

## Bridging global and microregional scales: ploidy distribution in *Pilosella echiioides* (Asteraceae) in central Europe

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- **Background and Aims** A detailed knowledge of cytotype distribution can provide important insights into the evolutionary history of polyploid systems. This study aims to explore the spatial distribution of different cytotypes in *Pilosella echiioides* at various spatial scales (from the whole distributional range to the population level) and to outline possible evolutionary scenarios for the observed geographic pattern.
- **Methods** DNA-ploidy levels were estimated using DAPI flow cytometry in 4410 individuals of *P. echiioides* from 46 populations spread over the entire distribution range in central Europe. Special attention was paid to the cytotype structure in the most ploidy-diverse population in south-west Moravia.
- **Key Results** Five different cytotypes (2x, 3x, 4x, 5x and 6x) were found, the last being recorded for the first time. Although ploidy-uniform (di- or tetraploid) sites clearly prevailed, nearly one-quarter of the populations investigated harboured more (up to all five) cytotypes. Whereas penta- and hexaploids constituted only a minority of the samples, a striking predominance of the triploid cytotype was observed in several populations.
- **Conclusions** The representative sampling confirmed previous data on cytotype distribution, i.e. the spatial aggregation of mixed-ploidy populations in south-west Moravia and Lower Austria and the predominance of ploidy-uniform populations in other parts of the area investigated. Recurrent origin of polyploids from diploid progenitors via unreduced gametes and their successful establishment are considered the key factors promoting intrapopulation ploidy mixture ('primary hybrid zones'). As an alternative to the generally accepted theory of cytotype co-existence based on the development of different means of inter-ploidy reproductive isolation, it is suggested that a long-term ploidy mixture can also be maintained in free-mating populations provided that the polyploids originate with a sufficient frequency. In addition, the prevalence (or subdominance) of the triploid cytotype in several mixed-ploidy populations represents the first evidence of such a phenomenon in plant systems with exclusively sexual reproduction.

**Key words:** *Pilosella echiioides*, cytotype co-existence, cytotype distribution, flow cytometry, free mating, ploidy variation, triploid, sympatry.

### INTRODUCTION

A substantial fraction of angiosperm species (30–70 %) are at least ancient polyploids (Stebbins, 1950; Grant, 1981; Masterson, 1994; Bennett, 2004; Soltis, 2005), but contemporary studies point to the presence of a whole duplication genome event, i.e. polyploidization, at the base of almost all angiosperms (Buggs *et al.*, 2009; Soltis *et al.*, 2009). Principle questions in the research of polyploids relate to the mechanisms of their origin, establishment relative to putative diploid progenitors, and coexistence of the different cytotypes (Petit *et al.*, 1999). There is increasing evidence that rates of polyploidization are often high and recurrent origins of both allo- and autopolyploids within diploid taxa are common (e.g. Brochmann *et al.*, 1992; Soltis and Soltis, 1993, 1999, 2000; Wendel, 2000; Sharbel and Mitchell-Olds, 2001; Ramsey and Schemske, 2002; Guo *et al.*, 2005; Wu *et al.*, 2010). Special

attention is also paid to the coexistence of cytotypes, namely within complexes of diploids and their autopolyploid derivatives, that is usually explained as either (a) a transitional stage in which one cytotype will outcompete the other, e.g. as a result of frequency dependent mating (cf. the minority cytotype exclusion principle; Levin, 1975; Fowler and Levin, 1984; Rodriguez, 1996; Petit *et al.*, 1999; Husband, 2000; Kennedy *et al.*, 2006), or (b) a more stable system with different cytotypes maintained by their different responses to spatial environmental variation ('ecogeographic preferences'; Lewis, 1980). The latter can include ecological sorting, e.g. better adaptation of polyploids to harsh environment (cold, drought), competitive superiority of polyploids associated with higher growth rates (Lewis, 1980; Lumaret *et al.*, 1987; Maceira *et al.*, 1993; Felber-Girard *et al.*, 1996; Husband and Schemske, 1998; Johnson *et al.*, 2003), divergence in flowering time, divergence in behaviour and preferences of pollinators,

mechanical isolation due to differences in floral morphology (van Dijk *et al.*, 1992; Petit *et al.*, 1997; Nuismer and Cunningham, 2005; Jersáková *et al.*, 2010), and increased self-fertilization in polyploids (e.g. Husband and Schemske, 1997; Cook and Soltis, 2000; Barringer, 2007), all of which might lead to reproductive isolation and separation in space and time. Nonrandom insect attacks on genotypes with different ploidy levels can also affect cytotype frequencies, although it is strongly affected by the population size, site and herbivores (e.g. Münzbergová, 2006; Halverson *et al.*, 2008a).

Distribution patterns of ploidy variation differ considerably among species. Some can consist of vicariant parapatric or allopatric populations of a single cytotype arranged along geographical or ecological gradients (e.g. Borgen and Hultgård, 2003; Mandáková and Münzbergová, 2006; Mráz *et al.*, 2008; Trávníček *et al.*, 2010). In other cases, mixed-ploidy populations occur with various frequencies across the geographic range of the species, often in variously wide contact zones between cytotypes (Borrill and Lindner, 1971; Husband and Schemske, 1998; Garnatje *et al.*, 2001; Weiss *et al.*, 2002; Keeler, 2004; Stuessy *et al.*, 2004; Kao, 2008; Schönswetter *et al.*, 2007; Suda *et al.*, 2007b; Halverson *et al.*, 2008b; Duchoslav *et al.*, 2010). Hybrid zones arising in the area of inter-cytotype contact are traditionally divided into primary and secondary. A primary hybrid zone is formed as a direct consequence of the emergence of a neopolyploid within a diploid population, secondary hybrid zones arise following contact of cytotypes after phases of geographic separation (for review, see Petit *et al.*, 1999).

Recent cytotype distributions can reflect the evolutionary past of particular species and various environmental or man-made factors that have co-determined it. Therefore knowledge of exact cytotype distribution patterns on geographical scales is helpful for understanding environmental conditions or evolutionary processes affecting such patterns (allopatric vs. sympatric occurrence; primary vs. secondary hybrid zone, etc.), coexistence of cytotypes within mixed populations (e.g. niche differentiation, assortative mating), and the frequency of inter-cytotype crossing (gene flow between cytotypes). The use of flow cytometry has allowed for cytotype evaluation of several-fold more individuals than conventional chromosome counting (Doležel, 1997) and is the most efficient tool for large screening studies of cytotype distribution (for review, see Kron *et al.*, 2007).

*Pilosella echioides* is a perennial sexual allogamous and partly agamosperous species. Its geographic range stretches from south Russia westwards to central Europe (Bräutigam, 1992) where the occurrence is split into more or less isolated areas of various sizes. Distribution of *P. echioides* is closely associated with steppe grasslands that reached their widest distribution repeatedly in cold periods of Pleistocene glaciation, but it often occupies open sands, sandy grasslands, heathlands, rocks and light pine forests. Previous studies on *P. echioides* revealed the presence of four ploidy levels from diploids ( $2n = 18$ ) to pentaploids ( $2n = 45$ ) (Rotreklová *et al.*, 2002, 2005) with diploid populations prevailing in Europe while mixed-ploidy populations with two to four cytotypes were found in south-west Moravia (Rotreklová *et al.*, 2002, 2005), in the adjacent region of Lower Austria (Schuhwerk and Lippert, 1997) and in central and north-west Bohemia

(Rotreklová *et al.*, 2002). Diploids and triploids have also been shown to be sexual, tetraploids either sexual or (in some populations in north-west Hungary and south Slovakia) agamosperous, whereas the reproductive strategy of pentaploids is still poorly understood (Rotreklová *et al.*, 2002, 2005; Peckert *et al.*, 2005; Peckert and Chrtek, 2006; P. Trávníček *et al.*, unpubl. res.). Both sexual and agamosperous populations (morphologically slightly different from each other) were reported from the Saratov region in Russia (Kashin and Cherishova, 1997).

The main goal of this study was to explore the spatial distribution of different cytotypes of *P. echioides* at various spatial scales (from the European part of the total geographic area to the population level) and to outline possible evolutionary and biogeographic scenarios for the observed geographic pattern. Special attention was paid to relatively large mixed-ploidy populations in south-west Moravia consisting exclusively of sexual plants (Rotreklová *et al.*, 2002, 2005), which can serve as a model system for studies concerning mating interactions between diploids and polyploids, fitness differences among ploidy levels in various ontogenetic stages, and modelling dynamics of mixed-ploidy populations.

## MATERIALS AND METHODS

### *Sampling of populations*

A total of 46 Central European populations and 4410 individuals of *Pilosella echioides* (Lumn.) F.W. Schultz & Sch. Bip. (*Hieracium echioides* Lumn.) were surveyed for cytotype variation (Table 1). The scope of this study covered almost all contemporary known populations from Austria, Czech Republic, Germany, Hungary, Poland and Slovakia, comprising the geographic range 46°14' to 53°54'N and 13°56' to 21°48'E (Table 1). Plants from the rest of the distribution area (i.e. Asia and a part of the eastern Europe) were not included in this study due to their uncertain origin (hybridogenous origin cannot be ruled out) associated with taxonomical complexity (*P. echioides* s.l. is split into several minor species here). All samples included in this study could be straightforwardly identified with taxonomic concept adopted for this species in central Europe. At all but one site, fresh leaves of 1–108 (45 in average) randomly selected plants were sampled, according to the abundance of plants at a particular locality and previously assessed cytotype composition (Table 1).

The large population at the Havranické vřesoviště heathland (a former pasture) is currently fragmented into more or less isolated subpopulations (at least in terms of pollinator movements). The fragmentation is due to terrain ruggedness and the invasion of *Arrhenatherum elatius* (L.) J. Presl & C. Presl or trees and shrubs. Altogether 19 subpopulations and 2388 individuals (54.1 % of total amount) were selected here and screened for small-scale ploidy level variation (Table 2). Depending on the abundance of plants, approx. 29–179 individuals per subpopulation were sampled to assess cytotype composition. The only exception was subpopulation Ha13, where a fine-scale distribution analysis was performed and altogether 979 plants were screened for ploidy level with their position recorded on an orthogonal grid (Table 2 and Fig. 4A).

TABLE 1. List of studied *Pilosella echinoides* populations complemented by brief locality descriptions, geographical coordinates, altitude, DNA-ploidy levels, number of plants and the relative frequencies for particular cytotypes.

Locality code	Locality details	Geographic coordinates (WGS 84)	Altitude (m a.s.l.)	DNA ploidy level	No. of plants	Relative frequency	Cited ploidy levels
D1	Germany, Brandenburg, distr. Barnim: village of Niederfinow, waste sandy places and heathland along the road near the Schiffhebewerk	52°50'50"N 13°56'50"E	30–80	2x	70	1.00	2x (Rotreklová <i>et al.</i> , 2002)
D2	Germany, Brandenburg, distr. Barnim: Falkenberg Hills, pastures above the road from village of Amalienhof to village of Struvenberg ca 0.4 km NW of Amalienhof	52°49'30"N 13°56'10"E	40	2x	50	1.00	2x (Rotreklová <i>et al.</i> , 2002)
PL1	Poland, Wyżyna Małopolska upland, distr. Sandomierz. Góry Pieprzowe nature reserve near Sandomierz, loess slopes above the Wisła River, 4-8 km E of the city center	50°41'03"N 21°48'15"E	200	2x	51	1.00	2x (Rotreklová <i>et al.</i> , 2005)
PL2	Poland, Wyżyna Małopolska upland, distr. Busko-Zdrój, Skorocice village, Skorocice nature reserve, grassland on gypseous rocks	50°25'40"N 20°40'06"E	210	2x	51	1.00	2x (Rotreklová <i>et al.</i> , 2005)
PL3	Poland, Lower Odra valley, distr. Szczecin, Cedynia village, sandy soils near the road between villages of Bielinek and Dolny Lubiechów ca. 0.8 km of Bielinek	52°55'40"N 14°09'30"E	50	2x	40	1.00	2x (Rotreklová <i>et al.</i> , 2002)
PL4	Poland, Lower Wisła valley, distr. Chełmno, Góra św. Wawrzyńca 1.3 km WNW of the village Kaldusy, sandy grassland	50°19'38"N 18°22'55"E	80	2x	15	1.00	
PL5	Poland, Lower Wisła valley, distr. Sztum, Biała Góra nature reserve, ca. 0.8 km SE of the village Biała Góra, sandy slopes, very rare (1 plant)	53°54'43"N 18°54'03"E	32	2x	1	1.00	
PL6	Poland, Wyżyna Małopolska upland, distr. Radom, Iłża, 0.2 km E of the castle ruins, mesophilous grassland (pasture) on loess	51°09'47"N 21°14'31"E	210	2x	15	1.00	
PL7	Poland, Wyżyna Małopolska upland, distr. Ostrowiec Świętokrzyski, Ćmielów, 0.5 km S of the village, loess slopes on the road to Krzczonowice	50°53'06"N 21°30'27"E	185	2x	15	1.00	
PL8	Poland, Wyżyna Małopolska upland, distr. Ostrowiec Świętokrzyski, Goździelin, 0.6 km S of the village, loess slopes on the road to Moczydło	50°53'50"N 21°21'31"E	190	2x	15	1.00	
SK1	Slovakia, distr. Trebišov: Streda nad Bodrogom village, Tarbucka hill (277 m), sands on the NW slope ('Veterné piesky')	48°22'41"N 21°46'59"E	150	2x	52	1.00	2x (Rotreklová <i>et al.</i> , 2005)
SK2	Slovakia, distr. Malacky: Borský Mikuláš village, a pinewood along the road from Borský Mikuláš village to Šaštín village	48°37'45"N 17°11'17"E	200	2x	49	0.98	2x (Rotreklová <i>et al.</i> , 2005)
SK3	Slovakia, Nitriansky kraj, distr. Nové Zámky: Čenkov, grassland on sand (nature reserve), 0.8 km NW of the village	47°46'07"N 18°31'11"E	116	4x	4	1.00	4x (Peckert <i>et al.</i> , 2005)
SK4	Slovakia, Nitriansky kraj, distr. Nové Zámky: Jurský Chlm, near the road 0.5 km S of the village, grassland	47°47'59"N 18°32'08"E	150	4x	2	1.00	4x (Peckert <i>et al.</i> , 2005)
A1	Austria, Burgenland, Leithagebirge: Winden am See, Mt. Zeilerberg (302 m) ca 2 km N of the village of Winden am See, grassland	47°58'18"N 16°45'25"E	260	2x	32	1.00	2x (Rotreklová <i>et al.</i> , 2002)
A2	Austria, Niederösterreich: Retz, Windmühlenberg ca 0.7 km NW of the town Retz, dry grassland	48°45'42"N 15°56'27"E	317	2x 3x 4x	11 27 25	0.17 0.43 0.40	2x, 3x, 4x (Schuhwerk and Lippert, 1997)
A3	Austria, Niederösterreich, distr. Gänserndorf: slopes above the road near the railway station Helmahof	48°18'37"N 16°35'47"E	160	2x	50	1.00	
A4	Austria, Niederösterreich, distr. Gänserndorf: open sandy sites ca. 1 km N of the village of Markhof	48°16'43"N 16°50'19"E	150	2x 3x 4x	100 1 7	0.93 0.01 0.06	2x, 4x (Rotreklová <i>et al.</i> , 2002)
A5	Austria, Niederösterreich: Retz, hill Gollitsch (325 m) ca 0.5 km SW of the town of Retz, dry grassland	48°45'15"N 15°56'37"E	320	2x 3x 4x 5x	81 17 4 1	0.79 0.16 0.04 0.01	
A6	Austria, Niederösterreich: Retz, slopes of Talberg hill (302 m) ca 1.0 km W of the village of Obernalb, dry grassland	48°44'51"N 15°55'17"E	390	4x	50	1.00	
H1	Hungary, Komárom-Esztergom megye: Tokod-altáró, N margin of a sand-pit 1 km S of the village (Oldal-földek), sandy grassland	47°43'23"N 18°41'42"E	200	4x	52	1.00	
H2	Hungary, Komárom-Esztergom megye: Dorog, Arany-hegy ca 2 km SE of the railway station, dry grassland	47°42'20"N 18°44'22"E	170	4x	50	1.00	
H3	Hungary, Komárom-Esztergom megye: Dorog, Sátor-kő hill, 1 km SE of the railway station in the town	47°43'13"N 18°45'05"E	165	4x	50	1.00	
H4	Hungary, Komárom-Esztergom megye: Leányvár, Kalap-hegyi-dűlő NW of the village, near Vaskapuszta (Tapétagyár)	47°41'40"N 18°45'35"E	170	4x	50	1.00	

Continued

TABLE 1. *Continued*

Locality code	Locality details	Geographic coordinates (WGS 84)	Altitude (m a.s.l.)	DNA ploidy level	No. of plants	Relative frequency	Cited ploidy levels
H5	Hungary, Somogy megye: Nagybajom, Homokpuszta (near the road to Böhönye), 3.5 km NW of the village, sandy grassland	46°24'16"N 17°28'34"E	150	2x	50	1.00	
H6	Hungary, Baranya megye: Nagyharsány, Villányi hegység, Mt. Szársomlyó, Szoborpark, upper margin of a quarry, along a path	45°51'25"N 18°25'45"E	150	4x	20	1.00	
H7	Hungary, Veszprém megye: Lesenceistvánd, near a main road to Sümeg, near a sand-pit, ca 1.5 km NE of the village, sandy grassland	46°52'58"N 17°21'35"E	140	2x	30	1.00	
H8	Hungary, Bakony Mts.: Fenyőfő village, the sands on the southwest border of the village	47°17'N 17°45'E	320	2x	65	1.00	2x (Rotreklová <i>et al.</i> , 2005)
H9	Hungary, distr. Heves, Bükk Mts: stony slopes 0.8 km NW of the village of Szarvaskő (railway station)	47°59'35"N 20°19'48"E	340	2x	53	1.00	2x (Rotreklová <i>et al.</i> , 2002)
H10	Hungary, Borsod-Abaúj-Zemplén megye: Tállya, Patócs hill, grassland on the top, 2.7 km NW of the village	48°14'56"N 21°12'05"E	200	4x 5x 6x	43 2 3	0.90 0.04 0.06	
H11	Hungary, Pest megye: Újhartyán, disturbed grassland at the highway exit (Exit 44) NE of the village	47°13'29"N 19°24'27"E	118	4x	50	1.00	
H12	Hungary, Székesfehérvár: Balinka, bottom of a deserted limestone quarry near the road from Balinka to Bodajk	47°19'00"N 18°12'24"E	172	4x	41	1.00	4x (Peckert <i>et al.</i> , 2005)
H13	Hungary, Komárom-Esztergom megye: Szomód, Les-hegy hill, around the top, 2.2 km NW of the village	47°41'56"N 18°19'24"E	230	4x 6x	49 1	0.98 0.02	
H14	Hungary, Bács-Kiskun megye: Kelebia, pine forest margin on sand 5 km NW of the village	46°14'09"N 19°38'21"E	130	4x	25	1.00	
H15	Hungary, Pest megye, Szentendrei sziget: Tótfalu, dry grassland on sand dunes 4.3 km NNW of the village center	47°47'30"N 19°04'42"E	103	4x	26	1.00	
CZ1	Czech Republic, distr. Mělník: village of Tišice, the sands along the railway Tišice – Neratovice, ca 100 m from railway station Tišice	50°15'57"N 14°33'07"E	160	2x	62	1.00	2x (Rotreklová <i>et al.</i> , 2005)
CZ2	Czech Republic, České středohoří Mts., distr. Litoměřice: Kalvárie ('Tříkřížová hora') hill, rocks above the Labe river, 1.5 km NW of the village of Velké Žemoseky	50°32'50"N 14°03'01"E	214	3x 4x 5x	18 35 40	0.19 0.38 0.43	3x, 4x (Rotreklová <i>et al.</i> , 2002)
CZ3	Czech Republic, distr. Beroun: 'Trubínský vrch' hill at the W margin of the village of Trubín	49°56'37"N 13°59'47"E	325	2x	27	1.00	2x (Rotreklová <i>et al.</i> , 2002)
CZ4	Czech Republic, distr. Třebíč: nature reserve 'Mohelenská hadcová step', serpentine rocks near the village of Mohelno	49°06'30"N 16°11'10"E	350	2x	19	1.00	2x (Rotreklová <i>et al.</i> , 2002)
CZ5	Czech Republic, distr. Znojmo: hill called 'U Michálka' at the S margin of the village of Bohutice, dry grassland	48°59'03"N 16°21'31"E	270	4x	15	1.00	4x (Rotreklová <i>et al.</i> , 2002)
CZ6	Czech Republic, distr. Znojmo: hill at the NE margin of the village of Hoštěradice (place called 'U kapličky'), dry grassland	48°57'12"N 16°15'46"E	240	4x 6x	46 3	0.94 0.06	2x, 3x (Rotreklová <i>et al.</i> , 2002)
CZ7	Czech Republic, distr. Znojmo: slopes in the valley of the Dyje river SE of the village of Dyje	48°50'26"N 16°07'27"E	232	2x	37	1.00	2x (Rotreklová <i>et al.</i> , 2002)
CZ8	Czech Republic, distr. Znojmo: heathland and waste places S of the village of Konice (nature reserve 'Popické kopečky')	48°49'44"N 16°01'11"E	300	3x 4x 5x	4 10 1	0.27 0.67 0.06	3x, 4x (Rotreklová <i>et al.</i> , 2002)
CZ9	Czech Republic, distr. Znojmo: Skalky hill 0.6 km S of the church in the village of Havraníky	48°48'18"N 16°00'31"E	304	4x	100	1.00	4x (Rotreklová <i>et al.</i> , 2002)
CZ10	Czech Republic, distr. Znojmo: Hnanice, S slopes of the 'Staré vinice' hill (339 m) ca 0.8 km N of the village of Hnanice	48°48'23"N 15°59'28"E	318	3x 4x 5x	65 30 13	0.60 0.28 0.12	
CZ11	Czech Republic, distr. Znojmo: Havranické vřesoviště heathland 1.0 km W to NW of the village of Havraníky For more details and data on subpopulation level see Fig. 3 and Table 2.	48°49'10"N 16°00'10"E	325	2x 3x 4x 5x 6x	134 1745 483 25 1	0.06 0.73 0.20 0.01 0.00	2x, 3x, 4x (Rotreklová <i>et al.</i> , 2002)



TABLE 2. List of *Pilosella echioides* subpopulations from the Havranické vřesoviště heathland (population CZ11), their cytotype composition, number of plants and relative frequencies of particular cytotypes

Subpopulation code	DNA ploidy level	No. of plants	Relative frequency
Ha1	2x	18	0.13
	3x	108	0.80
	4x	8	0.06
	5x	1	0.01
Ha2	3x	36	0.72
	4x	14	0.28
Ha3	2x	9	0.05
	3x	145	0.81
	4x	20	0.11
Ha4	5x	5	0.03
	3x	19	0.38
	4x	31	0.62
Ha5	2x	6	0.09
	3x	57	0.85
	4x	4	0.06
Ha6	2x	3	0.05
	3x	61	0.94
	4x	1	0.02
Ha7	3x	4	0.08
	4x	46	0.92
Ha8	2x	2	0.03
	3x	44	0.67
	4x	20	0.30
Ha9	3x	71	1.00
Ha10	2x	10	0.17
	3x	24	0.41
	4x	25	0.42
Ha11	2x	47	0.96
	3x	2	0.04
Ha12	2x	20	0.69
	3x	9	0.31
Ha13	2x	8	0.01
	3x	874	0.89
	4x	97	0.10
Ha14	2x	1	0.01
	3x	93	0.85
	4x	15	0.14
	5x	1	0.01
Ha15	3x	13	0.35
	4x	15	0.41
	5x	9	0.24
Ha16	3x	55	0.80
	4x	13	0.19
	5x	1	0.01
Ha17	2x	10	0.08
	3x	57	0.46
	4x	57	0.46
	6x	1	0.01
Ha18	4x	49	1.00
Ha19	3x	73	0.49
	4x	68	0.46
	5x	8	0.05

Voucher specimens of selected plants from each population are deposited in herbarium PRA.

#### Flow cytometry

All 4410 collected plants were subjected to DNA-ploidy level estimation via flow cytometry (Suda *et al.*, 2006). Young, intact leaf tissue of the analysed plant(s) and an appropriate amount of leaf tissue of the internal reference standard

[*Bellis perennis* L., 2C-value set to 3.96 pg following Leong-Skorničková *et al.* (2007)] were co-chopped using a sharp razor blade in a plastic Petri-dish containing 0.5 mL of ice-cold Otto I buffer (0.1 M citric acid, 0.5 % Tween 20) (Otto, 1990; Doležal *et al.*, 2007). The crude suspension was filtered through a 0.42- $\mu$ m nylon mesh to remove tissue debris and then incubated for at least 10 min at room temperature. Isolated nuclei were stained with 1 mL of Otto II buffer (0.4 M Na<sub>2</sub>HPO<sub>4</sub>·12H<sub>2</sub>O) supplemented with the AT-selective fluorochrome 4',6-diamidino-2-phenylindole (DAPI) and  $\beta$ -mercaptoethanol at final concentrations of 4  $\mu$ g mL<sup>-1</sup> and 2  $\mu$ L mL<sup>-1</sup>, respectively. Immediately after staining, the relative fluorescence intensity of at least 3000 particles was recorded with a PA-II flow cytometer (Partec GmbH, Münster, Germany) equipped with a mercury lamp for UV excitation. Resulting histograms were evaluated with FloMax software (Partec GmbH) and DNA ploidy levels were determined on the basis of the sample/standard ratio. The *Pilosella* tissue was processed within 7 d of collection. Usually, bulk samples from three (but for ploidy-uniform populations up to ten) *Pilosella* plants (0.5 cm<sup>2</sup> of leaf from each plant) were measured. Previous trial analyses confirmed the reliability of such a protocol (i.e. the lack of endopolyploidy and the low mitotic activity in the *Pilosella* tissues selected for flow analyses). In addition, good congruency between the number of nuclei in particular peaks and the number of analysed individuals with different ploidy levels allowed the proportions of the cytotypes in mixed samples to be estimated with high accuracy. Only histograms with coefficients of variation of G<sub>0</sub>/G<sub>1</sub> peaks of both the bulked sample and the standard below 3.5 % were considered. If the quality of analyses did not meet this criterion, all plants from the bulked sample were re-analysed separately (to detect potential between-plant differences in fluorescence intensity).

Chromosome counts are available from all ploidy (but hexaploid) levels and a representative subset of populations included in the present study (Table 1). A standard procedure to correct evaluation of ploidy level via flow cytometry in concordance with chromosome counts has been already set (Rotreklová *et al.*, 2002, 2005). Genome size of di-, tri-, tetra- and pentaploids was published by Suda *et al.* (2007a).

#### Spatial analyses

For fine-scale spatial analysis of cytotype distribution, the position of all individuals in the mapped subpopulation (Ha13; population CZ11) was analysed using the *K*-function (Ripley, 1977) in the R-package 'spatstat' (Baddeley and Turner, 2005). Both, the intensity and the type (random, clumped or regular) of clustering of an individual cytotype distribution are detected with the *K*-function and the computation is based on comparison of the number of neighbours within a radius *r* of each individual from the plot with the expected unit density calculated from the number of all individuals and study plot area. For inter-cytotype comparison, the bivariate *K*<sub>12</sub>(*r*)-function (Cressie, 1993) was calculated to assess positive, neutral or negative associations based on counting all neighbours of the other cytotype within *r*. Transformation of *K*-functions to *L*-functions (Doležal *et al.*, 2006) is used to calculate the spatial patterns at various scales, i.e. for increasing

radius  $r$ . Graphic visualization of  $L_{12}(r)$  vs.  $r$  was determined by Monte Carlo permutations with 1000 replications to assess the type of individual association by comparing the behaviour of the L-function in relation to the 95% confidence interval. Values of  $L_{12}(r)$ -function above, within and below the limits of confidence interval point out positive, neutral and negative association of compared cytotypes, respectively.

## RESULTS

### *Cytotype distribution on a large scale*

Five DNA-ploidy levels were discovered among the 4410 plants within 46 populations. A representative histogram bearing the four most common ploidy levels is provided in Fig. 1. While diploids, triploids, tetraploids and pentaploids were known from previous research (Table 1), hexaploids are reported here for the first time. Altogether 11 populations (23.9%) were assessed as mixed and 35 (76.1%) as cytotypically uniform, possessing either diploids (21 populations) or tetraploids (14). Besides the two types of uniform populations (diploid or tetraploid), seven different population types (with respect to cytotype composition) were also found. They consisted of two (three populations), three (six), four (one) and five (one) cytotypes (for details, see Table 1). Whereas populations SK2, H13 and CZ6 had two intermingled cytotypes, where one was dominant and the second one in frequencies below 0.10, complex patterns and more or less balanced compositions of cytotypes were usually observed in populations with sympatric growth of three and more cytotypes (except for populations A4 and H10; Table 1). Overall the distribution patterns (Fig. 2) point to the accumulation of mixed-ploidy populations in south-west Moravia and adjacent regions of Lower Austria, several of which were rare in other parts of the area investigated (i.e. populations CZ2, H10 and H13). While cytotypically uniform populations prevail in the study area, diploid populations were assessed as ubiquitous and only tetraploid ones were shown to have some spatial ties to Hungary (Table 1).

### *Cytotype distribution on a small- and fine-scale*

Detailed investigation of the Havranické vřesoviště heathland (south-west Moravia, population CZ11) revealed a high degree of differentiation in cytotype composition and their relative frequencies at the subpopulation level (Fig. 3 and Table 2). Generally, out of 19 subpopulations examined only two were assessed as cytotype-uniform (i.e. subpopulations 9 and 18 hosting triploids and tetraploids, respectively). The majority of populations were of mixed ploidy levels with two (five subpopulations), three (eight) and four (four) cytotypes co-occurring. Altogether eight different patterns of cytotype composition were identified with the prevailing type of 2x, 3x and 4x individuals growing in sympatry (five subpopulations, 26.3%). Such extensive insight into this particular population enables reliable interpretations of cytotype composition at the individual level (across all subpopulations) and points to a huge overall predominance of triploids (73.1% out of almost 2400 plants analysed). In contrast, only 5.6%, 20.2% and 1.1% representation was ascertained for diploids,

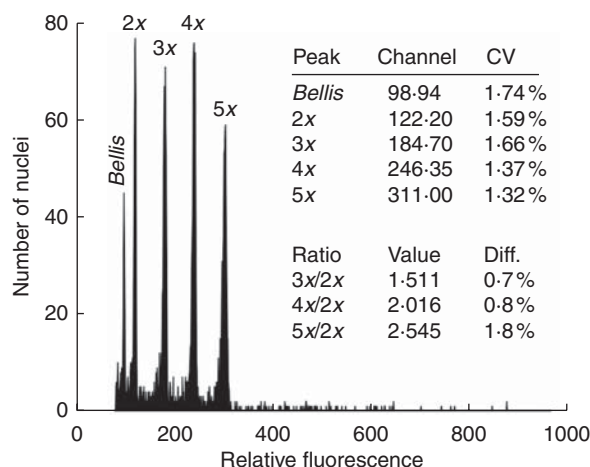


FIG. 1. Flow cytometric histogram of four most common cytotypes (2x, 3x, 4x and 5x) of *Pilosella echioides* from the Havranické vřesoviště heathland (populations CZ11) and the internal reference standard (*Bellis perennis*). Nuclei from all plants were isolated, stained with DAPI and analysed simultaneously. Note low coefficients of variance of resulting peaks and virtually identical size of the monoploid genome of all *Pilosella* cytotypes.

tetraploids and pentaploids, respectively. In addition just a single plant was estimated as hexaploid.

Although all subpopulations exhibit specific combinations and frequencies of particular cytotypes (Table 2), some tendencies should be highlighted. Unlike other parts of the area investigated, diploid and tetraploid cytotypes were predominant in only four subpopulations (two per ploidy level). In contrast, triploids, which were usually very rare, were detected as the dominant cytotype in ten subpopulations (52.6%), and in another five subpopulations they comprise the main admixture. In agreement with the generally observed pattern, pentaploids were a minority component (except for subpopulation Ha15, where there was almost equal representation of triploids, tetraploids and pentaploids; Table 2).

The fine-scale cytotype distribution was studied in one randomly chosen subpopulation (Ha13, one of the 12 subpopulations harbouring at least three different cytotypes) to reveal spatial patterns among ploidy levels at the individual level (Fig. 4A). Remarkably plants of the same cytotype were clumped together and thus a high degree of negative intercytotype association (even at distance of <5 cm) was documented (Fig. 4B and C).

## DISCUSSION

### *Large-scale cytotype distribution*

The diploid cytotype of *Pilosella echioides* prevails in central Europe and is the only one discovered so far in regions north of the Sudeten Mts and the Carpathians (north Germany, Poland). In contrast, both diploid and tetraploid populations with more or less pronounced differences in ecological preferences (sandy places and rocks vs. dry grasslands, respectively) and geographical distribution were found in the Pannonian Basin (samples from Slovakia, Austria and Hungary; Fig. 2 and Table 1). However, such spatial separation could be at least partially caused by the presence of exclusively

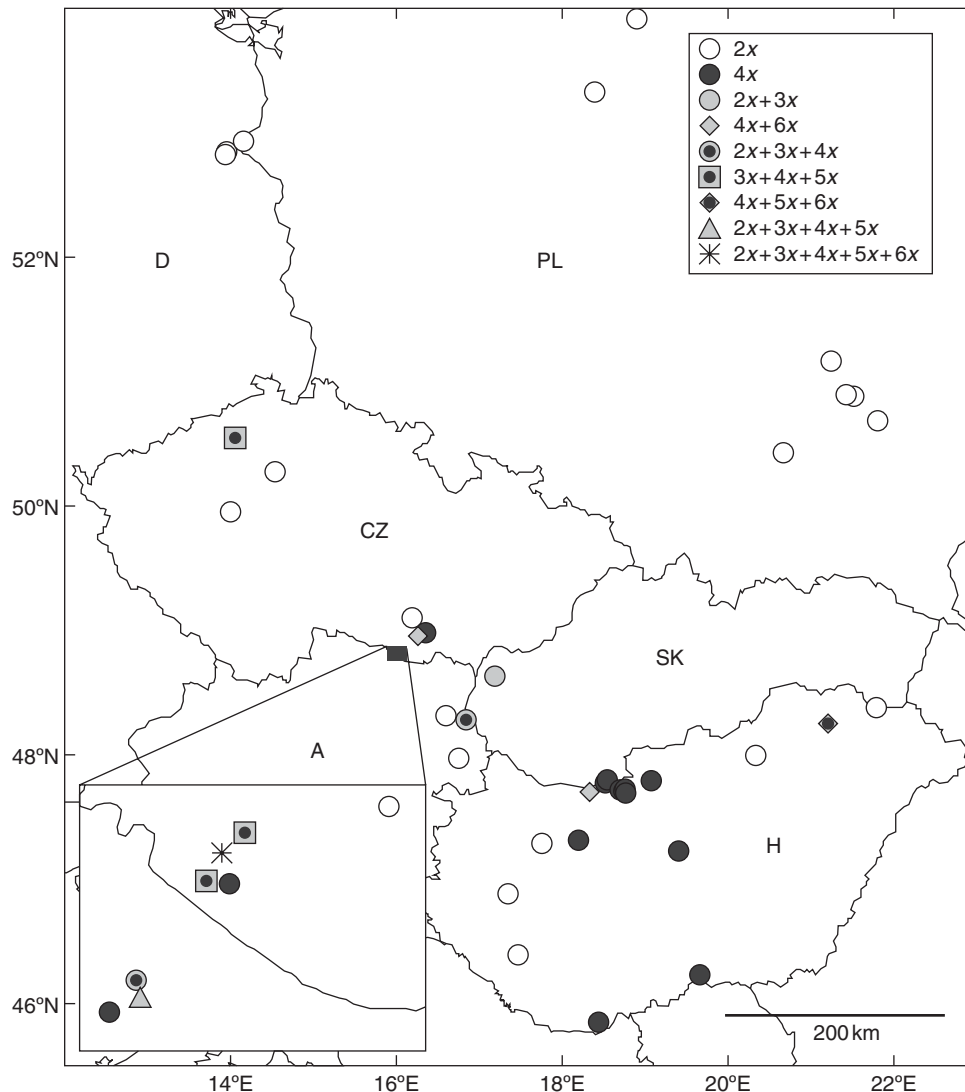


FIG. 2. Distribution of *Pilosella echioides* cytotypes in the area investigated, based on flow-cytometric analysis of 4410 individuals from 46 populations. The upper-right inset shows symbols used for populations with different cytotype composition. The lower-left inset shows the spatial distribution of populations in south-west Moravia (Czech Republic) and Lower Austria. Country abbreviations: A, Austria; CZ, Czech Republic; D, Germany; H, Hungary; PL, Poland; SK, Slovakia. Population CZ11, which is often mentioned in the text, is marked by an asterisk.

agamosperrmous tetraploid populations in this area (Peckert *et al.*, 2005). In agreement with previously published papers (Schuhwerk and Lippert, 1997; Rotreklová *et al.*, 2002, 2005), considerable cytotype variation and the common occurrence of mixed-ploidy populations were found in south-west Moravia and in the adjacent region of Lower Austria (Fig. 2 and Table 1). On the other hand, the presence of hexaploids (populations H10 and H13) and pentaploids (population H10) in otherwise tetraploid Hungarian populations was hitherto unknown. The overall distribution pattern showed only poor spatial structuring of cytotypes in contrast to previous studies from the same area examining polyploid complexes of *Vicia cracca* (Trávníček *et al.*, 2010), *Pilosella officinarum* (Mráz *et al.*, 2008), *Knautia arvensis* (Kolář *et al.*, 2009) and *Allium oleraceum* (Duchoslav *et al.*, 2010). Nevertheless, a similar pattern, showing the non-structured distribution of ploidy-uniform and the scattered presence of

mixed-ploidy populations, has previously been reported in *Gymnadenia conopsea* (Trávníček *et al.*, 2011). Moreover, *Pilosella echioides* with up to five ploidy levels within a single population joins *Gymnadenia conopsea* and *Senecio carniolicus* (Schönschwetter *et al.*, 2007; Suda *et al.*, 2007b; Hülber *et al.*, 2009) in being one of the most diverse polyploid complexes ever detected.

#### *Small-scale cytotype distribution and sympatric occurrence*

The present detailed research of the most diverse mixed-ploidy population (Havranické vřesoviště heathland, CZ11) revealed stochastic spatial arrangement of cytotypes at the entire population level and a pronounced cytotype coexisting ability in various types of composition and frequencies (Fig. 3 and Table 2). This challenges theoretical predictions suggesting that the co-occurrence of ploidy types should be





FIG. 3. Distribution of *Pilosella echioides* cytotypes in spatially isolated subpopulations in the Havranické vřesoviště heathland (population CZ11, south-west Moravia, Czech Republic). The lower-right inset shows symbols used for populations with different cytotypic composition. Numbers refer to detailed information on subpopulations in Table 2. (Orthophoto used with the permission of the Podyjí National Park Administration, ©GEODIS Brno 2008.)

unstable (minority cytotypic extinction) except for the evolution of reproductive barriers (e.g. Rieseberg and Willis, 2007) both pre-zygotic (e.g. differences in pollinator's assemblages and/or selective pollinator's foraging behaviour; Thompson *et al.*, 2004; Nuismer and Cunningham, 2005; Thompson and Merg, 2008; Husband and Sabara, 2004; Kennedy *et al.*, 2006) and/or post-zygotic (e.g. triploid block; Baack, 2005a; Köhler *et al.*, 2010). However, reproductive barriers and assortative mating have not evolved in *Pilosella echioides* since all cytotypes can hybridize and produce viable offspring without dramatically reduced fitness or pollen preferences (Peckert and Chrtek, 2006; P. Trávníček *et al.*, unpubl. res.). A high proportion (even dominance) of triploids indicates the frequent occurrence of inter-cytypic hybridization and the lack of triploid block. Moreover, the cytotypes do not differ from each other in their morphological characters, flower display and flowering time. Thus assortative mating caused by pollinator movements within rather than between cytotypes very likely should be ruled out. A similar system lacking pre-mating barriers, despite morphological differences between cytotypes, was found in *Gymnadenia conopsea s.l.* (Jersáková *et al.*, 2010), but post-zygotic mating barriers acting in those populations restricted the possibility of inter-cytypic hybrids (Trávníček *et al.*, 2011).

The fine-scale spatial distribution of cytotypes showed outstanding intra-cytypic aggregation and inter-cytypic separation (Fig. 4) that cannot be easily explained by one simple hypothesis. Vegetative spread by daughter rosettes could easily explain the intra-cytypic aggregation but it is rather rare (P. Trávníček *et al.*, unpubl. res.). The short-distance achene dispersal can be suggested as another possible explanation as it could contribute to cytypic coexistence (Baack,

2005b) and to accumulation of particular cytotypes (e.g. seeds from  $2x \times 4x$  crosses are almost exclusively triploid; Peckert and Chrtek, 2006). On the other hand, the achenes are adapted to long-distance dispersal due to the presence of a pappus. An alternative hypothesis is spatial separation (pre-mating barrier), i.e. microhabitat differentiation of cytotypes (Felber-Girard *et al.*, 1996; Šafařová and Duchoslav, 2010) which can significantly increase the probability of successful intra-cytypic mating and, thus, decrease the minority cytypic disadvantage (Levin, 1975; Felber, 1991). However, selection pressures leading to spatial separation are lacking due to the aforementioned free mating, producing viable and usually fertile inter-cytypic hybrids. In addition, aggregation of triploids is contrariwise due to the absence of recruitment ability by reciprocal hybridization (Peckert and Chrtek, 2006). Furthermore, separation on such a fine-scale is not likely to prevent pollen movement between cytotypes (the pollinators have longer cruising ranges). Last but not least, a preliminary study (P. Trávníček *et al.*, unpubl. res.) does not show correlations between cytypic occurrence and vegetation features (density, structure, species composition), although other factor(s) can drive the fine-scale distribution of cytotypes. Clearly the available data on cytypic coexistence in *Pilosella echioides* are insufficient to completely address such curious phenomena and future in-depth studies are needed.

#### Origin of populations with ploidal heterogeneity

Although the present study corroborates the occurrence of mixed-ploidy populations of *Pilosella echioides* in south-west Moravia and Lower Austria, their origin is still puzzling. Nevertheless, the hypothesis that the polyploids arose within



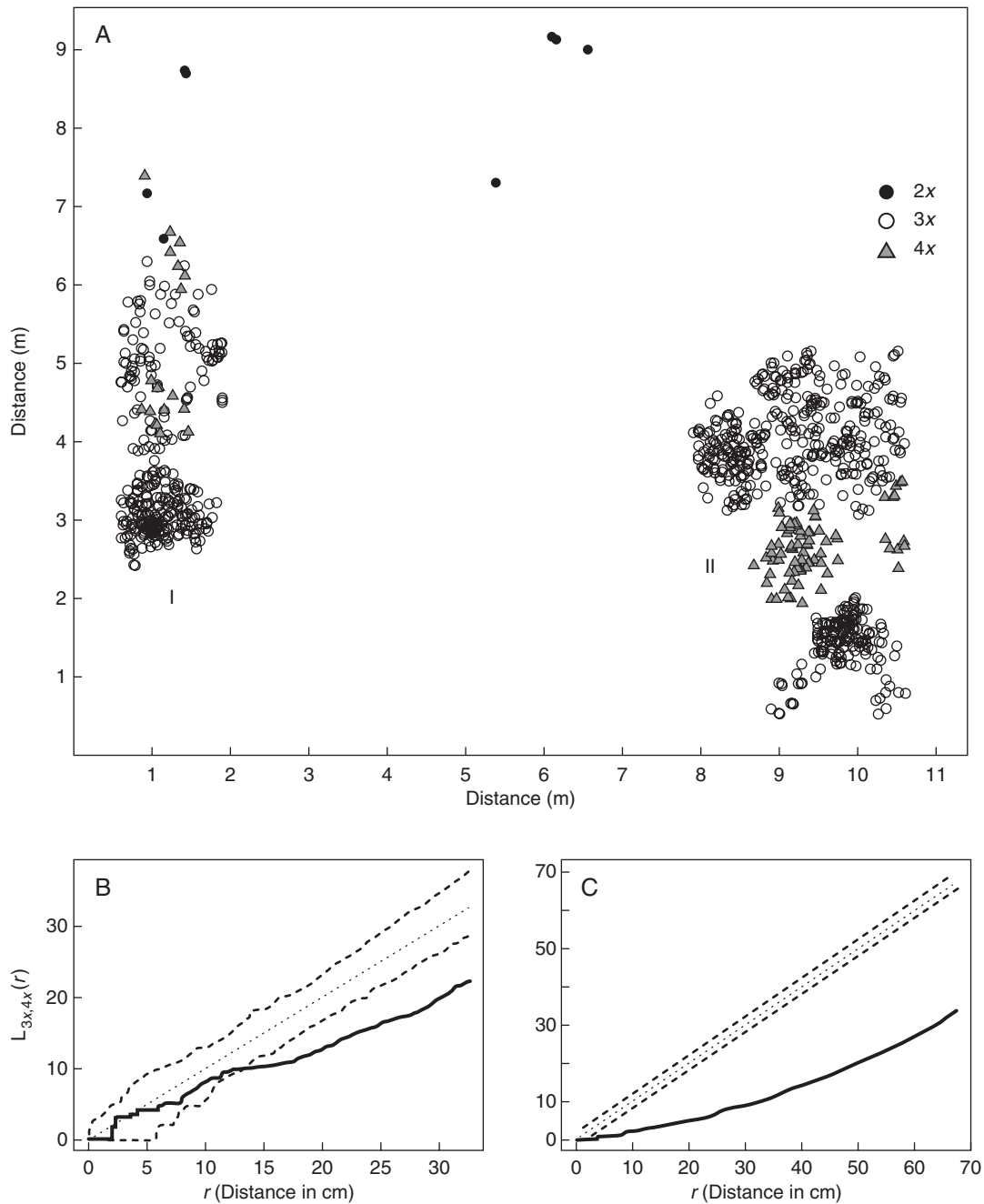


FIG. 4. Fine-scale distribution of *Pilosella echioides* cytotypes in subpopulation Ha13 from the Havranické vřesoviště heathland (population CZ11). (A) Spatial arrangement of *Pilosella* plants – note distinct intra-cytotype clustering and structuring of the subpopulation into separate areas I and II. (B, C) Pairwise inter-cytotype association between triploid and tetraploid plants in both areas (I in B and II in C). Values of  $L_{3x,4x}(r)$ -function are shown by a thick continuous line and dashed lines delimit a 95 % confidence interval. The negative association of cytotypes is indicated by smaller values of  $L(r)$ -function than is the lower threshold of the confidence interval which is obvious in both subplots. It means that both cytotypes are strongly clumped together and spatially separated from each other. Diploids were excluded from pairwise comparisons due to their low abundance.

diploid populations, i.e. establishment of a so-called ‘primary hybrid zone’ (e.g. Felber, 1991; Ramsey and Schemske, 1998; Petit *et al.*, 1999) appears to be likely. It is possible that the scarce presence of triploids and/or tetraploids in otherwise pure diploid populations outside this area (e.g. populations SK2 and A4) could point to the first stages of polyploid establishment (Husband, 2004; Yamauchi *et al.*, 2004; Baack, 2005a), analogous to past processes in populations in south-

west Moravia. However, an alternative explanation, namely long-distance seed dispersal (promoted by achenes with pappus) from polyploid populations, can be suggested. A detailed study of *Pilosella aurantiaca*, a species with seeds nearly identical to *P. echioides*, revealed that up to 95 % achenes were dispersed within 6 m distance from the mother plant (Stergios, 1976). Similar dispersal distances were observed in another Asteraceae species, e.g. in *Senecio*

*inaequidens* where 99.8 % of achenes were dispersed within 100 m with a maximum deposition rate of 5.2 m from the maternal plant (Monty *et al.*, 2008). Although short-distance achene dispersal seems to prevail within close relatives, the long-distance achene dispersal is inherently difficult to quantify and cannot be simply ruled out as a contributory element to gene flow in *Pilosella echioides*. It can also be argued that polyploid achenes (embryos) were observed in neither hand-pollinated diploids (Peckert and Chrtek, 2006) nor natural diploid populations (P. Trávníček *et al.*, unpubl. res.) therefore long-distance seed dispersal is a plausible explanation.

The 'primary hybrid zone' hypothesis can be supported by the overall cytotype distribution pattern, i.e. by the lack of a transitional zone between the two main ploidy levels (di- and tetraploids), either in the entire area investigated or in the vicinity of populations showing an increased incidence of mixed-ploidy (Fig. 2). Therefore, the hypothesis of enhanced occurrence of populations with cytotype variability in such zones (e.g. Husband and Schemske, 1998; Petit *et al.*, 1999, Trávníček *et al.*, 2010) seems to be unlikely. Nevertheless, different past cytotype distribution patterns could boost the establishment of a contact zone between cytotypes and contribute to the present state.

Population history *per se* can play a crucial role in the formation of cytotype co-occurrence. Several populations (including the cytotypically diverse CZ2, CZ8, CZ10 and especially CZ11) inhabit relict areas, such as rocks in canyons of big rivers (Labe, Dyje), where a permanent forest-free area has been hypothesized (Chytrý and Vicherek, 1995, 2003; Ložek, 2007). The long-term occurrence of different cytotypes in such habitats, leading to the evolution of mechanisms allowing sympatric growth is one possible explanation. This agrees with the unusual occurrence of sexual hexaploids in closely related *Pilosella officinarum* in the same habitats (Mráz *et al.*, 2008). Alternatively, relict habitats may have facilitated the long-term occurrence of diploids and tetraploids in close vicinity and their equal adaptation to local conditions. More recently, as new secondary habitats are created by man-made changes (including deforestation and maintenance of suitable forest-free areas by pasture) both cytotypes may have been able to spread out widely and form mixed-ploidy populations. If so, then the relatively short-term coexistence of cytotypes may explain the absence of any kind of pre-zygotic isolation. Clearly, all outlined scenarios need further in-depth study and no definitive conclusions can be drawn at present.

Besides the mixed-ploidy populations comprising diploids and tetraploids, it is noted that diploids are absent in several populations (e.g. populations CZ2, CZ8 and CZ10), and here only polyploids (tetraploids and higher) coexist (populations H10, H13 and CZ6). That such populations were founded by tetraploids is very likely and their further diversification may have been triggered by the fusion of reduced and unreduced gametes and subsequent backcrossing. Nevertheless, an allopolyploid origin of higher ploidy levels via introgression from coexisting *Pilosella* species cannot be ruled out completely despite the morphological identity of cytopes and the fact that *Pilosella* species differ distinctly in their genome size (and thus interspecific hybridization is detectable by flow cytometry; Suda *et al.*, 2007a).

#### *The role of triploids in mixed-ploidy populations*

One of the most challenging findings of the present study is the prevalence of fertile (though with lower fecundity in comparison with di- and tetraploids; Peckert and Chrtek, 2006) triploids in several cytotypically diverse populations. The role of triploids has hitherto been understood to be crucial in promoting autotetraploid establishment (Husband, 2004), despite the observation of lower fitness and fecundity of triploids (Felber, 1991; Burton and Husband, 2000; Baack, 2005a). This has resulted in the evolution of various pre-zygotic barriers between newly arisen tetraploids and their diploid progenitors constraining triploid genesis based on the principle of minority cytotype disadvantage (Levin, 1975; Husband, 2000; Burton and Husband, 2001; Baack, 2005a). Contrary to this broadly accepted model, the present research indicates that triploids are essentially equivalent to diploids and tetraploids, if free inter-cytotype mating and sufficient triploid fitness are ensured. Under these conditions triploids even produced the highest cytotype seed variation and contributed significantly to the maintenance of mixed-ploidy populations (Peckert and Chrtek, 2006).

#### *Conclusions and future prospects*

*Pilosella echioides*, with up to five different ploidy levels growing in sympatry, represents a species with one of the most complex patterns of cytotype coexistence so far detected. The uniqueness of this plant system also lies in (a) a presumable lack of inter-cytotype breeding barriers and (b) a high frequency of triploid individuals. These findings stand in stark contrast to generally accepted theories of cytotype coexistence and call for further research aimed at understanding the evolutionary forces governing formation, establishment and further fate of different cytotypes. The possibility of a long-term ploidy co-existence due to free inter-cytotype mating interactions reshapes our views on (a) the evolution of pre- and/or post-zygotic breeding barriers among cytotypes, (b) the patterns and dynamics of inter-cytotype interactions and (c) the processes maintaining mixed-ploidy populations. In addition, the abundance of triploid plants coupled with their fertility provides qualitatively new information on the evolutionary potential of this odd-ploidy level and its role in maintaining ploidy mixtures. Collectively, *Pilosella echioides* represents a unique system for detailed investigation of evolutionary dynamics of populations with ploidy heterogeneity.

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