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Anthropogenic disturbance as a driver of microspatial and microhabitat segregation of cytotypes of *Centaurea stoebe* and cytotype interactions in secondary contact zones

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• *Background and Aims* In a mixed-ploidy population, strong frequency-dependent mating will lead to the elimination of the less common cytotype, unless prezygotic barriers enhance assortative mating. However, such barriers favouring cytotype coexistence have only rarely been explored. Here, an assessment is made of the mechanisms involved in formation of mixed-ploidy populations and coexistence of diploid plants and their closely related allotetraploid derivates from the *Centaurea stoebe* complex (Asteraceae).
• *Methods* An investigation was made of microspatial and microhabitat distribution, life-history and fitness traits, flowering phenology, genetic relatedness of cytotypes and intercytotype gene flow (cpDNA and microsatellites) in six mixed-ploidy populations in Central Europe.
• *Key Results* Diploids and tetraploids were genetically differentiated, thus corroborating the secondary origin of contact zones. The cytotypes were spatially segregated at all sites studied, with tetraploids colonizing preferentially drier and open microhabitats created by human-induced disturbances. Conversely, they were rare in more natural microsites and microsites with denser vegetation despite their superior persistence ability (polycarpic life cycle). The seed set of tetraploid plants was strongly influenced by their frequency in mixed-ploidy populations. Triploid hybrids originated from bidirectional hybridizations were extremely rare and almost completely sterile, indicating a strong postzygotic barrier between cytotypes.
• *Conclusions* The findings suggest that tetraploids are later immigrants into already established diploid populations and that anthropogenic activities creating open niches favouring propagule introductions were the major factor shaping the non-random distribution and habitat segregation of cytotypes at fine spatial scale. Establishment and spread of tetraploids was further facilitated by their superior persistence through the perennial life cycle. The results highlight the importance of non-adaptive spatio-temporal processes in explaining microhabitat and microspatial segregation of cytotypes.

Key words: Assortative mating, Asteraceae, *Centaurea stoebe*, cpDNA, cytotype coexistence, disturbance, flow cytometry, microsatellites, polyploidy, reproductive isolation, triploid block.

INTRODUCTION

Mixed-ploidy populations consisting of different cytotypes of the same or closely related species offer a unique opportunity to study the intercytotype interactions and reproductive isolation mechanisms which may affect the establishment and coexistence of a newly emerged polyploid from sympatric diploids in primary contact zones or a new cytotype immigrated to the population of other cytotypes in secondary contact zones (Petit *et al.*, 1999). Regardless of the type of contact zone, the minority cytotype will be subjected to strong frequency-dependent mating with the more frequent cytotype. As a consequence, the rarer cytotype may be progressively eliminated from the mixed-ploidy population, because it will mainly produce aborted seeds or sterile hybrids due to strong postzygotic isolation frequently observed in diploid–polyploid crosses ('minority cytotype exclusion'; Levin, 1975).

Although frequency-dependent mating should lead to single-cytotype populations, recent studies have shown that the sympatric occurrence of cytotypes is more common than previously anticipated (Baack, 2004; Suda *et al.*, 2007; Halverson *et al.*, 2008; Li *et al.*, 2010; Šafářová and Duchoslav, 2010; Šingliarová *et al.*, 2011). The disadvantage of the minority cytotype may be overcome by several mechanisms that increase the probability of assortative mating. Such barriers include: (1) microspatial segregation (Baack, 2004; Kolář *et al.*, 2009; Šafářová and Duchoslav, 2010; Trávníček *et al.*, 2011*a*, *b*), which may be correlated with habitat differentiation (Lumaret *et al.*, 1987; Felber-Girard *et al.*, 1996; Hardy *et al.*, 2000; Hülber *et al.*, 2009); (2) flowering time displacement (Petit *et al.*, 1997; Husband and Sabara, 2004); (iii) shifts in floral phenotype and physiology, which may alter the spectrum of pollinators (Segraves and Thompson, 1999; Kennedy *et al.*, 2006; but see Jersáková *et al.*, 2010 or Castro *et al.*, 2011); (iv) pollen competition (Husband *et al.*,

2002; Peckert and Chrtek, 2006); (5) a shift in breeding system (e.g. switch from allogamy to autogamy, or from sexual reproduction to parthenogenesis; Kao, 2007); or (6) breakdown of a strict self-incompatibility system and subsequent induced autogamy due to a mixture of heteropolyploid pollen ('mentor effect'; Tas and van Dijk, 1999; Mráz, 2003; Brock, 2004; Hörandl and Temsch, 2009). In addition, superior competitive ability (e.g. Buggs and Pannell, 2007; Besnard and Baali-Cherif, 2009) or frequent immigration of the minority cytotype may enhance its establishment (Felber, 1991).

Given the high incidence of polyploidy in vascular plants (Levin, 2002) and despite a growing number of studies on natural mixed-ploidy populations in the last decade, we still know little about the mechanisms underlying the establishment and coexistence of cytotypes in diploid–polyploid contact zones (Soltis *et al.*, 2010). Here we addressed these issues by using the *Centaurea stoebe* complex as a model system.

The Eurasian *Centaurea stoebe* complex is represented by diploid and tetraploid cytotypes occurring in predominantly single-cytotype populations (Treier *et al.*, 2009; P. Mráz *et al.*, unpubl. res.). Interestingly, several mixed-ploidy populations have been recorded in Central Europe and were interpreted as a putative primary contact zone with recurrent formation of tetraploids (Španiel *et al.*, 2008). However, Mráz *et al.* (2012) provided molecular evidence that the tetraploid cytotype originated from hybridization between the diploid *C. stoebe* and one still unknown closely related taxon. An allopolyploid origin of the tetraploid cytotype is thus at odds with the hypothesis of *in situ* evolution of tetraploids and rather suggests a secondary contact zone in Central Europe. Despite the higher frequency of diploid populations in Europe and a largely sympatric distribution with tetraploid populations, only the latter have been recorded in the introduced range in North America (Treier *et al.*, 2009; Mráz *et al.*, 2011), where they have become highly invasive (Sheley *et al.*, 1998). Such a pattern may indicate either stochastic introduction or possible post-introduction selection that favoured the tetraploid cytotype (Müller-Schärer *et al.*, 2004). Importantly, two cytotypes differ in their life history. While the diploids are annual/biannual monocarpic plants, the tetraploids are predominantly short-lived polycarpic plants (Boggs and Story, 1987; Ochsmann, 2000; Mráz *et al.*, 2011). Such difference might provide tetraploids with a superior colonization ability when compared with diploids, possibly leading to replacement of the latter. Indeed, this process has recently been observed at the margin of the species range in Germany where tetraploids replaced the diploids in deluvial sediments (Welss *et al.*, 2008). The natural mixed-ploidy populations of *C. stoebe* thus provide a unique opportunity to study prezygotic barriers that allow cytotype coexistence and their potential interactions under natural conditions at small spatial scale.

The main goal of the study was to explore the microspatial distribution (from a few centimetres to several tens of metres) and frequency of cytotypes in narrow contact zones and to infer whether there is any correlation between cytotype distribution and microhabitat characteristics (soil moisture, vegetation density). We also assessed the relative fitness of the two cytotypes by measuring several vegetative (number of bolting stems, number of accessory rosettes) and reproductive traits (seed set, germination). In addition, by using two molecular markers we aimed to provide molecular evidence of a secondary contact zone in Central Europe and to assess inter-cytotype gene flow and the origin of intermediate cytotypes.

MATERIALS AND METHODS

Study species

Centaurea stoebe is a herbaceous species distributed from western-most Asia to Western Europe with the centre of the distribution confined to South-Eastern and Eastern Europe (Meusel and Jäger, 1992). It is represented by two cytotypes, diploid ($2n = 2x = 18$) and allotetraploid ($2n = 4x = 36$), which are sometimes treated as separate taxa (Ochsmann 2000), but their nomenclature is unresolved (Mráz *et al.*, 2011). The cytotypes are morphologically very similar, especially in the field (Španiel *et al.*, 2008). However, under uniform conditions most representatives of the cytotypes can be distinguished, as shown in a morphometric study based on plants grown from seeds and cultivated in the greenhouse (Mráz *et al.*, 2011). Besides differences in the shape of capitula and the number of inner florets and branching pattern, the most striking difference between cytotypes is their life history. Whereas the diploids have a predominantly annual–biannual monocarpic life cycle, the tetraploids are usually short-lived perennial polycarpic plants forming overwintering accessory rosettes (Boggs and Story, 1987; Ochsmann, 2000; Mráz *et al.*, 2011). Both cytotypes are pollinated by a wide spectrum of insects and are strictly self-incompatible (Harrod and Taylor, 1995; Beil *et al.*, 2008; Mráz *et al.*, unpubl.). The seeds of *Centaurea* species have only a very short pappus, not allowing effective wind dispersal. As a consequence, most of the seeds are dispersed within a few decimetres from their mothers by falling down from open dehydrated capitula or by flicking of the loosely held achenes due to movement of the stem by wind or passing animals (Sheldon and Burrows, 1973; Witztum *et al.*, 1996; Colas *et al.*, 1997). For longer distances (>1 m), *Centaurea* seeds may be dispersed by ants (Imbert, 2006; P. Mráz, pers. observ.), grazing animals (Wessels-de Wit and Schwabe, 2010) or anthropogenic activities (attached to undercarriages of vehicles, to shoes etc., Sheley *et al.*, 1998).

Sampling

The study was performed in August 2007–2009 in south-western Slovakia and north-eastern Austria where four mixed-ploidy populations out of 11 known in total (P. Mráz *et al.*, unpubl. res.) have been reported in the literature (Španiel *et al.*, 2008; Treier *et al.*, 2009). As we suspected more mixed-ploidy populations in this region, we performed additional field surveys of six other populations of which two were revealed to be mixed and four to be pure diploid populations. In total, ten populations occurring in various types of habitats and with different land-use history were studied for cytotype structure (Table 1, Fig. 1, Supplementary Data Fig. S1). In each site, we first performed an initial cytotype screening of 30 plants along a transect across a mosaic of heterogeneous microhabitats to determine cytotype frequencies and the

TABLE 1. *Cytotype structure, habitat description and historical and present use of mixed-cytotype sites of* Centaurea stoebe *and pure diploid populations screened in the initial transect study*

Site acronym	No. of plants/cytotype composition			Coordinates (°N/°E)	Altitude (m)	Site description
	2*x*	4*x*	3*x*			
SAND	198	116	1	48·201/16·974	192–196	Slovakia; Devínska Nová Ves, Sandberg hill: steppe on tertiary sands, Nature Reserve since 1964, from 1897 to 1960s exploited for sand, until 1950s intensively grazed, grazing definitely abandoned in 1964 (Klačka and Pokorný, 1995).
WEIT	77	34	0	48·194/16·980	200–204	Slovakia; Devínska Nová Ves, Weit quarry: grasslands at the bottom of old limestone quarry and rock crevices, Nature Reserve since 1964, intensively exploited from 1897 to 1932, surrounding intensively grazed until 1960s (Klačka and Pokorný, 1995).
KOP	88	49	0	48·097/17·161	134–136	Slovakia; Podunajské Biskupice, Kopáč island on the river of Danube: steppe grasslands on gravel sediments, the island had been formed during a flood around 1809, Nature Reserve since 1976, until 1960s grazed, in 1960–1970s the gravel exploitation for building of the private houses in neighbour villages (Pišút, 2002; Pišút and Timár, 2007).
TLM	76	41	0	48·297/18·537	180–190	Slovakia; Tlmače, Kusá hora hill: rocks, steppe and shrub vegetation on steep slope on andesite background, and ruderal vegetation along the asphalt road and railway to the still working andesite quarry open in the 1930s (Breznická, 2008).
MAR	40	83	1	48·273/16·888	140	Austria; Marchegg: ruderal vegetation along gravel road near the large gravel pit filled by water, Quaternary sediments of Morava river.
GLA*	27 112	3 0	0 0	48·204/16·986	160–162	Slovakia; Devínska Nová Ves, Glavica: ruderal vegetation at the foot of artificially created hill built in 2003–2005 from the exploited material from the road tunnel 'Sitina', recultivation in 2005–2006, the site was completely destroyed in 2009–2010 by building of a residential complex.
MER	30	0	0	48·182/16·984	230	Slovakia; Devín, 'Merice': steppe grasslands on calcareous bedrock, in the past grazed.
STOC1	30	0	0	48·202/17·006	217–220	Slovakia; Bratislava, Dúbravka: steppe grasslands on calcareous bedrock, until 1950–1960s grazed.
STOC2	30	0	0	48·204/17·003	181–191	Slovakia; Bratislava, Dúbravka, Stockerau quarry: rock crevices and ruderal vegetation at the bottom of old limestone quarry (active from 1891 to 1970s) (Klačka and Pokorný, 1995).
DEV	30	0	0	48·167/17·003	225–260	Slovakia; Devín, 'Mokrý jarok': ruderal vegetation along gravel road among the vineyards and gardens, and abandoned vineyards in late succession stage, granite bedrock.

* Number of plants in the first line refers to the transect sampling in 2008; the number in the second line refers to the sampling performed in 2009, when we took the exact position of 112 diploids, but the micro-site with rare co-occurring 4*x* plants found in 2008 was already destroyed (see Supplementary Data Fig. S1b).

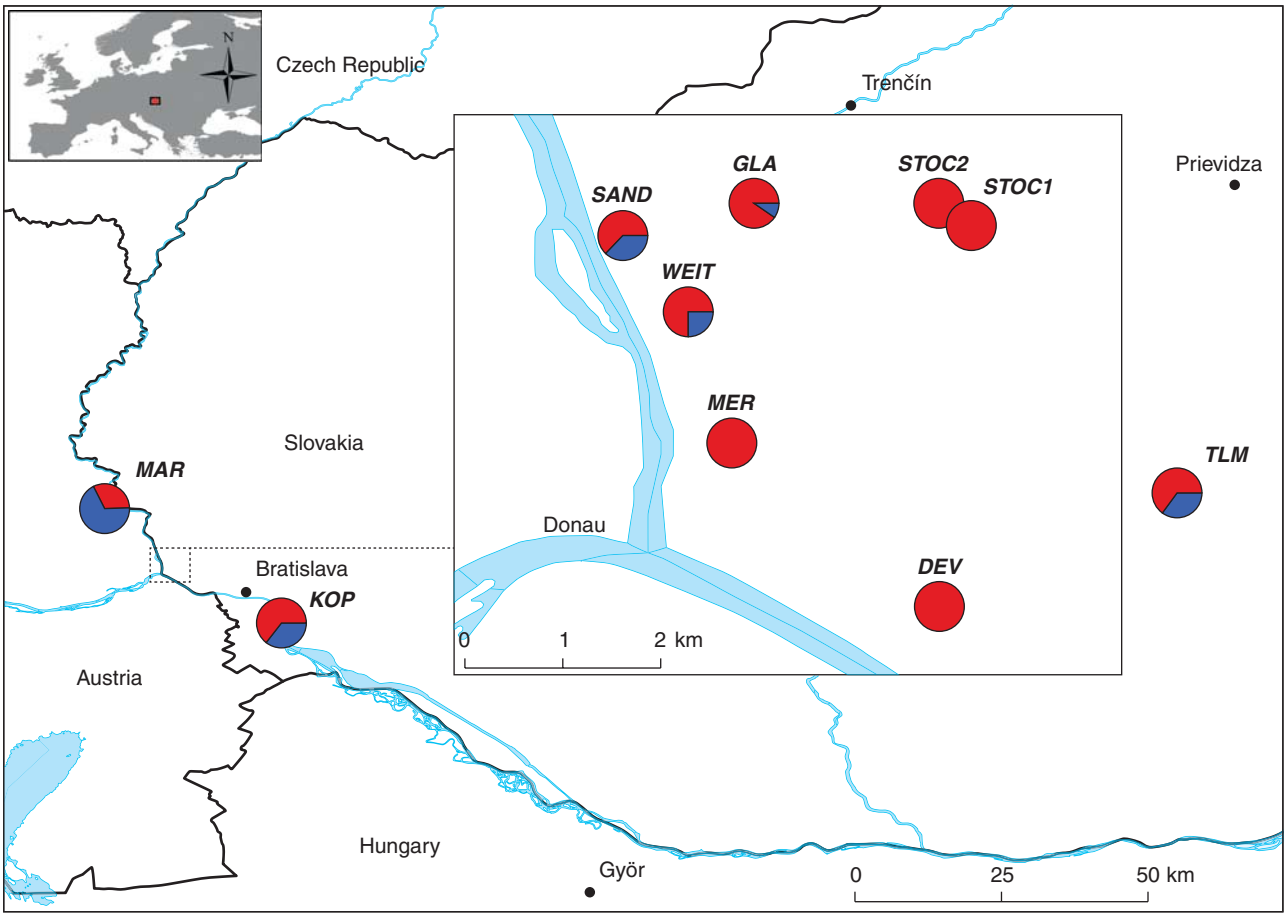

FIG. 1. Distribution of mixed-ploidy populations and pure diploid populations of *Centaurea stoebe* included in the present study. Red represents the diploid and blue the tetraploid cytotype. Population codes and exact proportions of cytotypes are given in Table 1.

position of the contact zone. Subsequently, in six mixed-ploidy populations we labelled at least 100 randomly selected plants at the flowering and rosette stage. The exact positions of plants were determined triangularly using a measuring tape. From each labelled plant we sampled fresh leaf tissue to determine its ploidy level. From a subset of plants, leaf material was also sampled and stored in silica-gel for molecular analyses. Microspatial cytotype distribution was studied in five populations only, as one site (GLA) was partially destroyed in 2009. For that population we noted only the approximate position of 30 plants sampled during the initial transect study in 2008. In 2009, we localized 120 plants at this site, but all plants were diploid because the small patch with tetraploid plants found in 2008 was destroyed (Table 1, Supplementary Data Fig. S1).

Traits and environmental variables

For each labelled plant at mixed-cytotype sites, with the exception of MAR, we recorded the presence (number)/absence of accessory rosettes and the number of stems of flowering plants as a proxy for perenniality and competitiveness, respectively. To estimate the reproductive output per plant we determined the seed set of all capitula that were mature at the sampling time at four sites. For environmental variables, we estimated vegetation cover around each individual (20 × 20 cm) using a semi-quantitative scale ranging from 1 (0–5 % vegetation cover) to 5 (75–100 %) corresponding to the Braun-Blanquet scheme for vegetation plots (Braun-Blanquet, 1928). In addition, at SAND, WEIT and KOP we took 2–3 measurements of soil moisture in the vicinity (20 × 20 cm) of randomly selected plants with known ploidy using a Theta Probe soil moisture sensor (Delta T Devices, Cambridge, UK) equipped with 12-cm-long rods. Soil moisture measurements were performed before 1200 h and lasted for 1–2 h maximum to avoid temporal variations in soil humidity at the study site. Only the mean value of soil moisture around each measured plant was used for statistical analyses. Because of strong differences in flowering phenology between the two cytotypes at KOP on the sampling day (14 August 2009), we recorded the number of withering plants (with no open capitulum or capitulum bud before anthesis), and the number of plants having at least one still flowering capitulum or capitulum bud before anthesis.

Germination experiment

To compare the rate and speed of germination between cytotypes, we sowed 20 seeds from each of 20 randomly selected mother plants per cytotype collected at SAND in 2008.

Seeds that were stored at room temperature were exposed to cold treatment (4 °C) for 3 weeks before sowing. On 7 January 2009 the seeds were planted in 2 × 2-cm cells at a depth of 1 cm in seedling trays filled with a 2 : 1 mixture of sterilized compost and sand. The seedlings were cultivated in a heated greenhouse with 16 h artificial light per day and 23 °C day/15 °C night temperature, and were watered every 3–4 d. Emergence time (i.e. the number of days from sowing to germination) was inspected every day between 1500 and 1800 h over a period of 60 d. At the end of the experiment (8 March 2009), we calculated the proportion of germinated seeds per plant and per cytotype.

Flow cytometry and karyology

The samples were cytometrically analysed using a Partec Cyflow cytometer (Partec GmbH, Münster, Germany) equipped with a mercury lamp in the Institute of Botany in Bratislava, while the ploidy of seedlings from the germination experiment was assessed using a Partec Cyflow SL cytometer equipped with a green laser at the Department of Biology in Fribourg. In the first case, samples consisting of fresh leaf tissue were prepared in a two-step procedure using Otto's extraction buffer and staining buffer containing 4′,6-diamidino-2-phenylindole (DAPI; Doležel *et al.*, 1989; Otto, 1990); in the second case, we used general-purpose buffer and propidium iodide (PI) as a stain following the protocol of Loureiro *et al.* (2007). *Bellis perennis* L. was used as an internal reference standard in both procedures (Španiel *et al.*, 2008). The DNA-ploidy level was inferred as a relative position of the sample G1 peak of to that of the internal standard. The exact chromosome number of one germinated seedling (DK-293-P10) produced by a triploid plant (DK-293) was determined by chromosome counting (for method see Mráz *et al.*, 2011).

DNA extraction and molecular analyses

DNA was extracted from 10–15 mg of silica-dried leaf tissue with a DNeasy 96 Plant Kit and DNeasy Plant Mini Kit (Qiagen) following the manufacturer's protocol.

The *trnT-trnL* and *atpB-rbcL* loci were usually sequenced in six plants per population and cytotype. The loci were amplified using primers developed by Taberlet *et al.* (1991) and Chiang *et al.* (1998), respectively. Amplifications were performed in a 25-μL volume containing 5 μL genomic DNA (4 ng μL^{-1}), 2·5 μM buffer (10× PCR buffer), 1 mM $MgCl_2$, 0·2 mM of each dNTP, 0·4 μM of each primer, 0·25 μM bovine serum albumin (BSA) and 1 U *Taq* polymerase (Qiagen). The cycle profile included initial denaturation at 94 °C for 3 min followed by 36 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min, ending with 72 °C for 5 min and 4 °C thereafter. Sequences were edited manually using Chromas Lite 2·01 (http://www.technelysium.com.au/chromas_lite.html) and assembled using Mega 4·01 (Tamura *et al.*, 2007).

Microsatellites were analysed in 248 plants collected at SAND to estimate the relatedness between the cytotypes as well as the origin of one triploid plant. In addition, single sequence repeats (SSRs) were used to determine the origin of progeny of two rare tetraploid plants found in the predominantly diploid GLA population in order to distinguish between induced autogamy ('mentor effect', see above) and allogamy. The details of four SSR loci that were developed using expressed sequence tags (Barker *et al.*, 2008) were kindly provided by R. Kesseli and D. Tsirelson (Boston) and are given in Supplementary Data Table S1. Amplifications were performed in 25 μL reaction volume containing 1× PCR buffer, 2·5 mM $MgCl_2$, 1× BSA, 0·25 mM of each dNTP, 0·08 μmol of forward primer with M13(−21) tail, 0·4 μmol of reverse primer and 0·32 μmol of fluorescently labelled M13(−21) universal primers, 1 U *Taq* polymerase (Qiagen), and 2 μL of diluted (20 ng μL^{-1}) genomic DNA. The cycle profile included initial denaturation at 94 °C for 5 min followed by 36 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, ending with 72 °C for 5 min and 4 °C thereafter. PCR products were multiplexed and analysed using an ABI 310 genetic analyser (Applied Biosystems). Only presence/absence of alleles and not their dosage were used to genotype individual plants.

Data analyses

Spatial aggregation of cytotypes within each site was assessed using Mantel tests where the correlation between pair-wise geographical distances of plants and pair-wise cytotype 'distances' (0 for plants of the same cytotype and 1 for different plants) was computed and statistically evaluated using 9999 randomizations. Association between cytotypes and the five vegetation cover classes was tested by chi-square tests for the whole dataset and separately for each site. The chi-square test was used to assess differences in flowering between cytotypes at KOP. Differences in soil moisture between microsites of diploids and tetraploids, as well as the germination rate from the germination experiment, were assessed using *t*-tests. Differences in the probability of forming accessory rosettes for each cytotype for the whole data set were tested using a generalized linear mixed-effects model, with binomial distribution and logit link function with cytotype as a main factor and population nested in cytotype as a random factor. Wilcoxon non-parametric tests were used to test differences between the cytotypes in seed production, number of shoots and accessory rosettes per plant, and germination speed from the germination experiment. To test whether the seed production of tetraploid plants depends on their proportions in mixed-ploidy populations, we used a linear mixed-effects model with log-transformed values of number of seeds per plant as response variable and ploidy and proportion of tetraploids per site and their interaction as explanatory variables, with population as a random factor. Genetic relatedness between cytotypes was assessed by principal component analysis based on presence/absence of the alleles at four SSR loci. All statistical analyses and plotting were carried out using several packages implemented in R (R Development Core Team, 2009). To visualize microspatial cytotype distribution in different vegetation classes at SAND we geographically interpolated the vegetation density plots using the inverse distance-weighted technique [IDW function in Spatial Analysts extension for $ArcGIS_{TM}$ (ESRI, 2011), with a variable search radius including 12 points (i.e. standard parameters)]. The microspatial distribution of cytotypes was then

overlaid onto this layer. A haplotype network based on substitution and insertion–deletion polymorphisms of two assembled cpDNA loci was constructed using the median-joining algorithm in Network v. 4·6·0·0 (www.fluxus-engineering.com; Bandelt *et al.*, 1999).

RESULTS

Cytotype frequency

Diploid plants were more abundant than tetraploids in five of the six mixed-ploidy populations studied (Table 1, Fig. 1). Although at MAR we sampled more tetraploids in 2007 (Table 1), inspection of this site in 2010 revealed that diploids were more common than tetraploids when also considering the area outside of the transect studied in 2007 (P. Mráz, pers. observ.). Frequent pure diploid populations (DEV, MER, STOC1, STOC2) in the close vicinity of mixed-cytotype sites confirm the general predominance of the diploid cytotype in the region (Table 1, Fig. 1). Single triploid plants were found at two sites (SAND and MAR) representing 0·3 and 0·8 % of plants analysed at these sites, respectively. These data are the first record of triploidy in the *C. stoebe* group.

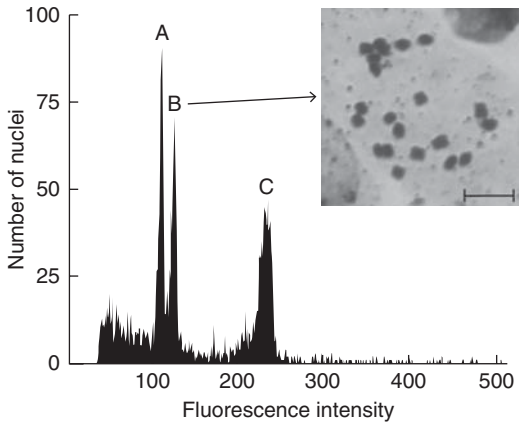

FIG. 2. Histograms of absolute DNA content of PI-stained nuclei of (A) a diploid plant ($2n = 2x = 18$) of *Centaurea stoebe*, (B) an aneuploid progeny (DK-293-P10, $2n = 21$; see insert image of its mitotic chromosomes, scale bar = 5 μm) of a triploid hybrid (DK-293) found at the SAND site, and (C) *Bellis perennis* used as an internal standard. Photo: P. Mráz.

The very low frequency of triploids was corroborated by analysing 449 seedlings from the germination experiment. Mother plants of the SAND site exclusively produced progeny of their own ploidy level, with the exception of one triploid seedling (DK-189-P4) from one tetraploid mother plant (DK-189). Similarly, only tetraploid offspring (24 in total) were produced by two tetraploid plants from the predominantly diploid GLA population. Flow cytometry analysis of one seedling (DK-293-P10) of a triploid mother plant (DK-293) from the SAND site indicated an aneuploid ploidy level (Fig. 2) and counting of the exact chromosome number showed $2n = 21$. Thus, the seedling had three extra chromosomes when compared with the diploids ($2n = 18$, Fig. 2).

Spatial and habitat segregation of cytotypes at the micro-scale

Spatial analyses revealed significant segregation of diploid and tetraploid cytotypes at all sites (Table 2, Fig. 3). Although not studied in detail due to destruction of the site, three tetraploids at GLA were also found clustered together within one patch. In general, the ratio between the proportion of diploid plants and the proportion of tetraploid plants increased significantly with increasing vegetation density (linear regression, $P = 0{\cdot}0055$, $r = 0{\cdot}96$; Fig. 4). However, when analysing each site separately, this pattern was significant only at SAND, where the 4*x* plants had a clear tendency to occupy more open sites (Table 2, Supplementary Data Fig. S2). Non-significant differences in other sites were probably caused by the lower numbers of plants analysed (cf. Table 1) and, in some cases, also by the absence of the densest vegetation classes (4 and 5). Importantly, the occurrence of 4*x* plants was strongly associated with human-induced disturbance (see details for each site in Table 1, Supplementary Data Fig. S1). Specifically, at SAND the 4*x* plants were most abundant on a sand pile – a remnant from ancient sand exploitation. At WEIT the 4*x* plants occurred in crevices of a limestone quarry wall and at disturbed sites in the close vicinity. At KOP the tetraploids were concentrated on and along a dirt road at the entrance to a Natural Reserve and in small gravel pits exploited in the 1970s. Furthermore, tetraploids were never found in non-disturbed parts of steppe meadows, where only diploids were present. At TLM the tetraploids were found exclusively along the road and railway tracks leading to an andesite quarry, but were completely

TABLE 2. *Tests with significance values* (P) *for spatial and habitat segregation, and differences in competitive, persistence and fitness traits between diploid and tetraploid plants from the mixed-ploidy populations of* Centaurea stoebe

Site	Spatial distribution: Mantel test	Vegetation density 2*x* vs. 4*x*: χ^2 test	Soil moisture 2*x* vs. 4*x*: *t*-test	*P* of rosettes formation 2*x* vs. 4*x*: Linear mixed effect model	No. of rosettes per plant 2*x* vs. 4*x*: Wilcoxon test	No. of shoots per plant 2*x* vs. 4*x*: Wilcoxon test	No. of seeds per plant 2*x* vs. 4*x*: Wilcoxon test
SAND	**<0·001**	**0·004**	0·07	NT	**<0·001**	**<0·001**	**<0·001**
WEIT	**0·015**	0·212	**<0·001**	NT	**<0·001**	**0·013**	**<0·001**
KOP	**<0·001**	0·521	0·1	NT	**0·01**	**0·026**	NM
TLM	**<0·001**	0·479	NM	NT	0·053	**<0·001**	**0·04**
MAR	**<0·001**	NM	NM	NM	**0·01**	**0·031**	NM
GLA	NM	NM	NM	NM	NM	NM	**0·02**
Total	NT	**<0·001**	NT	**<0·05**	**<0·001**	**<0·001**	**<0·001**

NM, not measured; NT, not tested. Bold type indicates statistically significant differences.

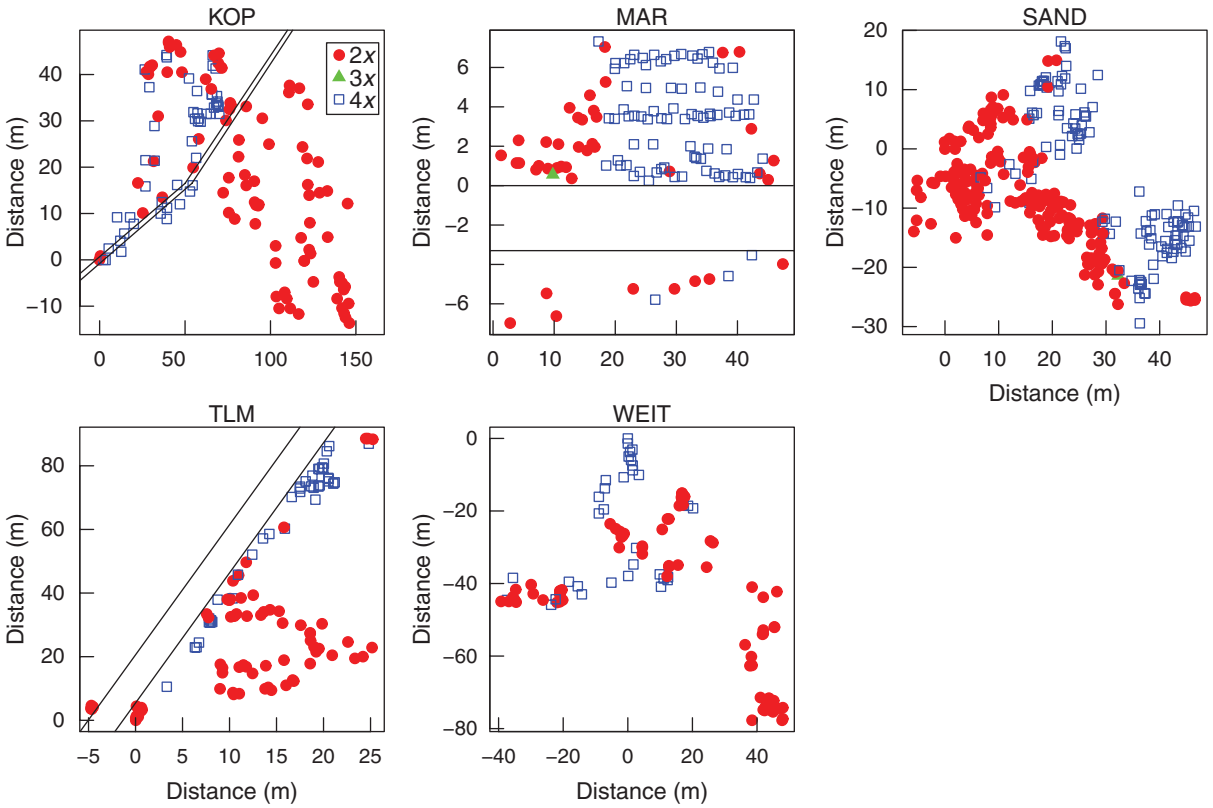

FIG. 3. Microspatial distribution of diploid, triploid and tetraploid plants of *Centaurea stoebe* in five mixed ploidy populations. Dirt (MARCH, KOP) and asphalt roads (TLM) are schematically drawn. For site abbreviations see Table 1.

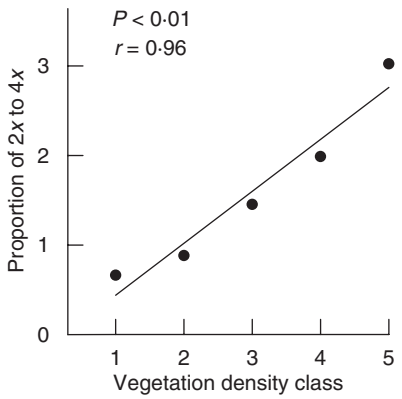

FIG. 4. The ratio between the proportion of diploid and the proportion of tetraploid plants of *Centaurea stoebe* occurring in five vegetation density classes corresponding to Braun-Blanquet's scale for phytosociological plots (1, 0–5 % vegetation cover; 5, 75–100 %; see Material and Methods) at five mixed-ploidy sites.

missing in semi-natural steppes and natural andesite rock outcrops situated above the road and rails, where diploids were abundant. Similarly, tetraploids at MAR were found along a dirt road near a gravel pit. Finally, at GLA three tetraploids were found at the start of a dirt road under a recently built hill. Lower soil moisture values measured around the 4*x* plants as compared with 2*x* plants at WEIT and SAND (only significant at WEIT) furthermore indicate an association of tetraploid plants with drier micro-sites (Table 2, Supplementary Data Fig. S3). At KOP no such pattern was found, probably because of the high level of groundwater on this Danube River island (Table 2, Supplementary Data Fig. S3).

Persistence, competitive and fitness traits

The production of accessory rosette(s) in flowering plants and thus the ability to reproduce polycarpically was significantly higher in tetraploid than in diploid plants (34·4 % of flowering tetraploids vs. 2·6 % of flowering diploids produced accessory rosettes; Table 2, Supplementary Data Fig. S4). Moreover, tetraploids produced on average significantly more shoots (4·45) and more accessory rosettes (1·18) per flowering plant than diploids (1·93 and 0·04, respectively) (Table 2, Supplementary Data Figs S5 and S6). The number of shoots per plant in diploids was negatively correlated with vegetation density (Spearman's test for the whole data set, $r = -0{\cdot}23$, $P < 0{\cdot}001$), indicating a possible environmental effect on this trait, but no such correlation was observed in tetraploids (Spearman's test for the whole data set, $r = 0{\cdot}02$, $P = 0{\cdot}8$). Diploids produced significantly more seeds not only per plant, but also per shoot and per capitulum (Wilcoxon test, $P < 0{\cdot}001$ for both traits) at each of the study sites (Table 2, Fig. 5). However, the seed production of tetraploid plants was dependent on the frequency of tetraploids in the population; two rare tetraploids of three in total found at GLA

produced significantly fewer seeds than the tetraploids from other mixed-ploidy populations where they were more common (linear mixed-effects model, $t = 2{\cdot}62$, $P = 0{\cdot}009$; Fig. 5). One triploid plant from SAND produced only 20 mature seeds of unusual rounded shape and they did not germinate with exception of one seed. The diploids and tetraploids from SAND did not differ in average germination rate per plant (57·5 and 54·2 %, respectively; *t*-test, $t = 0{\cdot}45$, $P = 0{\cdot}7$; Fig. S7), nor in the time of seedling emergence (median: 8 and 7 d, respectively; Wilcoxon test, $P = 0{\cdot}5$; Fig. S7). At KOP many more tetraploids than diploids were still flowering when visited on 9 August 2009 (93·5 and 54·8 %, respectively; chi-square test = 18·22, $P < 0{\cdot}001$; Fig. S8), while almost half of the 2*x* plants had already withered.

Haplotype distribution among cytotypes

In total, 12 cpDNA haplotypes were found in the 69 plants studied (Table 3, Supplementary Data Table S2). In diploids, five haplotypes with low frequency (0·015–0·07) were unique for 2*x*, while four haplotypes were shared with tetraploids. Of these shared haplotypes, three were very frequent (H1, H2, H12; Fig. 6). In tetraploids, three haplotypes of seven were unique for 4*x* (H9, H10 and H11), with one of them being frequent (H9, 0·15). No shared haplotypes between cytotypes were found at KOP and MAR. At the remaining sites some of the 2*x* and 4*x* plants shared one of the most common haplotypes (H1, H2 and H12), but with different proportions (Table 3). The triploid plant from MAR (Ma-134) had the same haplotype (H2) as the majority of co-occurring diploids, while the triploid plant from SAND (DK-293) shared its H9 haplotype with co-occurring tetraploid plants (Table 3, Supplementary Data Table S2).

Pattern of microsatellite variation

In total, 48 alleles were found among 247 plants in SAND, with 33 alleles shared between diploids and tetraploids

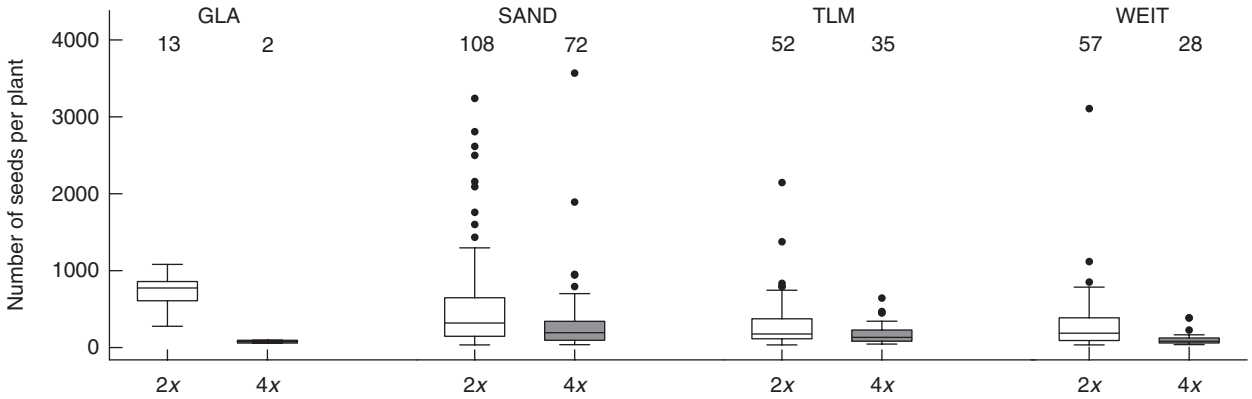

FIG. 5. Seed production per plant in four mixed-ploidy populations of *Centaurea stoebe*. The number of analysed plants per ploidy level and respective site is given above each box plot.

TABLE 3. *Chloroplast haplotype distribution of* Centaurea stoebe *per site and ploidy*

			Haplotype											
Site and ploidy	*N*	N_H	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12
SAND 2*x*	6	3	3	0	0	0	1	0	0	2	0	0	0	0
SAND 4*x*	6	2	1	0	0	0	0	0	0	0	5	0	0	0
SAND 3*x*	2*	2	1	0	0	0	0	0	0	0	1	0	0	0
GLA 2*x*	6	2	2	0	0	0	0	0	0	0	0	0	0	4
GLA 4*x*	2	2	1	0	0	0	0	0	0	0	0	0	0	1
WEIT 2*x*	6	4	0	2	0	0	0	1	1	0	0	0	0	2
WEIT 4*x*	6	2	0	2	0	0	0	0	0	0	4	0	0	0
KOP 2*x*	6	2	5	0	0	0	0	1	0	0	0	0	0	0
KOP 4*x*	4	1	0	0	0	0	0	0	0	0	0	4	0	0
TLM 2*x*	6	2	0	0	0	5	0	0	1	0	0	0	0	0
TLM 4*x*	6	2	5	0	0	0	0	0	1	0	0	0	0	0
MAR 2*x*	6	2	0	5	1	0	0	0	0	0	0	0	0	0
MAR 4*x*	6	3	2	0	0	0	0	0	0	0	0	0	3	1
MAR 3*x*	1	1	0	1	0	0	0	0	0	0	0	0	0	0

N, number of plants per site and cytotype; N_H, total number of cpDNA haplotypes per site and cytotype.

* In addition to one triploid adult plant (DK-293) found at the SAND site, another triploid (DK-189-P4), the progeny of a tetraploid mother plant (DK-189), found in the germination experiment was included in the analysis.

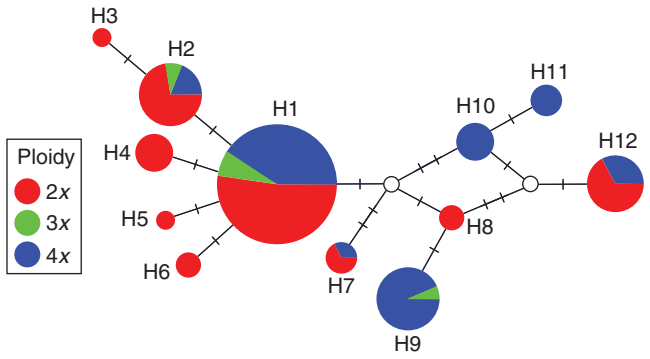

FIG. 6. Haplotype network of 12 haplotypes from 69 accessions of *Centaurea stoebe* based on combined *trn*T-*trn*L and *rbc*L-*atp*B sequences. Three different ploidies are marked by different colours; open black circles indicate missing haplotypes, and slashes represent mutational steps. The size of each pie plot is proportional to the number of accessions sharing the haplotype.

(Table 4). Seven private alleles were found in diploids, two at relatively high frequency (0·52 and 0·23). Similarly, tetraploids harboured eight private alleles, with one of them being very frequent (0·76) and two moderately frequent (0·18 and 0·14) (Table 4). One triploid plant (DK-293) was highly heterozygous, showing three different alleles at each locus (Table 4). Interestingly, of the three alleles found in this triploid at the CM-8337 locus, two alleles were private for tetraploids and one was unique for diploids, thus strongly suggesting a hybridogeneous origin of this triploid (Table 4). Principal component analyses revealed two slightly overlapping clusters of plants corresponding to diploids and tetraploids (Fig. 7). None of the 24 tetraploid offspring from the two rare tetraploid mother plants sampled at GLA arose from self-pollination, as all analysed offspring exhibited some non-mother alleles at least at one locus (Supplementary Data Table S3).

DISCUSSION

Evidence for secondary origin of narrow contact zones

In agreement with expectation, both molecular markers showed a certain level of genetic differentiation between cytotypes and hence corroborated that the contact zones were secondary. Some fraction of variation was, however, shared between cytotypes. This shared polymorphism might simply be explained by the close relationship between the diploid progenitor and its allotetraploid descendant (Mráz *et al.*, 2012), although further additional non-exclusive processes such as incomplete lineage sorting or occurrence of homoplasies are possible (Font *et al.*, 2009; Löser *et al.*, 2009; Mráz *et al.*, 2012).

Role of anthropogenic disturbances and other non-adaptive processes in microspatial and microhabitat segregation of cytotypes

We found strong microspatial cytotype segregation at every mixed ploidy population studied. Our data thus confirm results of a handful of recently published studies on microspatial

TABLE 4. *Allele frequencies computed from the presence or absence of alleles at four microsatellite loci in diploid, triploid and tetraploid plants of* Centaurea stoebe *in the Sandberg population (SAND)*

Locus	CM-730										CM-1922									
Allele size (bp)	140	143	147	150	154	157	163	169	173	176	197	195	198	201	204	207	210	213	215	219	222	225	228
2*x* (*n* = 168)	0·11	0·36	0·24	0·70	0·01	0·11	0	**0·01**	0·01	0	0	0·10	0·20	0·39	0·05	0·37	0·01	0·39	**0·01**	0·01	0·02	0	**0·01**
4*x* (*n* = 79)	0·11	0·09	0·54	0·89	0·25	0·54	**0·03**	0	0·03	**0·01**	**0·11**	0·44	0·01	0·41	0·11	0·46	0·54	0·35	0	0·04	0·03	**0·18**	0
3*x* (*n* = 1)		1	1	1										1			1	1					
Locus	CM-8337										CM-10060									
Allele size (bp)	231	234	237	240	243	246	249	255	258	206	209	212	215	218	221	224	227	230	233	237	240	242	245	248	250
2*x* (*n* = 168)	0	**0·52**	0·11	0	0·51	0·29	0·14	0·05	0	0·02	0·33	0·12	0·05	0·18	0·01	0·28	0	0·1	**0·23**	0·11	0·02	**0·02**	0·30	**0·03**	0·01
4*x* (*n* = 79)	**0·01**	0	0·13	**0·76**	0·86	0·13	0·20	0·15	**0·04**	0·46	0·39	0·39	0·10	0·05	0·10	0·11	**0·14**	0·37	0	0·67	0·23	0	0·15	0	0·03
3*x* (*n* = 1)	1	1		1							1						1			1					

After the cytotype abbreviation, the number of plants is given in parentheses. Frequencies in bold type denote private alleles for either diploid or tetraploid plants.

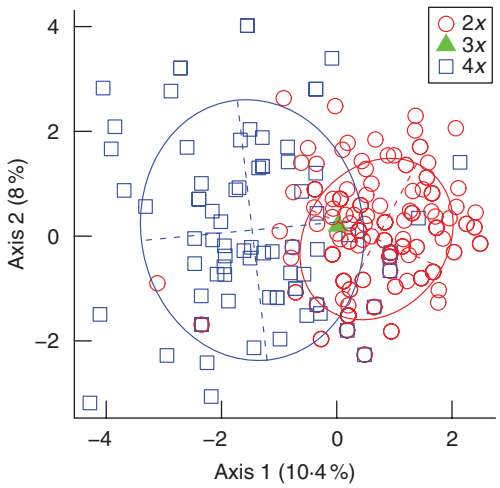

FIG. 7. Principal components analysis of 168 diploid, 78 tetraploid and one triploid plant of *Centaurea stoebe* from the SAND population based on the presence/absence of 48 SSR alleles. Confidence ellipses were defined by the gravity centre (centroid) of the cloud and 1·5 standard deviations. One tetraploid plant (DK-314) was removed from the analysis as it was an outlier due to the presence of one unique allele, but which was found to be relatively frequent in the tetraploid plants from Serbia (P. Mráz, unpubl. res.).

cytotype distribution in various diploid–polyploid complexes showing non-random patterns (Baack, 2004; Kolář *et al.*, 2009; Hülber *et al.*, 2009; Kao and Parker, 2010; Šafářová and Duchoslav, 2010; Trávníček *et al.*, 2011*a*, *b*; but see Halverson *et al.*, 2008). Moreover, microspatial segregation of the *C. stoebe* cytotypes was correlated with habitat differentiation. Tetraploids occurred more frequently in disturbed, open and drier micro-sites mainly created by human activities, but were found to be less common or absent in more natural grassland communities or relic rocky outcrops where they were replaced by diploids. Microhabitat segregation has been reported only in few diploid–polyploid aggregates, e.g. in *Anthoxanthum alpinum* (Felber-Girard *et al.*, 1996), *Senecio carniolicus* (Hülber *et al.*, 2009), the *Dactylorhiza maculata* group (Stahlberg, 2009) and *Solidago altissimma* (Richardson and Hanks, 2011), and these patterns were explained as differences in competitive ability and/or tolerance to stress (but see Hülber *et al.*, 2011).

Although the microspatial and microhabitat segregation observed in our studied mixed-ploidy *C. stoebe* populations might also indicate, at least to some extent, ecological differentiation between cytotypes, we suggest that this distributional pattern is rather the result of non-adaptive mechanisms. The observed patterns suggest a relatively recent immigration of tetraploids (perhaps $<$100–120 years; cf. indications on the anthropogenic activities carried out on the sites summarized in Table 1) into already established diploid populations favoured by human activities. Such activities not only created new open niches suitable for colonization by the tetraploid newcomers, but are also expected to selectively introduce tetraploid propagules through transport of material (see Table 1). Human-mediated introductions have frequently been observed not only in the invasive range in Northern America (Sheley *et al.*, 1998), but also in its native European range, where the tetraploids are frequently spreading along the transport corridors (Ochsmann, 2000; Korneck, 2004; Wells *et al.*, 2008). Thus, locally created disturbed niches and unintentional human-mediated dispersals of tetraploids into such sites probably contributed to the initial non-random distribution of cytotypes in mixed-ploidy populations. Historical, non-adaptive processes also may have driven habitat segregation in diploid–polyploid complexes of *Deschampsia caespitosa* in the British Isles (Rothera and Davy, 1986) and *Taraxacum* sect. *Ruderalia* in Switzerland (Meirmans *et al.*, 1999). In both cases, polyploids occur at recently disturbed sites while diploids were mainly found on more relict or semi-natural habitats.

With regard to our studied mixed-ploidy *C. stoebe* populations, additional non-adaptive processes may have been involved in the joint occurrence of the two cytotypes and the formation of the present cytotype distribution. (1) Prevailing short-distance dispersal in *Centaurea* spp. (see Material and Methods) will lead to cytotype clustering at a small scale, which in turn will enhance intraploid pollination and ultimately favour cytotype coexistence (see also Baack, 2005). (2) Absence of long-distance dispersal by a lack of grazing domestic animals (goats, sheep; cf. Wessels-de Wit and Schwabe, 2010) over the past 50 years at the study sites (see Table 1) may also have contributed to the restricted distribution of tetraploids. (3) Minority cytotype exclusion might indeed have reduced the fitness of the less frequent cytotype as observed at GLA (Fig. 5) and thus contribute to cytotype homogenization over small distances. (4) The observed significant asynchrony in flowering time between ploidies at the KOP site will substantially limit interploid gene flow. Interestingly, no obvious asynchrony in flowering was observed at the other sites, suggesting an influence of specific site conditions and/or differences in local genotypes.

Evidence for a strong postzygotic reproduction barrier

The frequency and fertility of an intermediate cytotype can provide evidence for the level of effective gene flow between cytotypes. Of 1307 cytometrically analysed plants and seedlings from six mixed-ploidy populations, only three triploids were found. Although triploids could arise also among diploid plants through unreduced gamete formation (Ramsey and Schemske, 1998), our combined molecular data (SSRs, cpDNA and internal transcribed spacer; present data and Mráz *et al.*, 2012) and the absence of triploids in pure diploid populations clearly indicate their origin through inter-cytotype crosses. Moreover, the cpDNA data show that these crosses were bidirectional, i.e. both diploid and tetraploid plants could serve as mother or pollen parents.

Although the rare occurrence of interploid hybrids could be explained by fine spatial segregation of diploids and tetraploids, many plants of both cytotypes grew intermingled or in very close proximity (Fig. 3). Therefore, intensive inter-cytotype pollen transfer can be expected in these plants, given: (1) that foraging pollinators usually move short distances between flowers, often visiting neighbouring plants (Mitchell *et al.*, 2009); (2) the generally observed synchrony in flowering between diploids and tetraploids of *C. stoebe*; and (3) the observation that pollinators readily move between the cytotypes of *C. stoebe* in common garden experiment and in the field

(M. Hahn, University of Fribourg, Switzerland, pers. comm.; P. Mráz, pers. observ.). Thus, together with the difficulties in obtaining viable triploid progeny from artificial heteroploid crosses (P. Mráz *et al.*, unpubl. res.), our field and experimental data indicate strong postzygotic isolation between the cytotypes, probably caused by an unbalanced ratio between male and female genomes in the endosperm, which becomes malfunctioned and triggers early seed abortion ('triploid block' mechanism; Marks, 1966). Strong postzygotic isolation between diploids and tetraploids has been frequently observed in other diploid–polyploid complexes, including *Centaurea* species (Hardy *et al.*, 2001; Koutecký *et al.*, 2011). The only progeny of the triploid plant from SAND was aneuploid and probably arose from fusion of an aneuploid ovule ($n = 12$) of a triploid mother with strongly irregular meiosis and haploid pollen ($n = 9$) probably from a diploid donor.

Differences in competitive and fitness traits

Tetraploids in mixed-ploidy populations produced a significantly lower number of seeds per plant and per flowering season but more shoots and more frequently produced accessory rosettes, resulting in a predominantly polycarpic life cycle as compared with diploids. These findings are well in line with results from common garden and greenhouse experiments and indicate strong differences in life cycle and in inflorescence structure (lower number of florets per capitulum and capitula per stem in tetraploids) between the cytotypes (Henery *et al.*, 2010; Mráz *et al.*, 2011). Importantly, reduction in seed set in tetraploids was most pronounced at GLA, the site with the lowest proportion of tetraploids among the mixed-ploidy sites. This might indicate a frequency-dependent mating disadvantage of the minority cytotype, which was indeed found in experimental populations of *Chamamerion angustifolium* (Husband, 2000).

Conclusions

This study suggests that recent anthropogenic disturbance and selected introductions of the tetraploid cytotype into established diploid populations are the main causes in creating the present spatial and habitat segregation of cytotypes, even at a fine spatial scale. Habitat cytotype segregation might thus not necessarily be associated with differences in physiologically determined habitat preferences of the cytotypes (e.g. Ramsey, 2011; Manzaneda *et al.*, 2012), but may result from various spatio-historical processes, and their timing and interactions (at larger spatial scales through different centres of speciation or refugia, colonization routes, anthropogenic disturbance; Stebbins, 1985; van Dijk and Bakx-Schotman, 1997; Mandáková and Münzbergová, 2006; Mráz *et al.*, 2008; Cosendai and Hörandl, 2010). Besides spatio-temporal processes, our results highlight additional non-adaptive mechanisms contributing to the establishment, coexistence and maintenance of distinct distribution patterns of *C. stoebe* cytotypes at small spatial scale. Most important are differences in the life cycle, limited seed dispersal and asynchronous flowering (at one site). Especially important is the increased longevity and overall greater lifetime seed production in polycarpic tetraploids as compared with monocarpic diploids (Broz *et al.*, 2009). These factors might especially favour colonization and persistence of the tetraploids in newly created disturbed areas and thus contribute to the observed spatial segregation of cytotypes in the studied mixed-ploidy sites. Furthermore, our study is, to our knowledge, the first demonstrating a relationship between the frequency of the less common cytotype and its fitness (seed set) in natural mixed-ploidy populations. Frequency-dependent mating thus could further contribute to the spatial aggregation of cytotypes at the small spatial scale. Given the higher persistence ability of the tetraploid cytotype as compared with the diploid cytotype, these natural contact zones of *C. stoebe* offer rewarding study sites to follow the spatio-temporal dynamics of the cytotypes over a longer time scale.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Figure S1: habitats of six mixed-ploidy populations of *Centaurea stoebe*. Figure S2: cytotype distribution at SAND across heterogeneous microhabitats with different vegetation density. Figure S3: averaged soil moisture around randomly selected diploid and tetraploid plants in three mixed-ploidy populations. Figure S4: estimated mean proportions of plants forming accessory rosettes for diploids and tetraploids. Figure S5: number of shoots in diploid and tetraploid plants. Figure S6: number of accessory rosettes in diploid and tetraploid plants. Figure S7: germination rate per plant and germination speed in diploid and tetraploid plants at SAND. Figure S8: proportions of plants that were still flowering and that had finished flowering at the mixed-ploidy KOP site on 14 August 2009. Table S1: details of the four microsatellite loci used in the present study. Table S2: list of sequenced diploid, triploid and tetraploid plants of *Centaurea stoebe* and their haplotypes and GenBank accession numbers. Table S3: allelic composition at four microsatellite loci of two tetraploid plants sampled at the GLA site and their progeny.

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