

POLYPLOIDY IN PHENOTYPIC SPACE AND INVASION CONTEXT: A MORPHOMETRIC STUDY OF *CENTAUREA STOEBE* S.L.

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The taxonomy of the *Centaurea stoebe* complex is controversial. Diploid and tetraploid plants occur in its native European range, but to date only tetraploids have been recorded from its introduced range in North America. We examined morphological differentiation of *C. stoebe* using multivariate and univariate approaches to clarify the taxonomic status of the known cytotypes. We measured more than 40 morphological traits on plants originating from 78 populations, grown from seed under uniform glasshouse conditions. The ploidy of almost 300 plants from 2 native and 20 introduced populations from Canada was assessed to test for the absence of diploids from North America. Finally, we explored whether postintroduction processes have resulted in phenotypic changes in introduced plants which may have contributed to the invasion success of *C. stoebe*. Morphometric analyses showed a clear separation of 2x and 4x plants and thus supported recognition of both cytotypes as separate taxa. Differences in the life cycle, the number of florets, the shape of capitula, and the shape of young rosette leaves were the best discriminant characters. Only minor differences were found between native and introduced tetraploids. All plants from the introduced range except for one hexaploid were found to be tetraploid. Rare diploids from Canada were identified as *Centaurea diffusa* or *Centaurea psamogenna*.

Keywords: Asteraceae, biological invasion, flow cytometry, karyology, multivariate morphometrics, polyploidy, spotted knapweed.

Introduction

Polyploidy, a state when an organism has more than two complete sets of chromosomes, is considered a major evolutionary mechanism in flowering plants (Müntzing 1936; Stebbins 1950; De Wet 1971; Grant 1981; Levin 1983; Otto and Whitton 2000). Genome duplication can lead to instantaneous multiple changes in organisms that are manifested at different structural, developmental, and functional levels, from the genes to phenotypic traits interacting directly with the surrounding environment. Polyploid cytotypes are often morphologically different from their diploid ancestors, but it is often difficult to separate the direct effect of polyploidization on morphology from other factors such as hybridization and/or postpolyploidization processes (Levin 1983). Two main groups of polyploids are commonly recognized on the basis of their origin; autopolyploids arise within populations of a single species, and allopolyploids arise from interspecific hybridization (Ramsey and Schemske 1998). With the exception of differences in chromosome behavior in meiosis, segregation ratios, and fertility, autopolyploids are usually morphologically more

similar to their diploid progenitors than allopolyploids (Grant 1981; Soltis et al. 2007). Morphological differences between cytotypes have traditionally formed the primary basis for appropriate taxonomic characterization. However, additional characteristics such as ecological and/or distributional shifts, genetic differentiation, and the presence and strength of reproductive barriers have also been used as supporting criteria (Soltis et al. 2007). Given the frequent coexistence of different cytotypes in nature, it is surprising that there are relatively few studies using rigorous multivariate techniques that examine morphological variation within diploid-polyploid complexes (Lihová et al. 2004; Koutecký 2007; Mandáková and Münzbergová 2008). A detailed knowledge of the morphological variation within and between polyploid taxa and their diploid ancestors is important for taxonomy, but it is also critical for identification of characters and life-history traits that could potentially be involved in adaptive evolution.

Centaurea L. is a species-rich and taxonomically intricate genus with a high proportion of polyploids. The genus is distributed across large parts of Eurasia and northern Africa, and phenotypic and cytotype variation contribute significantly to its taxonomic complexity (Hellwig 2004). The European and Mediterranean regions encompass more than 700 species and subspecies of *Centaurea* s.str. (Greuter 2006–2009). In spite of the recent progress in understanding intrageneric variation of

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Centaurea (Wagenitz and Hellwig 1996; Garcia-Jacas et al. 2006; Font et al. 2009), the low-level taxonomy of the genus remains largely unresolved. This taxonomic uncertainty is also present for the diploid-polyploid complex of *Centaurea stoebe* L. (spotted knapweed). Several intraspecific taxa within *C. stoebe* have been described from different parts of the distributional range (Španiel et al. 2008 and references therein) as well as some closely related species (*Centaurea reichenbachii* DC., *Centaurea trinifolia* Heuff.; Ochsmann 2000). Using the most recent taxonomic revision (Ochsmann 2000), *C. stoebe* includes three subspecies that differ in their morphology, ploidy level, and life cycle. The nominate subspecies *stoebe* (hereafter, *C. stoebe* s.str.) is diploid ($2n=2x=18$) and predominantly monocarpic, whereas the subspecies *micranthos* (Gugler) Hayek ($2n=4x=36$, hereafter, *C. stoebe* s.l.) is tetraploid and polycarpic. The third subspecies, subsp. *serbica* (Prodan) Ochsmann, is not well known; it is a diploid taxon distributed in the Balkan Peninsula with uncertain life cycle (Ochsmann 2000). Both subspecies *stoebe* and *micranthos* are distributed across Europe and the western part of Asia, with the tetraploid *micranthos* being more frequent in southern latitudes and almost absent in Western Europe (see Ochsmann 2000; Španiel et al. 2008; Treier et al. 2009). All three subspecies are reported to differ in overlapping morphological traits such as color, the width of involucre, pappus length, and the number of lateral fimbriae on involucre bracts (Ochsmann 2000). Recently Španiel et al. (2008) studied the morphological differentiation of diploids and tetraploids and their distribution in central Europe, using multivariate morphometrics and flow cytometry. In contrast to Ochsmann (2000), they proposed a single species concept with no recognition of intraspecific units because they observed a lack of morphological discrimination, a largely sympatric distribution of the two common cytotypes, and the presence of mixed-ploidy populations (Španiel et al. 2008).

Centaurea stoebe s.l. was introduced into North America more than 120 years ago as an alfalfa contaminant. It has subsequently become a highly successful invasive plant, especially in western North America (Sheley et al. 1998). To date only the tetraploid cytotype has been confirmed in the introduced range, even though the diploid cytotype dominates in Europe and there is an overlapping distribution of two cytotypes in the native range (table 1). Such a pronounced cytotype shift may be the result of stochastic founder event(s) or may be the result of possible postintroduction selection that favored the tetraploid cytotype (Treier et al. 2009). Niche modeling indicated a higher level of niche differentiation between tetraploids from the native and introduced range than between native diploids and tetraploids (Broennimann et al. 2007; Treier et al. 2009). However, native tetraploids still showed a small but significant shift in climatic niche toward a drier climate when compared to native diploids (Treier et al. 2009). Thus, a preadaptation of tetraploids to a drier and warmer climate in North America could represent a possible advantage over diploids if both cytotypes have been introduced.

Although several studies have compared life-history traits of *C. stoebe* cytotypes from widely distributed populations from both the native and introduced range (Müller 1989; Broz et al. 2009; Henery et al. 2010), none of them have considered morphological characters in detail. In this article we present a multivariate and univariate morphometric com-

Table 1

Published Chromosome Counts/DNA-Ploidy Level Estimations of *Centaurea stoebe* from Its Introduced Range in North America

Country (state/province)	N	Source
$2n=2x=18$: ^a		
CAN (BC)	1	Treier et al. 2009
$2n=4x=36$:		
CAN (BC), USA (WA)	2	Moore and Frankton 1954
CAN (BC), USA (MT)	2	Powell et al. 1974
CAN (BC)	2	Taylor and Taylor 1977
USA (AZ)	1	Morefield and Schaack 1985
USA (VA)	2 ^b	Hill 1995
USA (MT)	1	Ochsmann 1999
CAN (BC, OT), USA (AZ, CA, CO, CT, ID, MD, MN, MT, NV, NY, VA, VT, WI, WY)	48	Treier et al. 2009
CAN (BC)	20	Mráz et al., this article
$2n=6x=54$:		
CAN (BC)	1 ^c	Mráz et al., this article

Note. CAN = Canada, USA = United States; state/province names are shown as abbreviations. N = number of sites where *C. stoebe* was analyzed.

^a Diploid ploidy level estimation refers with high probability to either *Centaurea diffusa* or to *Centaurea psamogemma* (*C. stoebe* × *C. diffusa*); see “Discussion.”

^b Hill (1995) did not publish exact locality(ies), but only accession(s) originated from Virginia; therefore, this record is not mapped (fig. 1).

^c Site with 2 analyzed plants of which 1 was hexaploid and 1 was tetraploid.

parison between cytotypes that addresses the following questions: (i) Do diploids and tetraploids differ morphologically, and what are the taxonomic consequences? (ii) If they differ, which are the best morphological characters for their discrimination? (iii) Are there morphological differences between tetraploids from the native and introduced range? We hypothesize a more pronounced differentiation between cytotypes than between native and invasive tetraploids, as polyploidization is expected to have a stronger impact on morphology than founder events and other processes promoting evolutionary changes following a relatively recent introduction (Schlaepfer et al. 2010). However, even small phenotypic differences between invasive and native tetraploids are of interest, as these characters might be important for the successful invasion in North America. Finally, we assess the occurrence of a rare diploid cytotype that was recently found in the introduced range of British Columbia, Canada (Treier et al. 2009), by analyzing samples from an additional 20 populations from this province.

Material and Methods

Material and Morphological Measurements

We used pot-grown plants originating primarily from seeds collected during a 2005 field survey across both the native

Table 2

Details of Sampled *Centaurea stoebe* and *Centaurea vallesiaca* Populations with Number of Plants Used for Morphometric and/or Ploidy Analyses

Population code	Country	Locality	Coordinates	Collector	N_{tot}	N_{leaf}	FCM
Native range, diploid (2xEU)					202	352	0
A2	AT	Niederösterreich, Neckenmarkt	47.595°N, 16.515°E	Tr, No	12	16	...
A3	AT	Niederösterreich, Hainburg	48.153°N, 16.955°E	Br, Th	9	16	...
SAF-2x	AT	Niederösterreich, Marchegg	48.273°N, 16.890°E	Tr, Br	3	6	...
CH1b	CH	Basel, Basel	47.552°N, 7.642°E	Br, Th	3	16	...
SW4 ^a	CH	Wallis, Ausserberg	46.312°N, 7.845°E	Th	10	16	...
D1	DE	Bayern, Simbach am Inn	47.657°N, 7.545°E	Tr, No	3	15	...
DE10	DE	Sachsen-Anhalt, Meissen	51.195°N, 13.431°E	Br, Th	8	11	...
DE11	DE	Bayern, Kallmünz	49.171°N, 11.966°E	Br, Th	8	15	...
DE2	DE	Baden-Württemberg, Istein	47.662°N, 7.530°E	Br, Th	14	16	...
DE6	DE	Sachsen-Anhalt, Halle	51.509°N, 11.956°E	Br, Th	11	16	...
DE8	DE	Brandenburg, Ziesar	52.268°N, 12.299°E	Br, Th	6	15	...
H1	HU	Somogy, Visz	46.722°N, 17.769°E	Tr, No	13	16	...
H3	HU	Veszprém, Tapolca	46.914°N, 17.335°E	Tr, Br	6	13	...
H5	HU	Bács Kiskun, Batmonostor	46.109°N, 18.925°E	Tr, Br	7	14	...
H6	HU	Bács Kiskun, Kiskunfélegyháza	46.706°N, 19.896°E	Tr, Br	6	8	...
SHG	HU	Pest, Isaszeg	47.528°N, 19.384°E	Tr, Br	10	13	...
SRUD	RU	Moscow, Schurovo	55.052°N, 38.822°E	Sh	3	11	...
SRUG	RU	Samara, Perevoloki	53.253°N, 49.188°E	Sh	6	9	...
SUAG	UA	Zhytomyr	50.275°N, 28.911°E	Tr, Br	2	12	...
SUAH	UA	Poltava, Khorol	49.671°N, 33.701°E	Tr, Br	8	15	...
SUAI	UA	Poltava, Chutove	49.668°N, 34.948°E	Tr, Br	8	11	...
SUAJ	UA	Krivohrad, Novoarkhanhel'sk	48.646°N, 30.776°E	Tr, Br	4	13	...
UA1	UA	L'viv, Zolochiv	49.798°N, 24.711°E	Tr, Br	10	15	...
UA2	UA	L'viv, Olesko	49.930°N, 24.836°E	Tr, Br	9	15	...
UA3-2x	UA	Ivanofrankivsk, Czortova	49.400°N, 24.664°E	Tr, Br	12	13	...
UA5	UA	Khmelmitsky, Starokostyantyniv	49.772°N, 27.291°E	Tr, Br	11	16	...
Native range, tetraploid (4xEU)					188	254	30
A1	AU	Niederösterreich, Dürnstein	48.393°N, 15.532°E	Tr, No	5	10	...
BIE	CH	Vaud, Bière	46.526°N, 6.33°E	Bow	10	15	15
CH1	CH	Aarau, Gontenschwil-Zetwill	47.552°N, 7.642°E	Tr, No	14	16	...
DE3	DE	Bayern, Nürnberg	49.417°N, 11.086°E	Br, Th	8	14	...
DE4	DE	Bayern, Steinbach	49.994°N, 10.631°E	Br, Th	13	16	...
DE5	DE	Bayern, Coburg	50.298°N, 10.658°E	Br, Th	11	14	...
H2	HU	Veszprém, Devecser	47.117°N, 17.443°E	Tr, Br	13	16	...
H4	HU	Somogy, Barcs	45.965°N, 17.500°E	Tr, Br	6	11	...
SHE	HU	Somogy, Böhömye	46.402°N, 17.473°E	Tr, Br	16	16	...
SHF	HU	Baranya, Pécs	46.098°N, 18.220°E	Tr, Br	11	15	...
RO9	RO	Miercurea Ciuc	46.357°N, 25.797°E	Hä	8	11	15
PH2	RO	Sucaeva, Radaseni	47.475°N, 26.268°E	Hä	9	15	...
PH3	RO	Neamt, Moldova	47.233°N, 26.516°E	Hä	15	16	...
PH4	RO	Alba, Buru	46.509°N, 23.604°E	Hä	14	16	...
SAF-4x	AT	Niederösterreich, Marchegg	48.273°N, 16.890°E	Tr, Br	6	9	...
SUAA	UA	Transkarpatia, Vynohradiv	48.138°N, 23.077°E	Tr, Br	8	14	...
SUAD	UA	Chernivtsy, Laovanka	48.250°N, 25.896°E	Tr, Br	7	13	...
UA3-4x	UA	Ivanofrankivsk, Czortova	49.400°N, 24.664°E	Tr, Br	1	1	...
UA4	UA	Khmelmitsky, Khotyn	48.516°N, 26.466°E	Tr, Br	13	16	...
Introduced range, tetraploid (4xNA)					276	421	263
CW	CA	British Columbia, Clearwater	51.640°N, -120.077°W	Bo	16	20	19
Nakusp	CA	British Columbia, Nakusp	50.223°N, -117.787°W	Bo	0	0	16
LL	CA	British Columbia, Pemberton	50.133°N, -122.515°W	Bo	16	18	12
202	CA	British Columbia, Burton Lake	49.307°N, -115.155°W	Bo	3	3	3
61PT	CA	British Columbia, Chasm	51.247°N, -121.488°W	Bo	0	0	16
MS	CA	British Columbia, Courtenay	49.640°N, -125.002°W	Bo	10	19	19
HR	CA	British Columbia, Courtenay	49.799°N, -125.063°W	Bo	0	0	9
RavenPit	CA	British Columbia, Courtenay	49.696°N, -125.093°W	Bo	0	0	16
440	CA	British Columbia, Hope	49.377°N, -121.347°W	Bo	13	20	17
CS001	CA	British Columbia, Kamloops	50.661°N, -120.409°W	Bo	14	17	16
CS002	CA	British Columbia, Merritt	50.079°N, -120.650°W	Bo	0	0	16
CS003	CA	British Columbia, Nicola	50.169°N, -120.549°W	Bo	15	19	18

Table 2

(Continued)

Population code	Country	Locality	Coordinates	Collector(s)	N_{tot}	N_{leaf}	FCM
CS005	CA	British Columbia, Nicola	50.318°N, -120.375°W	Bo	0	0	16
ROSN	CA	British Columbia, Rosebery	50.045°N, -117.430°W	Bo	9	16	10
147	CA	British Columbia, Rosebud Lake	49.040°N, -117.273°W	Bo	0	1	1
CS004	CA	British Columbia, Savona	50.705°N, -120.880°W	Bo	0	0	16
153	CA	British Columbia, Wyndel	49.186°N, -116.567°W	Bo	6	8	8
380	CA	British Columbia, Yale	49.667°N, -121.403°W	Bo	0	0	16
411	CA	British Columbia, Yale	49.730°N, -121.367°W	Bo	12	16	17
URS	CA	British Columbia, Elko	49.292°N, -115.121°W	Bo	1	1	2
USCA1	US	California, Long Jam	41.010°N, -121.952°W	Hu	0	4	...
USCO2	US	Colorado, Breen	37.190°N, -108.080°W	Hu	0	2	...
USID2	US	Idaho, Coeur d'Alene	47.670°N, -116.680°W	Hu	0	2	...
USMT9	US	Montana, Simms	47.301°N, -112.126°W	Tr, Br	6	10	...
USMT4	US	Montana, Alder	45.324°N, -112.081°W	Tr, Br	13	16	...
USMT8	US	Montana, Big Timber	46.016°N, -110.088°W	Tr, Br	10	14	...
USMT11	US	Montana, Dixon	47.308°N, -114.300°W	Tr, Br	8	16	...
USMT2	US	Montana, Florence	46.584°N, -114.141°W	Tr, Br	8	16	...
USMT1	US	Montana, Missoula	46.820°N, -114.101°W	Tr, Br	6	14	...
USMT10	US	Montana, Missoula	46.999°N, -113.383°W	Tr, Br	8	14	...
USMT3	US	Montana, Ross Hole	45.835°N, -113.975°W	Tr, Br	9	15	...
USNY1	US	New York, West Point	44.278°N, -73.531°W	Hu	0	1	...
USOR8	US	Oregon, Bend	44.055°N, -121.244°W	Tr, Br	11	14	...
USOR11	US	Oregon, Cougar Reservoir	44.157°N, -122.262°W	Co	10	13	...
USOR3	US	Oregon, Dee Flat	45.590°N, -121.629°W	Tr, Br	10	16	...
USOR2	US	Oregon, Hood River	45.698°N, -121.506°W	Tr, Br	9	14	...
USOR10	US	Oregon, Klamath Falls	42.238°N, -121.796°W	Tr, Br	12	16	...
USOR4	US	Oregon, La Grande	45.323°N, -118.259°W	Tr, Br	13	14	...
USOR6	US	Oregon, Mt. Vermont	44.516°N, -118.990°W	Tr, Br	8	14	...
USOR1	US	Oregon, Portland	45.618°N, -122.770°W	Tr, Br	8	15	...
USVA1	US	Virginia, Middletown	38.900°N, -78.020°W	Hu	0	3	...
USWI1	US	Wisconsin, Necedah	44.020°N, -90.070°W	Hu	12	17	...
USWY2	US	Wyoming, Casper	46.810°N, -90.820°W	Hu	0	3	...

Note. Four diploid plants of *Centaurea diffusa* and one of *Centaurea psamogemma* found in three tetraploid populations from introduced range were not included in the list of plants analyzed for ploidy level. AT = Austria, CA = Canada, CH = Switzerland, DE = Germany, HU = Hungary, RO = Romania, RU = Russia, UA = Ukraine, US = United States; Bo = R. Bouchier, Bow = G. Bowmann, Br = O. Broenniman, Co = E. Coombs, Hä = P. Häfliger, Hu = R. Hufbauer, No = S. Normand, Sh = A. Shipunov, Tr = U. Treier. N_{tot} = number of plants/population with all traits measured and used for multivariate analyses; N_{leaf} = number of plants/population for which leaf traits were measured; FCM = number of plants/population analyzed with flow cytometry in our study.

^a Diploid *Centaurea vallesiaca* population.

and introduced range of *Centaurea stoebe* (for collection methods see Treier et al. 2009). Additional populations from Switzerland, Romania, and Canada were collected in 2006 and 2007 using similar methods. Plant populations included multiple representatives of the two cytotypes classified by geographic origin (hereafter referred to as geocytotypes: European diploids, 2xEU; European tetraploids, 4xEU; and North American tetraploids, 4xNA) and one population of *Centaurea vallesiaca* DC., a diploid species endemic to the Swiss Valais and the Italian Aosta Valley and closely related to the diploid *C. stoebe* s.str. (Ochsmann 2000). Details of sampled populations are given in table 2. At the beginning of October 2007, 5 seeds each, if available, from up to 16 sampled maternal plants per population, were directly sown in 1-L pots filled with sterilized and sieved compost. After germination, seedlings were reduced to 1 plant per pot. Plants were grown in a heated glasshouse, with 16 h artificial light per day and average temperatures of 23°C daytime and 15°C at night. Pots were watered approximately every 3–4 d. For

morphological comparisons, 31 quantitative, 4 binary, and 10 derived ratio characters of leaves, stems, and reproductive organs were measured, scored, or computed (table 3). In addition to the attributes traditionally used for taxonomic or cytotype identification (Ochsmann 2000; Španiel et al. 2008), we used several novel characters that we thought could be potentially important for cytotype-level determination (table 3).

Leaf measurements (table 3) were taken 2 mo after sowing because leaves emerging later were much more extensively dissected and thus could not be accurately scanned. All leaves were assessed over a 10-d period starting December 10, 2007. To standardize measurements between plants, the sixth true leaf (not counting cotyledons) including petiole was cut from an individual rosette. In some rare cases where the sixth true leaf was damaged by fungi or insects, the fifth, seventh, or eighth true leaf was substituted. Immediately after cutting, a binary image (black for leaf and white for background, 300 dpi) of each leaf was taken using a flatbed scanner. The leaves were then dried for a minimum of 48 h at 60°C, and the leaf

dry weight was recorded. Leaf images were analyzed with the software ImageJ (Rasband 1997–2009). The number of plants sampled for each population varied between 1 and 21 individuals because of limited availability of seeds and variable germination. The total sample size was large, consisting of more than 1000 plants originating from 78 populations, including two populations with mixed ploidy (table 2).

Measurements of reproductive plant parts were taken on the start date of flowering, which was defined as the day when three fully open capitula were observed. The number of accessory rosettes of flowering plants was also assessed on this day; however, this trait was checked again in mid-June 2008 because the formation of accessory rosettes continued after flowering. Outer and inner florets including ovaries with developing pappus and involucre bracts from the middle part of inflorescence were dissected and attached to paper using transparent tape. These plant parts were later scanned at high resolution (1200 dpi) and analyzed using ImageJ. Mean data for inflorescence traits (LOF, LIF, LP, LB, WB, WAP, LAP, LDP, LAM, LF, MEANOF, MEANIN, WCAP, LCAP; see table 3) were based on the three (sometimes two) largest capitula per plant. Stem height and the length of branches and peduncles were measured using a standard ruler with 1-mm precision; stem diameter was measured using a manual caliper with 0.1-mm precision. Further details on measured characters are given in table 3. The glasshouse experiment was finished at the end of June 2008. The plants that had flowered were mounted and kept as herbarium specimens. Plants that were still at the rosette stage were transferred from the glasshouse to field plots. When these plants flowered, additional measurements were taken as detailed above. Final measurements were taken at the beginning of September 2008. The total number of plants with data for both stem and reproductive organs was lower than the number of those with data for leaf traits because only a subset of cultivated plants flowered (table 2). In addition, 39 plants were removed from the final data set for the multivariate analyses because their inner and outer florets were damaged by thrips.

Ploidy Level Analysis and Chromosome Number Determination

Ploidy levels of more than 2000 seed families from most of the populations used in this study were taken from Treier et al. (2009). An additional 293 plants from 2 native (Switzerland and Romania) and 20 introduced (Canada) populations collected in 2006 and 2007 (table 2) were analyzed using flow cytometry. Bulk samples from 4–8 greenhouse-grown plants were prepared in a two-step procedure using Partec nuclei isolation and staining buffers following the manufacturer's protocol (for more details, see Treier et al. 2009). If more than one ploidy level was detected in the bulk sample, plants were reanalyzed individually. Flow cytometric analyses were performed with a CyFlow SL flow cytometer (Partec) equipped with a green laser functioning at 532 nm. Propidium iodide was used as a stain. Histograms were accumulated at a flow rate of ~20–50 particles per second for a total count of 1000–3000 nuclei. We used a previously counted diploid plant of *C. stoebe* (2x) as an external stan-

dard. *Glycine max* cv. Polanka was used as internal standard with a known genome size (2C = 2.5 pg of DNA; Doležel et al. 1994) for precise estimation of the genome size of the putative hexaploid plant.

Additional chromosome counts of one putative hexaploid plant were made on the root-tip meristems of pot-grown plants. Root-tip cuttings were pretreated with a 0.5% solution of colchicine for 1.5–3 h at room temperature, fixed in a mixture of ethanol and glacial acetic acid (3 : 1) for at least 1 h, and stored in 70% ethanol at 4°C until used. Hydrolysis was done in 1N HCl at 60°C for 7–10 min followed by the squash-and-smear method (Murin 1960) with cellophane replacing the glass cover. Giemsa solution in phosphate buffer was used as a stain.

Multivariate and Univariate Morphometric and Statistical Analyses

Several of the recorded traits were not included in morphometric analyses because they had extremely low levels of variation (NSTEM, FCOL) or because there were missing values for many tetraploid plants (ASL and ASW were not present in entire leaves). Thus, of the 45 measured characters, 40 were used in the multivariate analyses (table 3). Some characters were closely related (e.g., level of leaf dissection: NSEG, LPER, LSHAP; or polycarpy: ROS, NOROS). The removal of closely related characters (e.g., LSHAP, NSEG, ROS) did not change the pattern revealed by the principal component analyses (PCA; results not shown). Strong correlations might substantially distort the results of discriminant analyses; however, all characters used for multivariate analyses (table 3) had pairwise correlations <0.95 (Pearson or Spearman correlation coefficient), and thus none were omitted.

To visualize the relationships among individual plants and populations, we performed PCAs based on correlation matrices of measured characters standardized to 0 means and unit standard deviations. Populations with fewer than 5 available plants were excluded from the population-level multivariate analyses. Populations were represented by mean values of their characters. In the case of mixed-ploidy populations, both cytotypes were considered as different population units. To show the phenotypic space occupied by the different geocytotypes, we constructed confidence ellipses defined by the gravity center (centroid) of the cloud and 1.5 times the standard deviation.

While PCA extracts most of the overall variation along the first few components, canonical discriminant analysis (CDA) maximizes between-group differences and minimizes within-group differentiation. We used canonical discriminant analysis to reveal the most important characters contributing to the separation of diploid and tetraploid plants and tetraploids from the native and introduced range. Correct assignment of plants to predefined groups (either cytotypes or geocytotypes) was assessed using classificatory discriminant analyses (linear, quadratic, and a nonparametric cross-validation *k*-nearest-neighbor approach).

Using different algorithms (UPGMA, Ward method, complete linkage), we performed cluster analyses based on population means to assess hierarchical clustering at the population level. A Mantel test was used to infer correlation between spatial distribution and phenotypic differentiation of populations

Table 3

List of Characters Measured in *Centaurea stoebe*

Continuous quantitative characters:	
LOF	Length of outer florets (mm) from the base to the apex of the longest tip of floret
LIF	Length of inner florets (mm) from the base to the apex of the corolla tip
LP	Length of pappi (mm) measured on ovaries of inner flowers
LB	Length of middle involucre bracts (mm) from base to the apex
WB	Maximal width of middle involucre bracts (mm)
WAP	Width of appendages of middle involucre bracts (mm) measured at the base of the dark part, excluding lateral fimbriae
LAP	Length of appendages of middle involucre bracts (mm) excluding lateral fimbriae and apical mucro (mm)
LDP	Length of the dark part of appendages of middle involucre bracts (mm) measured from the apex of appendages (excluding apical mucro) to the color transition (from black or brown to the green color of involucre bract)
LAM	Length of apical mucros of appendages of middle involucre bracts (mm)
LF	Length of longest lateral fimbria of appendages of middle involucre bracts (mm)
MEANOF	Mean no. outer florets per capitulum
MEANIF	Mean no. inner florets per capitulum
WCAP	Width of capitulum (mm) measured just before or at the beginning of flowering
LCAP	Length of capitulum (mm) measured just before or at the beginning of flowering
DST	Stem diameter (mm) measured at the base of stem
HST	Stem height (cm) measured from the base to the basal part of the principal capitulum
LACL	Length of stalk of principal capitulum (akladium; cm),
LLBRA	Length of the longest lateral branch (cm) measured from ramification to the base of the principal capitulum of the longest lateral branch
LWEI	Dry weight of the sixth rosette leaf (mg)
LARE	Leaf area of the sixth rosette leaf (mm ²)
LL	Length of the sixth rosette leaf (mm)
LW	Width of the sixth rosette leaf (mm)
ASL ^a	Length of the apical segment of the sixth rosette leaf (mm) in the case of divided or pinatifid leaves
ASW ^a	Width of the apical segment of the sixth rosette leaf (mm) in the case of divided or pinatifid leaves
LPER	Leaf perimeter of the sixth rosette leaf (mm)
Discrete quantitative characters:	
NF	No. lateral fimbriae of appendages of middle involucre bracts measured on one side of appendages and only the highest value of three measured bracts was kept
NCAP	Total no. capitula counted on herbarium specimens, including only buds >3 mm
NSTEM ^a	No. stems per plant
NOROS	No. accessory rosettes
NDFLOW	No. days from sowing to flowering
NSEG	Total number of segments (lobes or leaflets) on the sixth rosette leaf measured on both sides
Binary characters:	
ROS	Accessory rosettes: present (1) or absent (0)
FCOL ^a	Flower color: violet/purple/dark red (0) or white/whitish (1)
FLOW ^a	Flowering in the first year: yes (1) or no (0)
LSHAP	Shape of the sixth rosette leaf: dissected/pinnatifid (1) or entire (0)
Ratio characters	LB/WB, LAP/LDP, LAP/WAP, LAM/LB, LCAP/WCAP, HST/LLBRA, NCAP/HST, LWEI/LARE (=SLA [specific leaf area]), LPER/LL, ASL/ASW ^a , LL/NSEG ^a , LL/LW

^a Characters were not included in multivariate morphometric analyses.

within each geocytotype separately. The robustness of the test was assessed using 9999 permutations.

Univariate statistics of quantitative characters (mean, standard deviation, minimum, maximum, and fifth and ninety-fifth percentiles) were computed for each geocytotype separately (appendix in the online edition of the *International Journal of Plant Sciences*). We assessed differences between cytotypes and tetraploid geocytotypes in selected characters related to putative reproduction success (MEANIN, NCAP), branching form (LLBRA), shape of florets and capitula (LOF, LCAP/WCAP), and leaf biomass accumulation (LWEI, LARE), using linear mixed effect models (LMM). The analyses were performed in two steps: (1) comparison of diploid and tetra-

ploid cytotypes and (2) comparison between tetraploids from the native and introduced range. Dependent variables were transformed as required, to address normality assumptions. Populations nested within cytotype or geocytotype were considered as a random factor. Differences in the probability of forming accessory rosettes (ROS) for each geocytotype were assessed using a generalized linear mixed effect model with a binomial distribution and a logit link function with geocytotype as a main factor and population nested in geocytotype as a random factor. We used nonparametric Wilcoxon rank sum test based on population means for the character NDTFLOW due to a strong violation of the normality assumption when applying LMM. Analyses and plotting were done within the

Results

Cyctotype Distribution

All but one of the flow cytometrically analyzed samples of *Centaurea stoebe* s.l. from the introduced and native ranges were tetraploid ($2n\sim 4x$) (introduced range: 263 plants from 20 Canadian populations; fig. 1, table 2; native range: 30 plants of two populations Romania and Switzerland). One plant (population URS, British Columbia) was hexaploid ($2n\sim 6x$), with 5.27 pg of DNA per genome (measured against *Glycine max* as an internal standard). Hexaploidy was confirmed by chromosome counts ($2n=6x=54$; fig. 2). In addition, 5 diploid plants ($2n\sim 2x$) were found as an admixture in otherwise tetraploid *C. stoebe* s.l. populations from Canada. However, they were identified as either *Centaurea diffusa* (2 plants from population URS, 1 plant from 153, and 1 plant from LL) or *Centaurea psamogemma*, a stable hybridogenous taxon between *C. stoebe* and *C. diffusa* (1 plant found in population 411; see table 2 for population codes).

Morphological Variation and Morphometric Analyses

The PCA analysis based on individual plants (fig. 3A) showed a fairly good separation of diploid and tetraploid plants along the first PCA axis but did not distinguish between the two ranges within the cloud of tetraploid plants. The separation of both cytotypes was also clear at the popu-

lation level (fig. 3B), but again, 4xNA populations did not separate from 4xEU populations. The characters that contributed most to the separation of the two cytotypes for the individual plant PCA were mean number of inner florets (MEANIF), presence/absence of accessory rosettes (ROS), and traits associated with leaf shape and its dissection level (NSEG, LSHAP, LW, LPER, LPER/LL, LL/LW; table 4, best correlations with the first PCA axes). Additional characters that were important for the discrimination at the population level were mean number of outer florets (MEANOF), starting day of flowering (NDTOFLOW), the density of flower heads on the stem (NCAP/HST), and number of accessory rosettes (NOROS; table 4). No obvious grouping of *Centaurea vallesiaca* plants within the diploids was found using a PCA based on individual plant characters (results not shown), although the SW4 population occupied a marginal position within the cloud of 2x populations (fig. 3B). This population had on average slightly larger values for LAP/WAP, LDP, LAM/LB, LF, NCAP/HST, and LPER and smaller values for LAP/LDP and MEANOF compared with other diploid *C. stoebe* populations (data not shown). Average population values for other traits of the SW4 population, including those contributing most to the cyctotype differentiation (see above), were in the range of the values for diploid *C. stoebe* populations.

CDA confirmed the results of the PCA; there was good separation of the two cytotypes with only a weak overlap (fig. 4A) but no separation between tetraploid geocytotypes (fig. 4B). The best characters for cyctotype discrimination were mean number of inner and outer florets (MEANIF, MEANOF), presence/absence and total number of accessory rosettes (ROS, NOROS), starting day of flowering (NDTOFLOW),

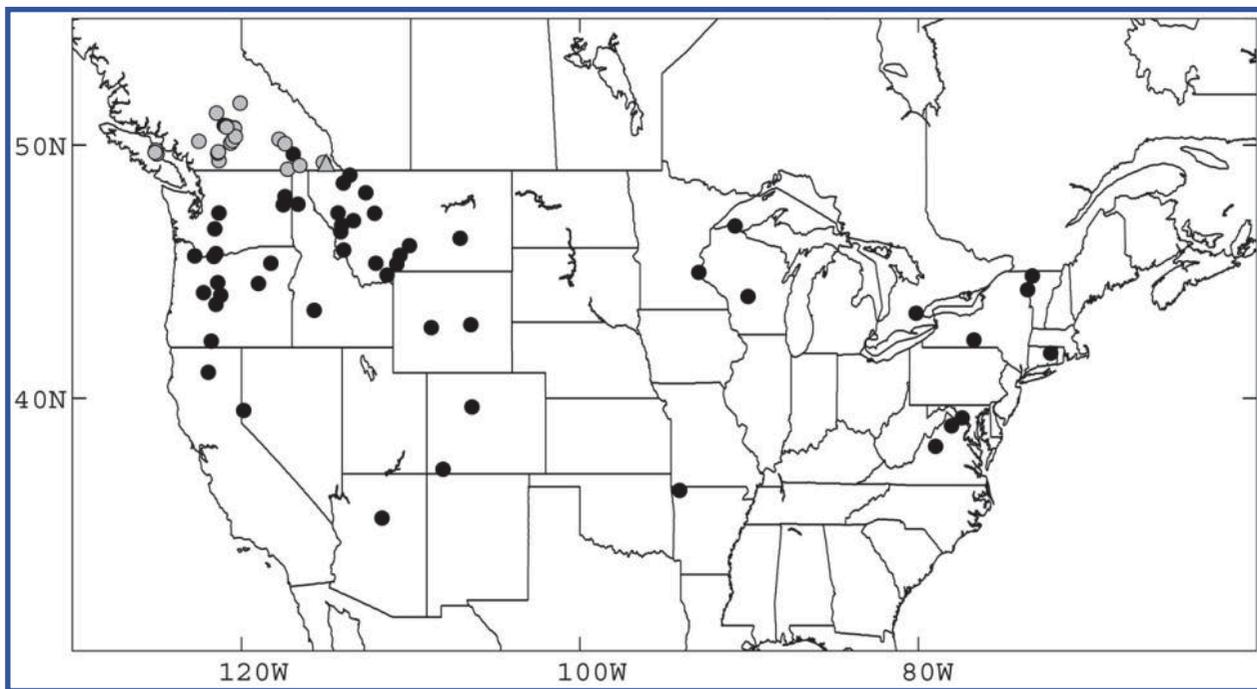


Fig. 1 Locations of tetraploid ($2n=4x=36$) populations of *Centaurea stoebe* s.l. in the introduced range in North America used for flow cytometry and/or karyological analyses. Black circles = literature data (table 1), gray circles = new data (table 2), gray triangle = population with a hexaploid plant ($2n=6x=54$; table 2). The map was created using DMAP software (Morton 2004).

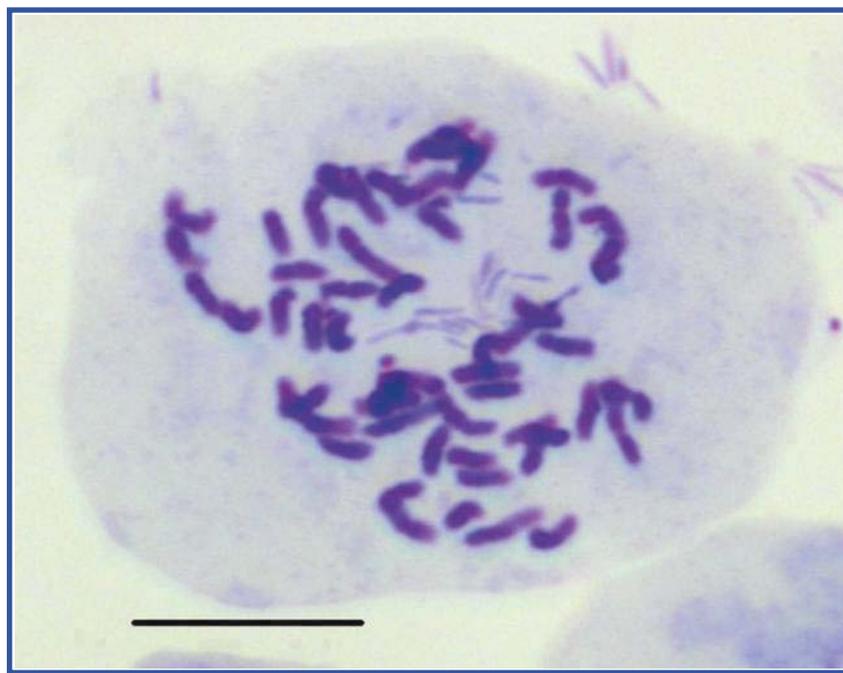


Fig. 2 Mitotic metaphase chromosomes of a hexaploid ($2n=6x=54$) *Centaurea stoebe* s.l. plant (plant 3 of population URS, British Columbia, Canada; 49.292°N, -115.121°W). Scale bar = 10 μ m.

and several leaf traits (NSEG, LSHAP, LW, LPER, LPER/LL, LL/LW; table 4; best correlation with canonical axis). All characters had lower correlations with the canonical axis when the analysis was limited to tetraploid plants and geographic range was used as a discriminant criterion (table 4). The num-

ber of capitula (NCAP) showed the highest correlation (table 4). To validate discrimination between cytotypes and tetraploid geocytotypes, we performed three types of classification analyses that showed essentially the same results. A highly successful assignment to the corresponding ploidy was achieved

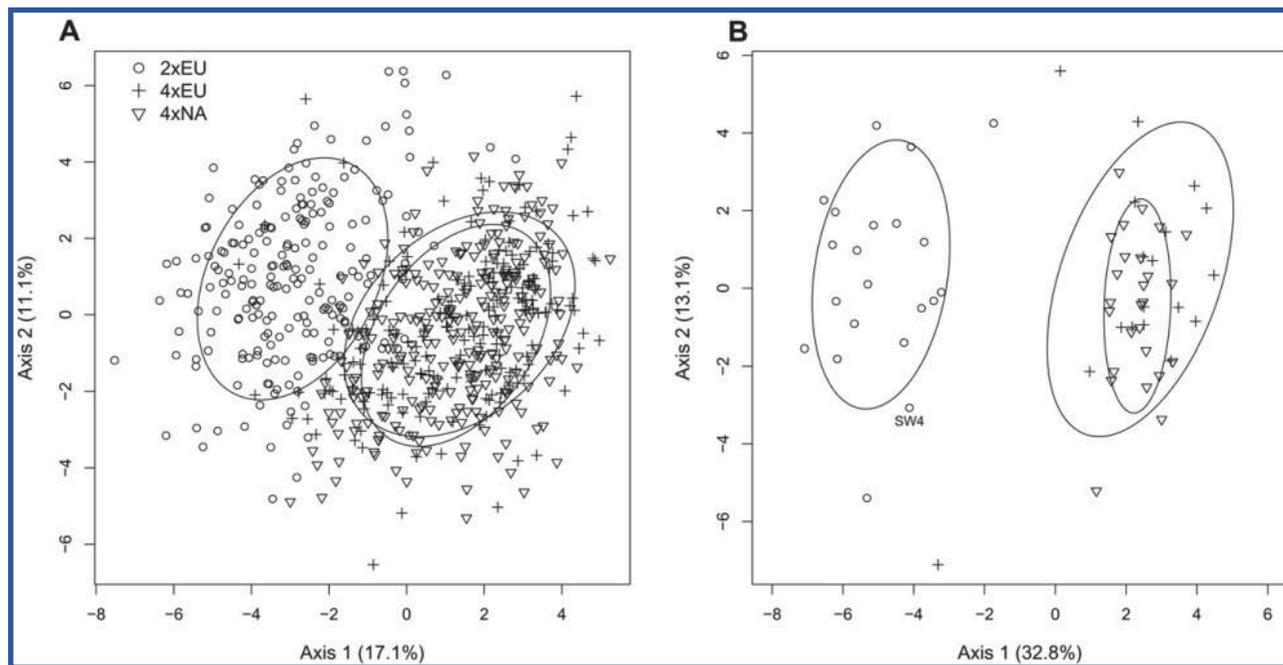


Fig. 3 Principal component analysis plots based on 40 morphological traits given for 678 plants (A) and 65 populations of *Centaurea stoebe* s.l. (B). Symbols represent the three geocytotypes: circles = native European diploids (2xEU), crosses = native European tetraploids (4xEU), triangles = invasive North American tetraploids (4xNA). The diploid *Centaurea vallesiaca* population (SW4) is indicated in B.

Table 4

Eigenvectors Showing Correlations of Characters with the First Two Principal Components for Analysis, PCA A and PCA B, and Correlations of Characters with the Canonical Axis from Canonical Discriminant Analysis, CAN A and CAN B

Character	PCA A		PCA B		CAN A	CAN B
	Axis 1	Axis 2	Axis 1	Axis 2	Can 1	Can 1
LOF	.006	<u>-.284</u>	-.105	-.244	.238	-.063
LIF	-.076	<u>-.254</u>	-.010	-.343	.034	-.113
LP	-.138	<u>-.118</u>	.144	-.212	-.290	-.048
LB	-.013	-.357	-.100	<u>-.286</u>	.150	.198
WB	-.186	-.073	.156	-.170	-.335	.292
LB/WB	.143	<u>-.256</u>	-.198	-.072	.403	-.066
WAP	-.166	<u>.044</u>	.156	-.065	-.295	-.128
LAP	-.060	<u>-.286</u>	-.003	<u>-.290</u>	.058	.200
LAP/WAP	.102	<u>-.279</u>	-.167	<u>-.171</u>	.308	.284
LDP	-.069	<u>-.259</u>	.020	<u>-.256</u>	-.031	.240
LAP/LDP	.020	<u>.020</u>	-.034	<u>.037</u>	.102	-.093
LAM	-.013	-.133	-.051	<u>-.250</u>	.085	.007
LAM/LB	-.013	.021	.006	<u>-.125</u>	.007	-.074
LF	-.070	<u>-.268</u>	.011	<u>-.235</u>	-.058	.262
NF	-.117	<u>-.042</u>	.146	<u>-.127</u>	-.181	-.005
MEANOF	-.184	.077	<u>.215</u>	.040	<u>-.548</u>	.121
MEANIF	<u>-.233</u>	-.007	<u>.242</u>	.045	<u>-.688</u>	-.045
WCAP	<u>-.199</u>	-.079	.190	-.035	-.390	.244
LCAP	-.059	-.191	.024	<u>-.293</u>	-.024	-.021
LCAP/WCAP	.135	-.048	-.157	<u>-.135</u>	.311	-.190
DST	-.022	-.128	.003	.107	-.098	.079
HST	.079	-.189	-.150	.102	.227	-.037
NCAP	-.086	-.008	.143	.145	-.374	-.381
LACL	-.044	-.043	.013	-.001	-.065	-.019
LLBRA	.079	<u>-.220</u>	-.172	.029	.340	-.016
HST/LLBRA	-.036	<u>.070</u>	.102	-.088	-.162	-.077
NCAP/HST	-.131	.114	<u>.208</u>	-.009	-.447	-.196
NOROS	.184	-.072	<u>-.232</u>	-.004	<u>.538</u>	-.260
ROS	<u>.210</u>	-.100	<u>-.250</u>	-.027	<u>.654</u>	-.206
NDTOFLOW	-.145	<u>.256</u>	<u>.206</u>	-.002	<u>-.564</u>	-.286
LWEI	-.012	-.151	-.122	-.186	<u>.326</u>	-.049
SLA	-.074	.012	.120	.125	-.239	.147
NSEG	<u>-.321</u>	.006	<u>.250</u>	-.014	<u>-.718</u>	-.042
LSHAP	<u>-.289</u>	-.019	<u>.237</u>	-.030	<u>-.566</u>	.010
LL	-.079	-.114	-.013	-.197	<u>.035</u>	-.294
LW	<u>-.306</u>	-.071	<u>.222</u>	-.149	<u>-.511</u>	-.095
LARE	-.047	-.158	-.098	-.171	<u>.243</u>	.030
LPER	<u>-.296</u>	-.051	<u>.211</u>	-.136	<u>-.531</u>	-.213
LPER/LL	<u>-.315</u>	-.004	<u>.242</u>	-.062	<u>-.674</u>	-.083
LL/LW	<u>.289</u>	.029	<u>-.234</u>	.053	<u>.532</u>	-.200

Note. PCA A based on individual plants, $n = 678$; PCA B based on populations, $N = 66$. Correlations of characters with the canonical axis from the canonical discriminant analysis CAN A (based on individual plants, ploidy level selected as discriminant factor: 2x vs. 4x) and CAN B (based on individual tetraploid plants, geographic origin selected as discriminant factor: native European vs. introduced North American range) of *Centaurea stoebe*. Values above 0.2 for PCA and 0.5 levels for CDA are underlined; for definitions of abbreviations, see table 1.

using (1) parametric linear discriminant functions (95.2% diploids and 98.7% tetraploids), (2) quadratic discriminant functions (92.1% diploids and 95.1% tetraploids), and (3) a nonparametric k -nearest-neighbor cross-validation procedure with training set based on $n - 1$ individuals and with $k = 1-31$ (68.9%–80.9% of diploid and 91.4%–92.5% of tetraploid). The same tests were applied on the tetraploid geocytotypes although with lower success of correct predictions: 58.7% of 4xEU and 78.5% of 4xNA (linear discriminate function), 47.1% of 4xEU and 73.5% of 4xNA (quadratic discriminate function), and 19.8%–45% of 4xEU and 63.9%–90% of 4xNA (k -nearest-neighbor cross-validation with $k = 131$).

Different cluster algorithms grouped populations according to their ploidy level at high levels of similarity (fig. 5; different clustering algorithms gave similar results; thus, only the dendrogram based on UPGMA is shown). At low levels of similarity, however, separation of diploid and tetraploid population clusters was not as clear (fig. 5).

There was a positive relationship between geographic location and Euclidean phenotypic distance based on mean characters' values of diploid European populations (Mantel test, $r = 0.2$, $P = 0.037$). This correlation remained statistically significant when the *C. vallesiaca* population was omitted ($r = 0.19$, $P = 0.0494$). However, after removing the five pop-

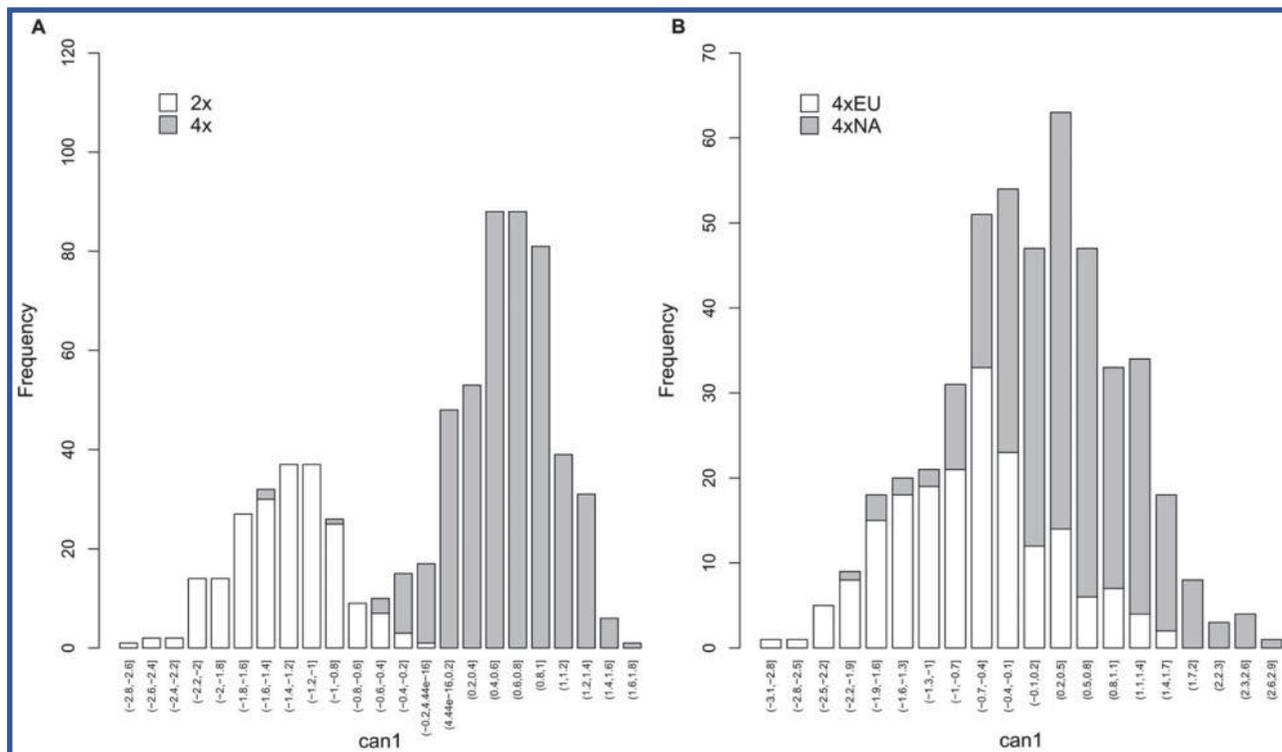


Fig. 4 Frequency histogram of linear discriminant analyses of diploid (2x) and tetraploid (4x) plants of *Centaurea stoebe* s.l. (A) and native European tetraploids (4xEU) and invasive North American tetraploids (4xNA; B).

ulations that had fewer than 5 available plants, the correlation was still positive but not significant ($r = 0.15$, $P = 0.1$). In tetraploid populations (including also those with fewer than 5 individuals), no correlation between morphological differentiation and geographical distance was found for either the native ($r = -0.08$, $P = 0.85$) or the introduced ($r = -0.02$, $P = 0.45$) range.

The proportion of tetraploid plants that flowered in the glasshouse was higher than that of the diploid plants (tetraploid: $n = 683$, 86% flowered, 87% 4xEU, 85% 4xNA; diploid: $n = 352$, 75% flowered). In the first year of cultivation tetraploid populations started flowering earlier than diploids (Wilcoxon nonparametric test based on populations means, $W = 1162$, $P < 0.001$). North American tetraploid plants flowered earlier than European tetraploids, although the difference was smaller than for the cytotype difference ($W = 359$, $P = 0.044$). Fewer than 3% of diploid plants formed accessory rosettes after flowering, indicating a fairly strict monocarpic life cycle for this cytotype. In contrast, the presence of accessory rosettes was much more frequent in tetraploid plants (65% on average, 74% for 4xEU, and 59% for 4xNA), indicating a predominantly polycarpic life cycle (fig. 6). Almost all of the diploids had their sixth rosette leaf dissected (97%), whereas less than half of the tetraploids showed this characteristic (42%; see appendix). All seven characters that were compared for diploid and tetraploid plants were significantly different (table 5; fig. 7). Specifically, diploid plants had a higher number of inner florets (MEANIN; fig. 7A) and capitula (NCAP; fig. 7B) but significantly fewer elongated capitula (LCAP/WCAP), shorter outer florets and

branches (LOF, LLBRA), and smaller and lighter leaves than tetraploids (LARE, LWEL; see table 5 and appendix). In addition to these statistical differences, there were also subtle but consistent differences in coloration between the cytotypes, observed by P. Mráz. Tetraploids frequently had darker (dark green with shades of violet) involucre bracts than did diploids (bright green). The same trend was observed in the color of florets, being darker in tetraploids than in diploids. Moreover, diploid plants more frequently had paler inner flowers (white or pinkish to violet) whereas tetraploids usually had darker inner flowers (dark violet). European tetraploids had significantly more capitula (NCAP) than North American tetraploids (table 5; fig. 7).

Discussion

Cytogeographic Pattern in Introduced Range

Our data confirm a considerable shift in ploidy level distribution of *Centaurea stoebe* between the native and introduced range (Španiel et al. 2008; Treier et al. 2009). All *C. stoebe* plants from North America analyzed in this study, except for one originating from British Columbia, were found to be tetraploid. Based on our analyses, published counts, and flow cytometric estimations, ploidy level has been determined for almost 1000 plants sampled from 77 populations across the entire introduced range in North America (table 1; fig. 1). In the introduced range, tetraploid *C. stoebe* s.l. sometimes co-occurs with two other highly invasive, but diploid,

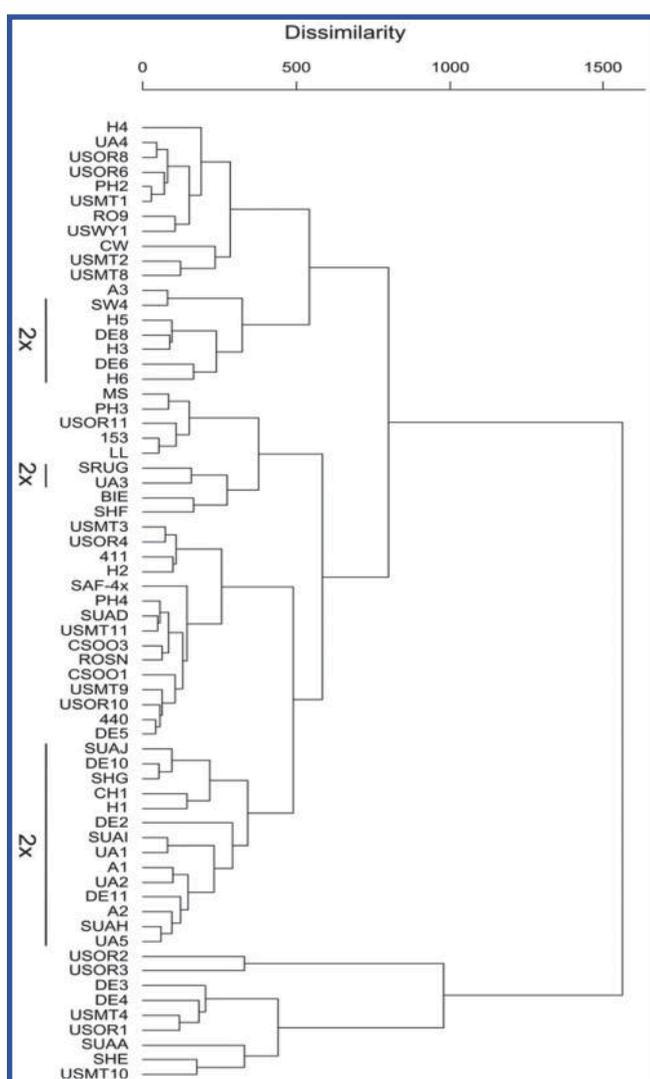


Fig. 5 Cluster analysis of 21 diploid (underlined) and 44 tetraploid populations of *Centaurea stoebe* s.l. using UPGMA method. For population codes see table 2.

European taxa, *Centaurea diffusa* and *Centaurea psamogenna* (Forcella and Harvey 1980). Our sample also contained five diploid plants from British Columbia that have been identified as *C. diffusa* or *C. psamogenna* (see “Results”). Treier et al. (2009) reported two *C. stoebe* diploids from one mixed-ploidy population in British Columbia; however, these two diploid plants were analyzed at an early rosette stage (U. A. Treier, personal communication). It is possible that these plants were misidentified because it is difficult to consistently distinguish diploid *C. stoebe* s.str. from *C. diffusa* or *C. psamogenna* at the rosette stage. This would explain this rare record of diploid *C. stoebe* from North America. Although we cannot completely exclude either historical or recent occurrence(s) of diploid *C. stoebe* s.str. in the introduced range, to date we have no convincing evidence for the presence of the diploid cytotype in North America.

Cytotype depletion in the introduced range has been reported for other invasive polyploid taxa such as *Lythrum salicaria* (Kubátová et al. 2008), *Senecio inaequidens* (Lafuma et al. 2003), and *Solidago gigantea* (Schlaepfer et al. 2008). Based on allopatric distribution and the frequency of particular cytotypes in the native range, an introduction of solely one polyploid cytotype has been suggested for two of these species (Lafuma et al. 2003; Kubátová et al. 2008). Even though diploid and tetraploid populations of *C. stoebe* s.l., are largely sympatric in their native range (Treier et al. 2009), several authors (Hufbauer and Sforza 2008; Mars et al. 2008) have suggested that the possible source area for North American populations of *C. stoebe* s.l. is the southeastern part of the native European range (the Balkans, Ukraine, and southeastern Russia). This is an area where tetraploid populations are more common than diploids (Treier et al. 2009; P. Mráz, unpublished data). Thus, even with a general situation of sympatric occurrence of diploids and tetraploids in Europe, a higher frequency of the tetraploid cytotype from the proposed source area could have increased the probability of its introduction into the new range, when compared to the diploid cytotype.

The single hexaploid plant that was found in population URS from British Columbia is the first record of hexaploidy within the complex of *C. stoebe* (Ochsmann 2000). While tetraploidy is a very common phenomenon in the genus *Centaurea*, hexaploidy is extremely rare (Phitos and Constantidi-

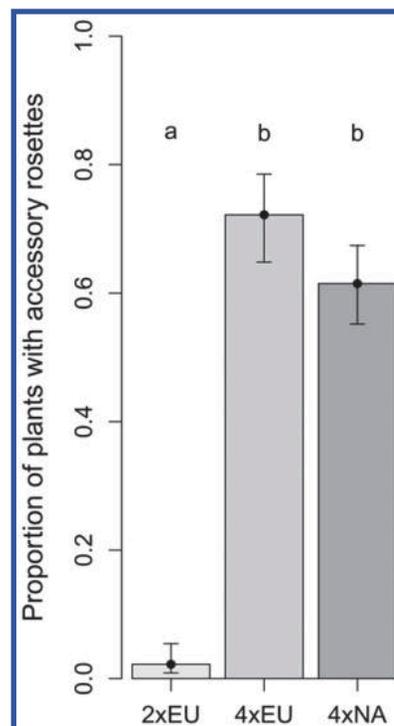


Fig. 6 Estimated mean proportions (with confidence intervals) of plants forming accessory rosettes for the three geocytotypes of *Centaurea stoebe* s.l.; European diploids (2xEU; excluding *Centaurea valesiaca*), European tetraploids (4xEU), and North American tetraploids (4xNA). Different letters above bar plots indicate significant difference between geocytotypes at $P < 0.05$.

Table 5

ANOVA Table of Linear Mixed Effect Models (LMM) for Seven Selected Variables of *Centaurea stoebe*

Traits/comparisons	2x vs. 4x	4xEU vs. 4xNA
Length of outer florets (LOF)	$F_{1, 70} = 21.3^{***}$	$F_{1, 45} = .19$
Mean no. inner florets (MEANIF)	$F_{1, 70} = 193.98^{***}$	$F_{1, 45} = .38$
Length/width of capitula (LCAP/WCAP)	$F_{1, 70} = 24.8^{***}$	$F_{1, 45} = 2.77$
No. capitula (NCAP)	$F_{1, 70} = 33.01^{***}$	$F_{1, 45} = 7.65^{**}$
Length of longest branch (LLBRA)	$F_{1, 70} = 33.47^{***}$	$F_{1, 45} = .06$
Leaf weight (LWEI)	$F_{1, 71} = 32.07^{***}$	$F_{1, 46} = .01$
Leaf area (LARE)	$F_{1, 71} = 19.52^{***}$	$F_{1, 46} = .03$

Note. Analyses were performed separately for diploid (2x; excluding *Centaurea valesiaca*) and tetraploid (4x) plants, and for European (4xEU) and North American tetraploids (4xNA). Populations nested within ploidy or geocytotype were included in the models as a random factor.

** $P < 0.01$.

*** $P < 0.001$.

nis 1993; Trigas et al. 2008; Garcia-Jacas et al. 2009). As the hexaploid plant was morphologically and genetically (nrDNA sequences and SSRs; P. Mráz, N. Garcia-Jacas, E. Gex-Fabry, A. Susanna, and H. Müller-Schärer, unpublished manuscript) indistinguishable from tetraploids of *C. stoebe* s.l., we suggest that it originated via fusion of reduced and unreduced gametes (2x + 4x) produced by tetraploid plants. This pathway is considered to be the most common in polyploid evolution of vascular plants (Ramsey and Schemske 1998).

Morphological Differentiation between the Diploid and Tetraploid Cytotype

Our multivariate morphometric data revealed strong morphological differentiation between the diploid and tetraploid cytotype of *C. stoebe*. This pattern was most clear at the population level (fig. 3B) but also was obvious for individual plants (fig. 3A). Španiel et al. (2008), looking at central European populations of *C. stoebe*, also found better separation of populations than individuals, but with a larger overlap of individual diploid and tetraploid plants than observed in our study. The reduced variation in our study may have resulted from our use of plants grown from the seeds under uniform conditions in a glasshouse whereas Španiel et al. (2008) measured traits on field-collected specimens. By growing plants from seed to maturity, we were able to include additional characters in our analysis, such as shape and size of young rosette leaves and onset of flowering in the first year of growth, that may have improved our discrimination between the two cytotypes. However, in spite of the clear separation by PCA, even the best discriminant morphological characters were still partially overlapping (see appendix). The observed large morphological variation within this complex was emphasized in the hierarchical clustering analyses (fig. 5), with the populations of the same ploidy level clustering together only at a high level of similarity. Thus, a combination of several characters rather than a single character is required for accurate determination of *C. stoebe* cytotypes. Similar high levels of phenotypic variation have been reported from other morphometric studies of closely related *Centaurea*

taxa, suggesting their relatively young origin and ongoing differentiation (Hardy et al. 2000; Vanderhoeven et al. 2002; Guarino and Rampone 2006; Koutecký 2007; Olšovská et al. 2009). In addition to postploidy processes, morphological differentiation observed between diploid and tetraploid cytotypes should result principally from either

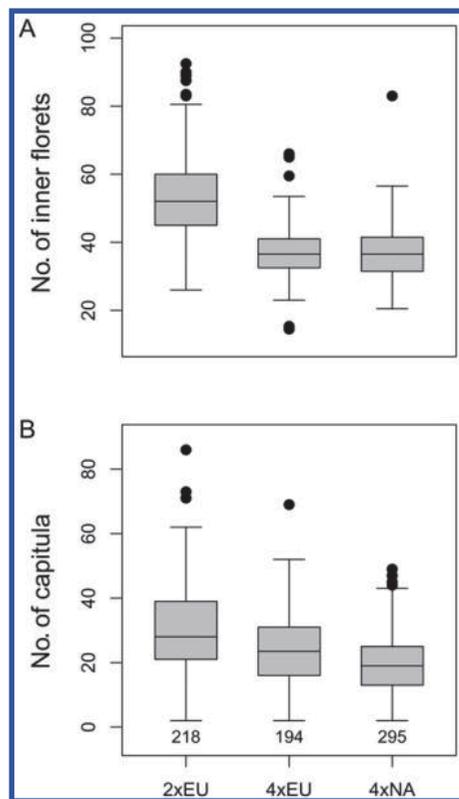


Fig. 7 Box plots of mean number of inner florets (MEANIF; A), number of capitula (NCAP; B) for European diploids (2xEU; excluding *Centaurea valesiaca*), European tetraploids (4xEU), and invasive North American tetraploids (4xNA) of *Centaurea stoebe* s.l. Number of plants per geocytotype are given above the horizontal axis in B.

direct autopolyploidization of the diploid cytotype or from allopolyploidization. Our preliminary results based on cloning and sequencing of two nuclear DNA regions favor the allopolyploid hypothesis (P. Mráz, N. Garcia-Jacas, E. Gex-Fabry, A. Susanna, and H. Müller-Schärer, unpublished manuscript). However, at this stage we cannot verify whether critical morphological changes manifested in tetraploids, such as a polycarpic life cycle, are the result of hybridization, because we have yet to find the second parental taxon (P. Mráz, N. Garcia-Jacas, E. Gex-Fabry, A. Susanna, and H. Müller-Schärer, unpublished manuscript).

In accordance with the literature (Dostál 1976; Boggs and Story 1987; Müller 1989; Ochsmann 2000; Story et al. 2001; Španiel et al. 2008; Treier et al. 2009; Henery et al. 2010) we have confirmed a pronounced shift in life cycle between cytotypes of *C. stoebe*. Most of the tetraploids were found to be polycarpic, as they formed accessory rosettes for bolting in the next season. In contrast, accessory rosettes were observed in fewer than 3% of diploids, indicating the prevalence of monocarpy. This character is thus the most reliable for discriminating between cytotypes and may also explain the invasion success of tetraploids in their introduced range (Müller-Schärer et al. 2004; Treier et al. 2009; Henery et al. 2010). For correct assignment of the cytotypes in the field, the presence/absence of renewing accessory rosettes on flowering plants needs to be assessed in late autumn, as the formation of accessory rosettes is stimulated by shoot withering. Eventually, this trait can be checked in spring of the subsequent year, if the withered shoots from the previous season are still present.

On average, diploid plants started flowering later than tetraploids. This shift, however, was primarily the result of a delayed onset of flowering for about one-quarter of diploid plants. We suspect that the observed late flowering peak in some of the diploid plants may have resulted from a prolonged growing season under unnatural but favorable glasshouse conditions. This may also explain the higher proportion of flowering diploids in the first year of our study, as compared to results from the common garden experiment reported by Henery et al. (2010).

Our data confirmed the previous observation (Ochsmann (2000) that diploid *C. stoebe* s.str. had a broader capitula than the tetraploid subsp. *micranthos*. Flower heads of diploids were generally more rounded (LCAP/WCAP) than those of tetraploids. This is likely associated with the increased number of both inner (MEANIF) and outer florets (MEANOF) in diploid plants (table 5; fig. 7A; Španiel et al. 2008). Diploids also produced more capitula than tetraploids in agreement with the results from common garden experiments (Henery et al. 2010). Considered together, the higher number of capitula and the higher number of inner florets per capitulum suggest a greater investment in seed reproduction in a given year for diploids than for tetraploids. Indeed, Henery et al. (2010) observed that diploids produced significantly more seeds per plant than tetraploids in a common garden experiment. However, with a polycarpic life cycle, the total lifetime reproductive output of tetraploids may be higher than that of diploid plants. Over the long term and during fluctuating conditions, a perennial life cycle might be more advantageous than monocarpy, as it assures greater local persistence through repeated,

albeit lower, annual seed production. Such a trade-off between a higher level of perenniality and lower annual seed production could have favored the establishment and persistence of the tetraploid cytotype in the introduced range, particularly if the risk of postflowering mortality is reduced due to a lack of natural enemies (Klinkhamer et al. 1997).

In contrast to previous data (Ochsmann 2000; Španiel et al. 2008), pappus length (LP) and the number of fimbriae (NF) were not different between cytotypes (results not shown). Homogeneity of pappus lengths among cytotypes may have resulted because plants were not pollinated under glasshouse conditions and we measured this trait on immature ovules. We also found this character to be extremely variable, especially in tetraploid plants, ranging from no pappus to very long ones, even within the same population (P. Mráz, unpublished data). Such phenotypic variation under uniform conditions suggests that this character should not be used to separate small endemic taxa within the *C. stoebe* group, such as *C. triniifolia* Heuff. or *C. reichenbachii* DC. (cf. Ochsmann 2000).

Leaves on young diploid rosettes were more dissected than on tetraploid rosettes (LSHAP, NSEG) because of differential timing of heteroblastic development of leaf shape (cf. Ashby 1948; Lynn and Waldren 2001). While the first 2–3 rosette leaves in diploids were completely entire, successive leaves (usually starting from the fourth leaf) became increasingly dissected until the mature stage of the plant (approximately the tenth to the eleventh leaf stage). This sequence was also observed in tetraploids, but with dissection starting usually only from the sixth to eighth leaf. These results indicate a genetic basis for faster leaf development in diploids than in tetraploids. Although we did not include mature leaves in our measurements because of the difficulties associated with accurate scanning, we observed that the segments/lobes of highly dissected mature leaves of diploids were usually narrower than those in tetraploids.

Diploid populations showed a positive correlation between geographic and phenotypic distances suggesting an isolation-by-distance pattern for population differentiation, possibly due to local adaptation. In contrast, no such correlations were found for tetraploid populations from either Europe or North America. The lack of a correlation within the introduced range can be explained using multiple stochastic introductions, mixing of plants from different sources, and a relatively short time since introductions (Hufbauer and Sforza 2008; Mars et al. 2008). An explanation for the lack of a correlation in native European tetraploid populations is not as clear. Floristic data indicate a recent and massive spread of tetraploids in Europe facilitated by increasing human disturbance (Ochsmann 2000; Korneck 2004; Wels et al. 2008; our unpublished observations). Such recent range expansion may have limited morphological and genetic differentiation and thus population structure of native tetraploids.

Morphological Differentiation of Tetraploids from the Native and Introduced Range

As hypothesized, there was very little differentiation in the morphology of tetraploids from the native and intro-

duced ranges. The time since the first introduction(s) into North America, ~120 years ago, has probably been too short for the accumulation of any significant phenotypic changes that would result in pronounced morphological differentiation between native and introduced tetraploid populations. However, we observed small but significant differences in some single traits between the two groups of tetraploids. Similar to results from a common garden experiment (Henery et al. 2010; see also Ridenour et al. 2008), introduced tetraploids produced a lower number of capitula than native tetraploids. North American tetraploid populations flowered earlier than European tetraploid populations. Early flowering may result from a greater accumulation of biomass during early growth in North American tetraploids (Henery et al. 2010). Cross-continental comparisons of native and introduced populations of many plant species have demonstrated shifts towards higher biomass production in introduced populations (Bossdorf et al. 2005). In contrast to Treier et al. (2009) and Henery et al. (2010), when we measured the proportion of plants forming accessory rosettes, we did not find a higher level of polycarpy in North American tetraploids when compared to the native European tetraploids.

Taxonomic Consequences and Nomenclatural Notes

The results of our morphometric study support separation of the two cytotypes into different taxonomic entities as proposed by Ochsmann (2000), and they challenge the single taxon concept adopted by some authors (Štěpánek and Koutecký 2005; Španiel et al. 2008). Španiel et al. (2008) argued that the combination of (1) the weak morphological differentiation at the individual level and (2) the existence of mixed-ploidy populations and the largely sympatric distribution of the cytotypes in Europe favor recognition of only one taxon, without further taxonomic treatment of cytotypes (either as species or subspecies). First, our data demonstrate a clear morphological discrimination of the two cytotypes. This may have resulted from using plants grown under a uniform environment rather than studying field and herbarium samples (Španiel et al. 2008). Secondly, a distinct distributional pattern is often considered as an additional argument for taxonomic separation when evaluating diploid-polyploid complexes (Marhold 1999; Soltis et al. 2007). Allopatric distribution providing a prezygotic reproductive barrier between cytotypes might result from either different evolutionary histories (stochastic range fragmentation, colonization, or extinctions) or from different ecological requirements of cytotypes, or from both processes. However, polyploid speciation regardless if auto- or allopolyploid is principally a sympatric process (Schemske 2000). Thus, some level of distributional overlap between parental diploid cytotype(s) and polyploid progeny should be expected at least during the first stages after polyploid formation. Furthermore, if the diploid and polyploid cytotypes are well reproductively isolated from each other by barriers other than geography, coexistence within the same range might be expected. Recent meta-analyses of diploid-polyploid congeners at a continental scale showed that there is no evidence for consistent range shifts following genome

duplication (Martin and Husband 2009), and thus, the distribution of closely related diploid and polyploid taxa can overlap.

Ochsmann (2000) suggested that the tetraploid cytotype arose in southeastern Europe and later colonized the current range. Recent observations have supported his hypothesis. There is increasing evidence that the largely sympatric distribution of both cytotypes in Europe is at least partially the result of a relatively recent spread of tetraploids, preferentially colonizing man-made habitats (railways, quarries, roadsides), as observed in the Czech Republic (P. Koutecký, personal communication), Germany (Ochsmann 2000; Korneck 2004; Welss et al. 2008); Switzerland (Ochsmann 2000); France and Slovakia (P. Mráz, personal observations). Such a pattern indicates that the tetraploid cytotype might not be a native floristic element for those countries (see also Ochsmann 2000; Greuter 2006–2009). Our recent ecological and genetic data from mixed-ploidy populations in central Europe (P. Mráz, unpublished data) indicate later arrival of tetraploids to the sites with established diploid populations resulting in secondary contact. This is the most common pattern observed in mixed-ploidy populations (Petit et al. 1999). In combination, these data suggest a recent increase in the level of range overlap between the two *C. stoebe* cytotypes compared to the past.

Once successfully established, polyploids are usually reproductively isolated from their diploid progenitor because of a ploidy barrier that causes seed abortion due to a so-called triploid block or because intercytotype hybrids that may arise are sterile (Marks 1966; Vinkenoog et al. 2003). Thus, effective gene-flow between cytotypes becomes substantially reduced and the cytotypes can diverge even under sympatric situations. We have recently observed a very strong reproductive barrier between the cytotypes of *C. stoebe*, using experimental crosses (P. Mráz and G. Bowman, unpublished data). As reproductive isolation is a prerequisite of speciation (Rieseberg and Willis 2007), it is considered an important criterion for taxon delimitation (Soltis et al. 2007). Thus, the existence of strong reproductive isolation of *C. stoebe* s.l. adds additional support for the recognition of the diploid and tetraploid cytotypes as separate taxa.

Based on these combined sources of evidence we propose to treat the two cytotypes as different species. While *C. stoebe* L. seems to be the appropriate name for the diploid cytotype (Greuter 2003; Španiel et al. 2008), the appropriate nomenclature for the tetraploid cytotype is not clear. Greuter (2003) argued that when treating the cytotypes as different subspecies, the name “*C. stoebe* subsp. *australis* (Pančić ex A. Kern.) Greuter” should be applied for the tetraploid cytotype as this name has priority over *C. stoebe* subsp. *micranthos*. However, as discussed by Španiel et al. (2008), one of two Hungarian populations of *Centaurea australis* mentioned in Kerner’s protologue and for which relevant syntype material exists was diploid (Španiel et al. 2008), and the ploidy level of the second population to date has not been checked. Thus, the interpretation of the name *C. australis* (or *C. stoebe* subsp. *australis*) depends on the choice of the syntype material. If the name is based on the diploid syntype, then it should not be used for the tetraploid cytotype. Alternatively, if the name *C. australis* is typified using the syntype from the

second locality and this turns out to be a tetraploid cytotype, this name can be used for the tetraploid taxon (either as species or subspecies). Because the name *Centaurea biebersteinii* DC. was validly published in 1838 (de Candolle 1838) and has been frequently applied to the tetraploid *C. stoebe* s.l. cytotype (see Greuter 2003, 2006–2009; and many papers focused on spotted knapweed in North America), it could have a priority over any use of *C. australis* that was described later (Kerner 1872). However, we believe that the name *C. biebersteinii* refers to diploid plants because original specimens in the herbarium of Genève (G) have very rounded capitula typical for diploid plants (inspection of the first author), and de Candolle noted that the plant is annual (=monocarpic; de Candolle 1838). This suggests that the name *C. biebersteinii* DC. is inappropriate for the tetraploid cytotype at any rank. Ongoing studies of both field and herbarium material hope-

fully will resolve the nomenclature of the tetraploid cytotype of *C. stoebe* s.l.

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Appendix

Discrimination Key for Diploid and Tetraploid *Centaurea stoebe* in the Field

Based on the results of this study, we propose the following key to discriminate between the diploid and tetraploid cytotypes of *C. stoebe* (values of number of inner florets are expressed as [minimum–]fifth percentile to ninety-fifth percentile[–maximum]):

A. Plants annual or biannual without formation of overwintering accessory rosettes after withering of the shoot(s) (monocarpic life cycle), usually one- to few-stemmed, number of inner florets per capitulum (26–)35 to 76(–93), capitula before anthesis more rounded (length/width ratio 1.2 on average), color of involucral bracts green to bright green: *C. stoebe* s.str. (diploid cytotype).

B. Plants short-lived perennial, forming overwintering accessory rosettes after withering of the shoot(s) (polycarpic life cycle), usually few- to many-stemmed, number of inner florets per capitulum (15–)25 to 50(–83), capitula before anthesis more elongated (length/width ratio 1.35 on average), color of involucral bracts dark green often with shades of violet: *C. stoebe* s.l. (tetraploid cytotype).

Literature Cited

- Ashby E 1948 Studies in the morphogenesis of leaves. I. An essay on leaf shape. *New Phytol* 47:153–176.
- Boggs KW, JM Story 1987 The population age structure of spotted knapweed (*Centaurea maculosa*) in Montana. *Weed Sci* 35:194–198.
- Bossdorf O, H Auge, L Lafuma, WE Rogers, E Siemann, D Prati 2005 Phenotypic and genetic differentiation between native and introduced plant populations. *Oecologia* 144:1–11.
- Broennimann O, UA Treier, H Müller-Schärer H, W Thuiller, AT Peterson, A Guisan 2007 Evidence of climatic niche shift during biological invasion. *Ecol Lett* 10:701–709.
- Broz AK, DK Manter, G Bowman, H Müller-Schärer, JM Vivanco 2009 Plant origin and ploidy influence gene expression and life cycle characteristics in an invasive weed. *BMC Plant Biology* 9:33.
- de Candolle AP 1838 *Prodromus systematis naturalis regni vegetabilis*. Vol 6. Treuttel & Würtz, Paris.
- De Wet MJM 1971 Polyploidy and evolution in plants. *Taxon* 20: 29–35.
- Doležel J, M Doleželová, FJ Novák 1994 Flow cytometric estimation of nuclear DNA amount in diploid bananas (*Musa acuminata* and *M. balbisiana*). *Biol Plant* 36:351–357.
- Dostál J 1976 *Centaurea* L. Pages 254–301 in TG Tutin, VH Heywood, NA Burges, DM Moore, DH Valentine, SM Walters, DA Webb, eds. *Flora Europaea*. Vol 4. Cambridge University Press, London.
- Font M, N Garcia-Jacas, R Vilatersana, C Roquet, A Susanna 2009 Evolution and biogeography of *Centaurea* section *Acrocentron* inferred from nuclear and plastid DNA sequence analyses. *Ann Bot* 103: 985–997.
- Forcella F, SJ Harvey 1980 New and exotic weeds of Montana. II. Migration and distribution of 100 alien weeds in northwestern USA, 1881–1980. Montana State University, Bozeman.
- Garcia-Jacas N, PS Soltis, M Font, DE Soltis, R Vilatersana, A Susanna 2009 The polyploidy series of *Centaurea toletana*: glacial migrations and introgression revealed by nrDNA and cpDNA sequence analyses. *Mol Phylogenet Evol* 52:377–394.
- Garcia-Jacas N, T Uysal, K Romashchenko, VN Suárez-Santiago, K Ertuğrul, A Susanna 2006 *Centaurea* revisited: a molecular survey of the *Jacea* group. *Ann Bot* 98:741–753.
- Grant V 1981 *Plant speciation*. Columbia University Press, New York.
- Greuter W 2003 The Euro+Med treatment of Compositae (Compositae): generic concepts and required new names. *Willdenowia* 33: 49–61.
- 2006–2009 Compositae (pro parte majore). In W Greuter, E von Raab-Straub, eds. *Compositae*. Euro+Med Plantbase: the information resource for Euro-Mediterranean plant diversity. <http://ww2.bgbm.org/EuroPlusMed/> (accessed May 5, 2010).
- Guarino C, S Rampone 2006 A morphometric analysis of *Centaurea* sect. *Dissectae* (Compositae). *Bocconea* 19:77–88.
- Hardy OJ, S Vanderhoeven, M de Loose, P Meerts 2000 Ecological,

- morphological and allozymic differentiation between diploid and tetraploid knapweeds (*Centaurea jacea*) from a contact zone in the Belgian Ardennes. *New Phytol* 146:281–290.
- Hellwig FH 2004 Centaureinae (Asteraceae) in the Mediterranean: history of ecogeographical radiation. *Plant Syst Evol* 246:137–162.
- Henery ML, G Bowman, P Mráz, UA Treier, E Gex-Fabry, U Schaffner, H Müller-Schärer 2010 Evidence for a combination of pre-adapted traits and rapid adaptive change in the invasive plant *Centaurea stoebe*. *J Ecol* 98:800–813.
- Hill LM 1995 IOPB chromosome data 9. *IOPB Newsletter* 24: 19–20.
- Hufbauer RA, R Sforza 2008 Multiple introductions of two invasive *Centaurea* taxa inferred from cpDNA haplotypes. *Divers Distrib* 14:252–261.
- Kerner AJ 1872 Die Vegetations: Verhältnisse des mittleren und östlichen Ungarns und angrenzenden Siebenbürgens. *Oesterr Bot Z* 22:117–121.
- Klinkhamer PGL, T Kubo, Y Iwasa 1997 Herbivores and the evolution of the semelparous perennial life-history of plants. *J Evol Biol* 10:529–550.
- Korneck D 2004 *Centaurea stoebe* subsp. *micranthos*, Kleinköpfige Flockenblume, eine verkannte Sippe unserer Flora (vorläufige Mitteilung). *Hess Florist Briefe* 51:1–5.
- Koutecký P 2007 Morphological and ploidy level variation of *Centaurea phrygia* agg. (Asteraceae) in the Czech Republic, Slovakia and Ukraine. *Folia Geobot* 42:77–102.
- Kubátová B, P Trávníček, D Bastlová, V Čurn, V Jarolímová, J Suda 2008 DNA ploidy-level variation in native and invasive populations of *Lythrum salicaria* at a large geographical scale. *J Biogeogr* 35:167–176.
- Lafuma L, K Balkwill, E Imbert, R Verlaque, S Maurice 2003 Ploidy level and origin of the European invasive weed *Senecio inaequidens* (Asteraceae). *Plant Syst Evol* 243:59–72.
- Levin DA 1983 Polyploidy and novelty in flowering plants. *Am Nat* 122:1–25.
- Lihová J, K Marhold, A Tribsch, TF Stuessy 2004 Morphometric and AFLP re-evaluation of tetraploid *Cardamine amara* (Brassicaceae) in the Mediterranean. *Syst Bot* 29:134–146.
- Lynn DE, S Waldren 2001 Morphological variation in populations of *Ranunculus repens* from the temporary limestone lakes (turloughs) in the west of Ireland. *Ann Bot* 87:9–17.
- Mandáková T, Z Münzbergová 2008 Morphometric and genetic differentiation of diploid and hexaploid populations of *Aster amellus* agg. in a contact zone. *Plant Syst Evol* 274:155–170.
- Marhold K 1999 Taxonomic evaluation of the tetraploid populations of *Cardamine amara* (Brassicaceae) from the eastern Alps and adjacent areas. *Bot Helv* 109:67–84.
- Marks GE 1966 The origin and significance of intraspecific polyploidy: experimental evidence from *Solanum chacoense*. *Evolution* 20:552–557.
- Mars RA, R Sforza, RA Hufbauer 2008 Evidence for multiple introductions of *Centaurea stoebe micranthos* (spotted knapweed, Asteraceae) to North America. *Mol Ecol* 17:4197–4208.
- Martin SL, BC Husband 2009 Influence of phylogeny and ploidy on species ranges of North American angiosperms. *J Ecol* 97:913–922.
- Moore RJ, C Frankton 1954 Cytotaxonomy of three species of *Centaurea* adventive in Canada. *Can J Bot* 32:182–186.
- Morefield JD, CG Schaack 1985 Chromosome number reports 86. *Taxon* 34:159–164.
- Morton A 2004 DMAP: distribution mapping software. <http://www.dmap.co.uk>.
- Müller H 1989 Growth pattern of diploid and tetraploid spotted knapweed, *Centaurea maculosa* Lam. (Compositae), and effects of the root-mining moth *Agapeta zoegana* (L.) (Lep.: Cochyliidae). *Weed Res* 29:103–111.
- Müller-Schärer H, U Schaffner, T Steinger 2004 Evolution in invasive plants: implications for biological control. *Trends Ecol Evol* 19: 417–422.
- Müntzing A 1936 The evolutionary significance of autopolyploidy. *Hereditas* 21:263–378.
- Murín A 1960 Substitution of cellophane for glass covers to facilitate preparation of permanent squashes and smears. *Stain Technol* 35: 351–353.
- Ochsmann J 1999 Chromosomenzahlen einiger europäischer *Centaurea*-Sippen (Asteraceae). *Haussknechtia* 7:59–65.
- 2000 Morphologische und molekularsystematische Untersuchungen an der *Centaurea stoebe* L.-Gruppe (Asteraceae-Cardueae) in Europa. *Diss Bot* 324:1–242.
- Olšavská K, M Perný, P Mártonfi, I Hodálová 2009 *Cyanus triumfettii* subsp. *triumfettii* (Compositae) does not occur in the western Carpathians and adjacent part of Pannonia: karyological and morphological evidence. *Nord J Bot* 27:21–36.
- Otto SP, J Whitton 2000 Polyploid incidence and evolution. *Annu Rev Genet* 34:401–437.
- Petit C, F Bretagnolle, F Felber 1999 Evolutionary consequences of diploid-polyploid hybrid zones in wild species. *Trends Ecol Evol* 14: 306–311.
- Phitos D, Constantinidis T 1993 A new species of *Centaurea* sect. *Phalolepis* from Greece. *Flora Medit* 3:273–275.
- Powell AM, DW Kyhos, PH Raven 1974 Chromosome numbers in Compositae. 10. *Am J Bot* 61:909–913.
- R Development Core Team 2009 R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna.
- Ramsey J, DW Schemske 1998 Pathways, mechanisms, and rates of polyploid formation in flowering plants. *Annu Rev Ecol Syst* 29: 467–501.
- Rasband W 1997–2009 ImageJ. National Institutes of Health, Bethesda, MD. <http://rsb.info.nih.gov/ij/>.
- Ridenour WM, JM Vivanco, YL Feng, J Horieuchi, RM Callaway 2008 No evidence for trade-offs: *Centaurea* plants from America are better competitors and defenders. *Ecol Monogr* 78:369–386.
- Rieseberg LH, JH Willis 2007 Plant speciation. *Science* 317:910–914.
- Schemske DW 2000 Understanding the origin of species. *Evolution* 54:1069–1073.
- Schlaepfer DR, PJ Edwards, R Billeter 2010 Why only tetraploid *Solidago gigantea* (Asteraceae) became invasive: a common garden comparison of ploidy levels. *Oecologia* 163:661–673.
- Schlaepfer DR, PJ Edwards, JC Semple, R Billeter 2008 Cytogeography of *Solidago gigantea* (Asteraceae) and its invasive ploidy level. *J Biogeogr* 35:2119–2127.
- Sheley RL, JS Jacobs, MF Carpinelli 1998 Distribution, biology and management of diffuse knapweed (*Centaurea diffusa*) and spotted knapweed (*Centaurea maculosa*). *Weed Technol* 12:353–362.
- Soltis DE, PS Soltis, DW Schemske, JF Hancock, JN Thompson, BD Husband, WS Judd 2007 Autopolyploidy in angiosperms: have we grossly underestimated the number of species? *Taxon* 56: 13–30.
- Španiel S, K Marhold, I Hodálová, J Lihová 2008 Diploid and tetraploid cytotypes of *Centaurea stoebe* (Asteraceae) in central Europe: morphological differentiation and cytotype distribution patterns. *Folia Geobot* 43:131–158.
- Stebbins GL 1950 Variation and evolution in plants. Columbia University Press, New York.
- Štěpánek J, P Koutecký 2005 *Centaurea* L. Pages 426–449 in B

- Slavík, J Štěpánková, eds. Flora of the Czech Republic. Vol 7. Academia, Praha.
- Story JM, L Smith L, WR Good 2001 Relationship among growth attributes of spotted knapweed (*Centaurea maculosa*) in western Montana. *Weed Technol* 15:750–761.
- Taylor RL, S Taylor 1977 Chromosome numbers of vascular plants of British Columbia. *Seysis* 10:125–138.
- Treier UA, O Broennimann, S Normand, A Guisan, U Schaffner, T Steinger, H Müller-Schärer 2009 Shift in cytotype frequency and niche space in the invasive plant *Centaurea maculosa*. *Ecology* 90: 1366–1377.
- Trigas P, T Constantinidis, T Touloumenidou 2008 A new hexaploid species of *Centaurea* section *Acrolophus* (Asteraceae) from Evvia Island, Greece. *Bot J Linn Soc* 158:762–774.
- Vanderhoeven S, O Hardy, X Vekemans, C Lèfebvre, M de Loose, J Lambinon, P Meerts 2002 A morphometric study of populations of the *Centaurea jacea* complex (Asteraceae) in Belgium. *Plant Biol* 4:403–412.
- Vinkenoog R, C Bushell, M Spielman, S Adams, HG Dickinson, RJ Scott 2003 Genomic imprinting and endosperm development in flowering plants. *Mol Biotechnol* 25:49–184.
- Wagenitz G, F Hellwig 1996 Evolution of characters and phylogeny of Centaureinae. Pages 491–510 in DJN Hind, HJ Beentje, eds. *Compositae-systematics*. Royal Botanical Gardens, Kew.
- Wells W, P Reger, W Nezdal 2008 Zur Verbreitung von *Centaurea stoebe* L. subsp. *stoebe* und *Centaurea stoebe* subsp. *australis* (A. Kern) Greuter (Asteraceae) im Nürnberger Becken. *Regnitz Flora* 2:44–53.