 2c).

To the best of our knowledge, the position of ribosomal genes has not yet been documented for any *Centaurea* species. Our study showed that the rDNA sequence might be a very useful tool for *Centaurea* karyotype characterization. It delivered markers for nine or ten chromosome pairs in the tetraploid and five in the diploid karyotype. A comparative study of chromosomal rRNA genes distribution suggested a similarity between genomes of *C. jacea* and *C. oxylepis*. The number of rDNA loci, as well as their locations was very similar in both analyzed tetraploid species. The chromosomal organization of rDNA sequences was also similar in the diploid *C. phrygia*. This genome exhibited half of the loci number observed in tetraploids although some variations in the localization of 5S rDNA loci were revealed.

A comparison of the results obtained with FISH and silver staining showed that not all 45S rRNA genes loci were usually transcriptionally active (Table 3, Fig. 2).

Table 3. *Number of chromosome, genome size (2C DNA, 1Cx-values) and CMA+ bands, total number of 5S and 45S rDNA sites and number of transcriptional active 45S loci (AgNOR) in genomes of Centaurea species.*

No.	Taxon	2n	2C-value (pg)	1Cx-value (pg)	Number of sites			
					CMA_3	5S rDNA	45S rDNA	AgNOR
1	*C. phrygia*	22	2.14±0.05	1.07	6	4	6	4
2	*C. phrygia*	22	x	x	6	4	6	4
3	*C. oxylepis*	44	x	x	10	8	12	10
4	*C. oxylepis*	44	3.99±0.04	1.00	10	8	12	10
5	*C. jacea*	44	3.82±0.13*	0.96	10	8	12	8
6	*C. jacea*	44	4.01±0.13	1.00	10	8	10	8
7	*C. jacea*	44	3.96±0.17	0.99	10	8	12	10
8	*C. jacea*	44	4.03±0.22	1.01	10	8	12	8
9	*C. jacea*	44	4.01±0.16	1.00	10	8	10	10
10	*C. jacea*	44	x	x	10	8	12	8
11	*C. jacea*	44	3.98±0.10	1.00	10	8	10	10
12	*C. jacea*	44	x	x	8	8	12	8

*Significance at $P < 0.05$.

Transcriptionally inactive rDNA loci have been reported for many species for example *Lupinus consentinii* (Hajdera et al. 2003) where only loci linked to secondary construction were transcriptionally active whereas all others did not express possibly due to DNA methylation or another expression-regulating mechanism.

Additionally, FISH revealed an intraspecies variation in the rDNA sequences chromosomal organization among *C. jacea* populations. Variation in chromosome patterns of rDNA loci is not uncommon and has been reported in many other plant species (Williams et al. 2001; Hasterok et al. 2006; Pedrosa-Harand et al. 2006). The rRNA gene copy number is known to evolve quickly and it is possible that some sites were lost or the number of repeats decreased below the limit of detection with FISH. Different mechanisms have been proposed to explain this phenomenon,

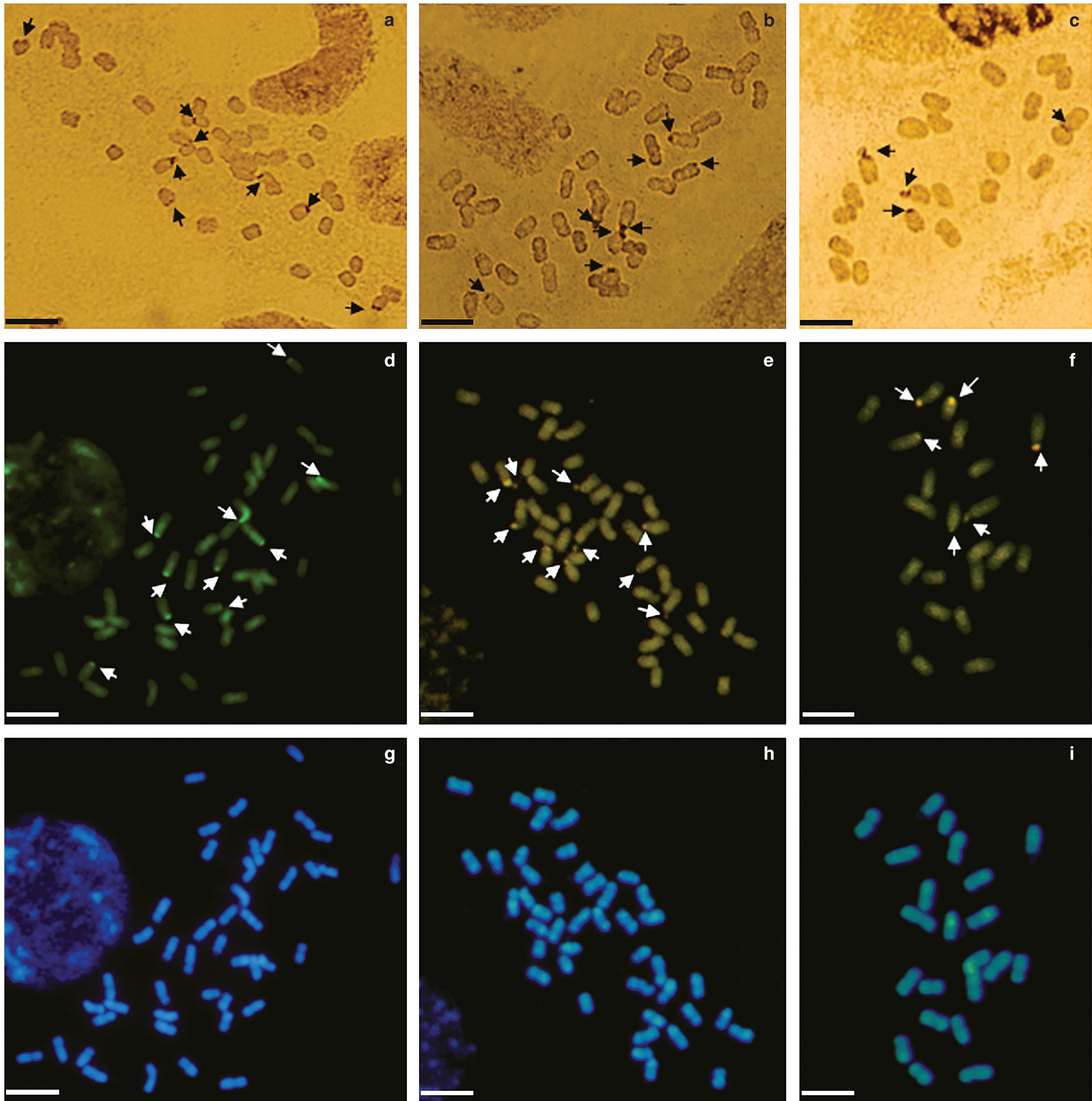

Fig. 2a–i. Somatic metaphase chromosomes of (**a**, **d**, **g**) *C. jacea* (population 5), (**b**, **e**, **h**) *C. oxylepis* and (**c**, **f**, **i**) *C. phrygia*; (**a**, **b**, **c**) detection of active 45S rRNA genes by silver staining, arrows showed AgNOR; (**d**, **e**, **f**) differential fluorescent staining with chromomycin A3, CMA^+ bands (arrows) indicate large GC rich regions of chromatin; (**g**, **h**, **i**) DAPI staining. Bar = 10 µm.

such as chromosome rearrangement, unequal crossing-over, gene conversion or a transpositional event (THOMAS et al. 2001, RASKINA et al. 2004). According to HARDY et al. (2000), tetraploid *C. jacea* probably is an autopolyploid which suggests that the original number of 45S rDNA loci was six pairs. It is probable that the deletion of one pair of minor 45S rDNA loci has occurred in some populations of *C. jacea*. Rearrangements of repetitive sequences during the evolution of polyploid genomes was described earlier for many species and is thought to be a part of diploidization process (MISHIMA et al. 2002). However, for a better understanding of the evolution of rDNA loci in polyploidy *C. jacea* it is necessary to check the organization of the sequence in diploid genotypes.

Differential fluorescence staining DAPI/CMA_3 allowed for the localization of the region rich in AT/GC (SCHWEIZER 1976). In all three species it was impossible to locate positive DAPI bands, however negative DAPI bands co-localized with CMA^+ bands (Fig. 2 d–i). Diploid *C. phrygia* showed six CMA^+ regions which differed in fluorescent intensity. One pair of chromosomes carried brighter bands than two others (Table 3, Fig. 2d). Tetraploid *C. oxylepis* exhibited ten chromosomes with CMA^+ bands (Table 3, Fig. 2e). Similarly in most of the *C. jacea* population analyzed, ten CMA^+ bands (eight bright and two week) were visible, however, one population exhibited fewer – eight bright CMA^+ bands (Table 3, Fig. 2f). In all the analyzed species CMA^+ bands were always located terminally and some of them were associated with secondary construction, which closely corresponds to transcriptionally active 45S rRNA genes. The 45S rDNA sequence in plant is CG-rich (TAKAIWA et al. 1984; KISS et al. 1988) and the presence of CG-rich DNA at NOR's has already been reported for numerous plant species, for example *Helianthus annuus* L. or *Lycopersicon esculentum* Mill. (CUELLAR et al. 1996; XU and EARLE 1996). In species with small chromosomes the secondary construction is more difficult to visualize especially if it is very small or terminally located in a chromosome (GUERRA 2000). This phenomenon could explain the smaller number of chromosomes with secondary construction revealed by CMA_3 than the number of AgNOR positive bands in *Centaurea* species.

Genome size

The genome size of the *Centaurea* species was estimated using flow cytometry and internal standards. The C-value for *C. phrygia* and *C. oxylepis* was estimated for first time as 2.14 pg/2C and 3.99 pg/2C respectively (Table 3). The genome size of *C. jacea* was measured in individuals belonging to six populations collected in different region of Poland. The mean 2C value obtained for *C. jacea* was 3.995 pg and this result is in agreement with data obtained earlier by BANCHEVA and GREILHUBER (2006). Flow cytometry indicated that the populations differ in genome size and that the variation in C-value among populations was 1.055-fold (Table 3). Among the analyzed populations one represent lowlands (5) and five represents highlands and mountains (6, 7, 8, 9, 11). The highland and mountain populations varied in genome size (from 3.96 to 4.03 pg/2C) but the genome size variation among the populations was not statistically significant. However, a significant difference was observed between these populations (6, 7, 8, 9, 11) and the lowland one (5), which had the lowest 2C value (3.82 pg) (Table 3). The intraspecies variations in genome size found in *C. jacea* are probably related to the specific microclimate conditions in each habitat. Intraspecies variations in genome size are not unique as they have been reported earlier for other *Centaurea* species such as *C. nervosa* Willd., *C. glaberrima* Tausch or *C. davidovii* Urum. (SILJAK-YAKOVLEV et al. 2005; BANCHEVA and GREILHUBER 2006). It has also been reported for other wild taxa such as *Arabidopsis thaliana* (L.) Heynh. (SCHMUTHS et al. 2004). Intraspecific genome size differences were found especially among geographically distant populations or among populations growing in different macroclimatic conditions. Significant relationships for intraspecies genome size variation have been found for example with altitude, latitude and temperature; however, the trends in different taxa may differ (KNIGHT et al. 2005). Intraspecies variations in genome size found between *C. jacea* population together with the polymorphism found in a number of 45S rRNA gene loci, AgNOR and CMA^+ bands support the idea that *C. jacea* is a very variable species that comprises different cytotypes.

For taxonomical considerations the Cx-value (monoploid genome size) is more relevant than the C-value because with the Cx-value the multiplying effect of polyploidy on DNA content is compensated for (GREILHUBER et al. 2005). We found a very narrow Cx variation among the analyzed species (Table 3). The Cx-value found for *C. oxylepis* was within the range represented by the *C. jacea* population. The Cx-value of the diploid *C. phrygia* was only slightly higher than that for tetraploids. A similar Cx-value for species belonging to the subgenus *Jacea* was reported by BANCHEVA and GREILHUBERT (2006) suggesting a low level of interspecies variation in the Cx-value in this subgenus.

The similar genome size of *C. jacea* and *C. oxylepis* reinforced the data obtained by karyotype analysis, which showed an overall similarity in their chromosome morphology as well as in the number of rDNA loci, AgNOR and CMA^+ bands. This might indicate a close relationship between these two species. This is in agreement with an earlier study, which reported that these two species cross-hybridize relatively easily. The hybrid plants are known as *C. x fleischeri* Hayek and have been reported from numerous localities in the

Sudetes and western Carpathian Mountains (Hayek 1901; Madalski and Ciaciura 1972).

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