Cytotype distribution in mixed populations of polyploid *Allium oleraceum* measured at a microgeographic scale

Rozšíření ploidií v cytotypově smíšených populacích *Allium oleraceum*

Lenka Šafářová & Martin Duchoslav

Department of Botany, Faculty of Science, Palacký University, Šlechtitelů 11, CZ-783 71 Olomouc, Czech Republic, e-mail: safarova@vcm.cz, martin.duchoslav@upol.cz

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Despite the substantial knowledge of the variation in cytotypes at large spatial scales for many plants, little is known about the rates at which novel cytotypes arise or the frequencies and distributions of cytotypes at local spatial scales. The frequency distribution, local spatial structure, and role of habitat differentiation of tetra-, penta- and hexaploid cytotypes of the bulbous geophyte *Allium oleraceum* were assessed in 21 populations sampled in the Czech Republic. The ploidy levels determined by flow cytometry confirmed that there was a mixture consisting of two or three cytotypes (i.e. 4x+5x, 4x+6x, 5x+6x, 4x+5x+6x). In addition, mixtures of cytotypes were found at sites previously considered to be cytotype-homogeneous. At all sites previously found to contain a mixture of two cytotypes, no plants with the third ploidy level were found. Although the relative frequencies of cytotypes varied considerably both among and within populations, mixed populations consisting of tetra- and hexaploids were usually dominated by tetraploids. This suggests that there are secondary contacts among cytotypes but there is little gene flow among them except for the rare formation of hexaploids in tetraploid populations. Cytotypes were not randomly distributed over the study area but were spatially segregated at either 47.6% or 61.9% of the sites investigated, depending on the statistical test (Mantel test or average distance test) used. When the composition of habitats at each of the sites is taken into account, cytotypes were more frequently spatially segregated at sites with a heterogeneous environment than a homogeneous environment. This implies that the cytotypes are ecologically differentiated. The frequent co-occurrence of cytotypes, with or without significant spatial segregation, at many sites with heterogeneous or homogeneous environments, however, suggests that niche differentiation alone is probably ineffective in determining co-occurrence. It is supposed that the prevailing vegetative reproduction associated with local dispersal, a high population density of the species in a landscape, and non-equilibrial processes influencing the establishment and extinction of *A. oleraceum* populations can also support the local co-occurrence of cytotypes.

K e y w o r d s: *Allium oleraceum*, clonal reproduction, coexistence, cytotype, flow cytometry, habitat differentiation, local spatial structure, ploidy

Introduction

Polyploidy is a highly dynamic process, which has had an important role in the evolution and speciation of angiosperms and evolutionary history of other eukaryotes (Grant 1981, Thompson & Lumaret 1992, Wendel 2000, Soltis et al. 2003). It is reported that the increased genetic buffering provided by having extra genome copies and changes in gene expression (Soltis & Soltis 1999, Otto & Whitton 2000, Wendel 2000, Soltis et al. 2003) produces novel characters that enable polyploids to adapt to new environments (Levin 1983, Brochmann & Elven 1992, Bretagnole & Thompson 1996, Levin 2002). Consequently, polyploid populations often occupy habitats intermediate between those of their

progenitors or colonize a wider or different range of habitats (Lewis 1980, Petit et al. 1999, Levin 2002, Soltis et al. 2003). This 'adaptive evolutionary scenario' predicts trends towards: (i) the parapatry or allopatry of cytotypes at large spatial scales if the fitness of the cytotypes is a function of an environment that is gradually changing at a geographical scale (Engen et al. 2002, Johnson et al. 2003), or (ii) partial or complete sympatry with ecological isolation between cytotypes if the environmental factors have a mosaic structure (Levin 2002). Forces producing macrogeographic polyploid variation also operate at a local scale. As most ecological variables are spatially structured (Legendre & Legendre 1998, Koenig 1999), ecological differentiation between cytotypes leads to the expectation of either a patchy distribution of the cytotypes at a site (Meirmans et al. 2003) or cytotype-homogeneous populations (Baack 2004).

Some studies have investigated the spatial patterns of cytotypes at mixed-cytotype sites (Keeler et al. 1987, Lumaret et al. 1987, Keeler 1992, 2004, van Dijk et al. 1992, McArthur & Sanderson 1999, Meirmans et al. 1999, 2003, Hardy et al. 2000, Husband & Schemske 2000, Suda 2002, Baack 2004, Suda et al. 2004, Schönswetter et al. 2007, Halverson et al. 2008, Hülber et al. 2009, Kolář et al. 2009) and the results are inconsistent. Husband & Schemske (2000), Meirmans et al. (2003), Baack (2004), Suda et al. (2004), Schönswetter et al. (2007), Hülber et al. (2009) and Kolář et al. (2009) found a microspatial structure in the distribution of cytotypes of *Chamerion angustifolium*, *Taraxacum* sect. *Ruderalia*, *Ranunculus adoneus*, *Empetrum* spp., *Senecio carniolicus* and *Knautia arvensis* agg. On the other hand, Lumaret et al. (1987), Keeler (1992, 2004), van Dijk et al. (1992), McArthur & Sanderson (1999), Meirmans et al. (1999), Hardy et al. (2000), Suda (2002) and Halverson et al. (2008) found either no or only a weak local spatial aggregation of cytotypes of *Dactylis glomerata*, *Andropogon gerardii*, *Plantago media*, *Artemisia* subgen. *Tridentatae*, *Taraxacum* sect. *Ruderalia*, *Centaurea jacea*, *Vaccinium* sect. *Oxycoccus* and *Solidago altissima*.

The effect of environmentally independent processes ('non-adaptive scenario') might account for the contradictory nature of the results. Polyploids must originate within populations of their progenitors, thus, at least the establishment of a new cytotype must occur in sympatry with its progenitors. Subsequent spatial separation between the new cytotype and its progenitors is usually directed by frequency-dependent mating success, which gradually leads to the elimination of the minority cytotype ('minority cytotype exclusion'; Levin 1975, Ramsey & Schemske 1998). The coexistence of cytotypes may therefore be of a temporary nature (Husband & Schemske 1998, Baack 2005). Recent studies (Burton & Husband 1999, Mandáková & Münzbergová 2006, Dorken & Pannell 2007) indicate that 'the minority cytotype exclusion' mechanism works not only in primary hybrid zones but also in zones of secondary contact between cytotypes (sensu Petit et al. 1999). This mechanism is based on many strict assumptions (Levin 1975, Petit et al. 1999) and mixed populations often occur despite the reproductive disadvantage of the minority cytotype (Husband 2004). Several theoretical models evaluating the fate of autotetraploids that arise within populations of their diploid progenitors show that the coexistence of the cytotypes is maintained by partially fit triploids (Husband 2004), selfing (Levin 1975), greater vegetative reproduction of polyploids (Gibby 1981, Rodriguez 1996), asynchronous flowering and shifts in pollinator preferences (Fowler & Levin 1984, van Dijk & Bijlsma 1994, Segraves & Thompson 1999). Moreover, the models developed by Felber (1991) and Rodriguez (1996) show that when the diploid cytotype produces a rather high

frequency of 2n gametes and/or cytotypes differ in fitness, fecundity, longevity and levels of self-compatibility (but see Mable 2004), tetraploids can become established and survive in populations. Li et al. (2004) and Baack (2005), using simulation models, show that over a short distance, seed and pollen dispersal, polyploid establishment and persistence should be possible even in the absence of niche separation or recurrent polyploid formation via unreduced gametes. A combination of the above mentioned factors may also lead to the spatial segregation of cytotypes regardless of niche differentiation between cytotypes. Finally, Halverson et al. (2008) suggest that theoretical predictions of unstable cytotype coexistence may simply be irrelevant in many cases because plant populations have not reached the equilibrium at which all cytotypes but one are locally excluded.

Allium oleraceum L. is a wide-ranging clonal bulbous geophyte, which occupies a multitude of different habitats in Europe (Duchoslav 2001a, Hæggström & Åström 2005, Karpavičienė 2008). It comprises triploid (2n = 3x = 24), tetraploid (2n = 4x = 32), pentaploid (2n = 5x= 40) and hexaploid (2n = 6x = 48) cytotypes (Krahulcová 2003, Åström & Hæggström 2004, Karpavičienė 2007). Recently the distribution and ecological differentiation among cytotypes in a sample of 325 populations in the Czech Republic were studied (Šafářová 2004, Duchoslav et al. 2010). All cytotypes (4x, 5x, 6x) except triploids were recorded. The distribution of tetra- and hexaploids is largely parapatric, while that of pentaploids with other cytotypes is sympatric. The results provide evidence for niche differentiation among cytotypes. Tetraploids occur equally in both natural and ruderal habitats but are usually confined to sites with a high content of organic carbon, a high pH and often under stress, e.g. shaded. Pentaploids occur in a wide range of habitats on soils that are usually intermediate chemically between those where the 4x and 6x cytotypes grow. Hexaploids apparently occupy a different ecological niche than the other cytotypes since they inhabit mostly human-influenced and often disturbed and exposed habitats with soils rich in phosphorus (Duchoslav et al. 2010). Ecological differentiation among cytotypes therefore accounts for the predominance of cytotype-uniform populations (77%) in this survey. However, 22% and 1% of the populations consisted of two and three cytotypes, respectively. Though larger populations and areas with environmental conditions intermediate between those found in uniform populations of respective cytotype pairs were found in mixed populations (Duchoslav et al. 2010), there is no information on the spatial structure and habitat differentiation of cytotypes at mixed-ploidy sites of *A. oleraceum*.

The local spatial structure of cytotypes within mixed-cytotype sites of *A. oleraceum* were investigated in this study. The aim was to determine whether: (i) cytotypes are spatially segregated within sites, (ii) differences in ecological niche observed among cytotypes at a regional geographical scale (Duchoslav et al. 2010) occur at a microgeographic scale at a site, and (iii) microhabitat differentiation is a major driving force determining spatial segregation. In addition, detailed sampling was used to check whether previously observed cytotype combinations (i.e. 4x+5x, 4x+6x, 5x+6x; Šafářová 2004, Duchoslav et al. 2010) were a consequence of the sampling procedure failing to detect rare cytotypes, which is suggestive of inter-cytotype gene flow within populations.

Material and methods

Plant material

Allium oleraceum L. (*Alliaceae*) is a bulbous geophyte occurring throughout most of Europe (Meusel et al. 1965). It mainly occurs in western, central and eastern Europe and southern Scandinavia. In the Czech Republic, the species is common and its distribution is concentrated between 300 and 500 m a.s.l. (Duchoslav 2001a). It grows in a wide range of natural and human-influenced habitats ranging from rocky ground and dry grasslands through field margins and road ditches to scrub and deciduous forests (Duchoslav 2001a, b, Karpavičienė 2002, 2004, 2008, Hæggström & Åström 2005).

The plant has 1–4 leaves, which are linear to filiform, with fistular bases that ensheath the lower half of the scape. The terminal bulb of non-flowering plants and the major offset bulb of flowering plants replace the parent bulb at the end of the growing season. Plants often form a non-dormant daughter bulb. At the top of the scape of sexually mature plants there is a loose lax umbel with a few (0–30) hermaphrodite, protandrous flowers and many bulbils (10–60). Each flower can potentially produce six seeds (Stearn 1980), but seed production varies considerably and seedling establishment is low (Duchoslav 2000, Karpavičienė 2002, Åström & Hæggström 2004, Ohryzek 2007).

Sampling

Twenty-one sites selected from a database based on previous research on this species (Šafářová 2004, Duchoslav et al. 2010) were sampled in the Czech Republic in early spring in 2005–2007 (for details of the sites see Appendix 1). This selection included three sites with penta- and tetraploids, four with tetra- and hexaploids, eight with penta- and hexaploids, two with tetra-, penta- and hexaploids, and four (one tetraploid, one hexaploid and two pentaploid sites) that were initially considered to be single-cytotype sites (Šafářová 2004) but subsequently a genetic study identified them as cytotype-mixed sites (Staňková 2005). Except for the cytotype-mixed sites with tetra-, penta- and hexaploids, the numbers of sites sampled for each cytotype combination roughly corresponded to the frequencies of sites with these cytotype combinations in the Czech Republic (Duchoslav et al. 2010).

At each site, population size, population area and spatial pattern of individuals was determined. Because previous research based on sampling and analysing all the plants within a few randomly located plots of ca 30×30 cm revealed that 95% of plots were cytotype-homogeneous (Duchoslav et al. 2010) a modified preferential sampling procedure was adopted. Sampling was adjusted to include all the area of the population but avoid collecting other individuals < 10 cm from a sampled plant in order to minimize the probability of sampling multiple ramets of individual genets. Overall, 778 plants were sampled. The numbers of plants sampled ranged from 24 to 83 plants per site (mean 37 per population) and were proportional to the size of the respective populations. At each site the exact position of all the individuals sampled were mapped onto a sketch map and the distances between neighbouring sampled plants and/or clumps of plants were measured. Fresh leaf tissue was collected from each plant sampled, stored in a plastic bag and transported to the laboratory for flow cytometric analysis.

When sampling the distribution of habitats at the sites were recorded along with the habitat in which each plant occurred. Habitats were defined according to system of habitat classification used in the NATURA 2000 mapping of the Czech Republic (Chytrý et al. 2001, see also Appendix 1). Subsequently, the environment of each population was classified as either homogeneous, if it inhabited just one habitat, or heterogeneous, if it inhabited two or more adjacent habitats.

Estimate of DNA ploidy level

Approximately 5 cm length of leaf tissue of individual plants of *A. oleraceum* and the appropriate amount of the reference standard (*Triticum aestivum* 'Saxana'; 2C = 34.24 pg based on repeated measurements through 2007-2008, and calibrated against *Hordeum vulgare* with 2C = 10.43 pg, cf. Doležel et al. 1989) were chopped with a new razor blade in a Petri dish containing 1 ml of ice-cold LB01 buffer (Doležel et al. 1989). The solution was filtered through nylon mesh (42 μm mesh size) and the samples stained with DAPI (2 $\mu g.ml^{-1}$ final concentration). The relative fluorescence intensity of the stained nuclei was analysed using a Partec PAS flow cytometer (Partec GmbH, Münster, Germany) with an HBO-100 mercury arc lamp. In each sample, 1000–2000 nuclei in each of the standard and the test plant G1 peaks were analysed. The DNA ploidy level (Suda et al. 2006) of the samples was characterized by the ratio of the relative position of their G1 peak and that of the internal standard. Tetraploid (10 plants from 5 populations), pentaploid (17 plants from 7 populations) and hexaploid (11 plants from 5 populations) individuals with known chromosome numbers were used to define the ratio between the relative DNA content of the *Allium* cytotypes and the internal standard.

Data analysis

Each sketch map was converted into electronic form using CorelDRAW 9 (CorelDRAW, version 9.397; Corel Corporation) and exported to ArcView GIS software (ArcView GIS, version 3.1; Environmental Systems Research Institute, Inc.). The distances between the plants sampled at each site were measured using Bearing & Distance Extension 1.1 in ArcView GIS. The aggregation of cytotypes was estimated using two different randomization analyses. In the first, the correlation between the cytotype identity and spatial distribution of the individuals sampled at each site was evaluated using the Mantel test (Manly 1991, Fortin & Gurevitch 2001). The inputs were two matrices: (i) a binary matrix of cytotype identities, and (ii) the matrix of the mutual distances between individuals. The null hypothesis was that the relationships between the two matrices could have been obtained by any random arrangement of cytotype identities of the plants. The statistical significance of the standardized Mantel statistics (r_M) was assessed by performing 999 random permutations (Legendre & Legendre 1998). In the second analysis, a spatial test developed by Halverson et al. (2008) was applied, i.e. the average distance between plants of the same cytotype was calculated and then compared with the distance obtained using similar calculations for 999 data sets in which cytotype labels were shuffled randomly among plants. For these calculations, Resampling, Monte Carlo analysis and Mantel test functions in PopTools software (version 2.7.5; Hood 2006) were used.

Mantel correlograms (Legendre & Legendre 1998) were used to identify the scales of variation at six sites where more individuals were sampled. We used 11 distance classes of unequal widths to overcome the problem of the low number of pairs of observations in some classes and to improve the power of the tests. Each class has at least 20 pairs of observations. The standardized correlation coefficients (r_M) were computed for each distance class and the statistical significance of the coefficients was adjusted by sequential Bonferroni correction (Legendre & Legendre 1998).

The associations between cytotypes and habitats were tested either by a two-tailed Fisher exact test for 2×2 tables or a chi-square test for > 2×2 contingency tables, respectively (Zar 1996). Only sites with heterogeneous environments were analysed.

Results

Cytotype composition of populations

DAPI staining yielded histograms with coefficients of variance (CV) of both the standard and sample below 5% in the majority of flow cytometric measurements. The ratios between the nuclei fluorescence intensity of the samples and the internal standard were 2.4–2.6, 2.8–3.0 and 3.3–3.4 for tetraploids, pentaploids and hexaploids, respectively (Fig. 1).

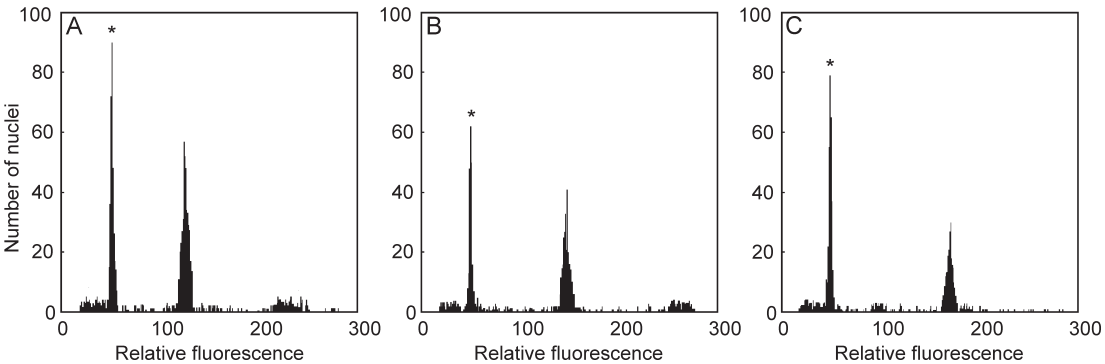

Fig. 1. – Fluorescence histograms of individual DNA ploidy levels of *Allium oleraceum*. Nuclei were simultaneously isolated from fresh leaf tissue of *Allium oleraceum* and an internal reference standard, *Triticum aestivum* 'Saxana', stained with DAPI and analyzed on a flow cytometer. A: tetraploid plant (population no. 7), B: pentaploid plant (population no. 16), C: hexaploid plant (population no. 9). The reference peak is marked with an asterisk.

Table 1. – Cytotype structure of populations and tests for spatial segregation and habitat differentiation of cytotypes of *Allium oleraceum* at 21 sites. Habitats are labelled with habitat names that are supplemented by more accurate habitat codes, following Chytrý et al. (2001), in Appendix 1. Preferential occurrence of a cytotype in some habitats at heterogeneous sites is associated with ploidy level (in parenthesis). Last four columns contain tests for spatial aggregation of cytotypes within sites, i.e. standardized Mantel statistics (r_M) measuring the correlation between spatial distances and cytotype identities and appropriate P values while the P-values in the following column (P_A) are based on average distance between plants of the same cytotype. The last column contains the results of two-tailed Fisher exact test or chi-square test (*) testing the association between habitat types and cytotypes. The Fisher exact test/chi-square was used in the case of sites with a heterogeneous environment. Statistically significant values (at $P \leq 0.05$) are in bold. At site no. 20, three separate analyses were performed, each for a different combination of cytotypes (i.e. 4x+5x, 4x+6x, and 5x+6x). At site no. 21, incidences of minority cytotypes (4x, 6x) were pooled before all analyses due to the low number of plants with these cytotypes. For detailed site locations, see Appendix 1. ▶

Site no.	Observed cytotypes	n	Frequency of cytotypes (%)			Percentage (%) of individuals sampled in different habitats						Spatial segregation of cytotypes			Association cytotype-habitat
			4x	5x	6x	Habitat 1		Habitat 2		Habitat 3		r_M	P	P_A	P
1	4[1], 5	25	56.0	44.0		forest	100					–0.067	0.098	0.174	–
2	4, 5[1]	24	67.0	33.0		forest	100					–0.071	0.155	0.127	–
3	4, 5	34	35.0	65.0		grassland (5x)	74	forest	26			–0.360	**0.001**	**<0.001**	**0.004**
4	4, 5	35	89.0	11.0		forest	100					0.151	0.980	**<0.001**	–
5	4, 5	39	64.0	36.0		scrub	92	grassland	8			–0.051	0.170	0.078	0.292
Mean			62.2	37.8											
CV (%)			31.4	51.6											
6	4, 6	27	85.0		15.0	forest	100					0.051	0.730	0.100	–
7	4, 6	27	89.0		11.0	grassland	70	*Robinia* forest	30			0.137	0.919	**0.005**	1.000
8	4, 6	24	29.0		71.0	field margin	100					–0.566	**0.001**	**<0.001**	–
9	4, 6	80	92.0		8.0	*Robinia* forest	71	grassland (6x)	29			–0.025	**0.001**	**0.050**	0.340
Mean			73.8		26.2										
CV (%)			40.6		114.2										
10	5, 6	29		21.0	79.0	grassland	100					0.103	0.242	**0.008**	–
11	5, 6	32		69.0	31.0	*Robinia* forest (5x)	78	field margin (6x)	22			–0.458	**0.001**	**<0.001**	**<0.001**
12	5, 6	37		65.0	35.0	scrub	95	field margin (6x)	5			0.002	0.340	0.485	0.117
13	5, 6	33		15.0	85.0	forest	100					–0.493	**0.020**	**<0.001**	–
14	5, 6	24		42.0	58.0	forest	54	grassland	46			0.025	0.360	0.419	0.408
15	5, 6	24		83.0	17.0	forest	100					0.083	0.266	0.097	–
16	5, 6	25		40.0	60.0	forest	100					–0.245	0.070	0.065	–
17	5, 6[1]	26		42.0	58.0	grassland	92	field margin	8			–0.113	**0.043**	**0.002**	0.169
18	5, 6	52		67.0	33.0	orchard (6x)	27	forest (5x)	73			–0.715	**0.001**	**<0.001**	**<0.001**
19	5[1], 6	62		82.0	18.0	steppe (5x)	58	field margin	42			–0.150	**0.024**	**<0.001**	0.094
Mean				52.6	47.4										
CV (%)				45.8	50.8										
20	4, 5, 6	36	47.0	28.0	25.0	grassland (4x)	72	forest (5x)	19	ruderal scrub (6x)	8				
	4, 5	27	63.0	37.0		scrub	74	forest	26			–0.616	**0.001**	**<0.001**	**<0.001**
	4, 6	26	65.0		35.0	scrub	92	forest	8			–0.817	**0.001**	**<0.001**	**0.032**
	5, 6	19		47.0	53.0	scrub	47	forest	37	ruderal scrub	16	–0.672	**0.001**	**<0.001**	**0.004***
21	4, 5, 6	83	2.0	89.0	9.0	field margin	52	forest	31	grassland	17	–0.322	**0.001**	**<0.001**	**0.001***

[1] Cytotype newly detected at a site previously considered to be cytotype-uniform (Šafářová 2004).

Accordance between the results of previous research (Šafářová 2004) and the present study was found in the composition of cytotypes at cytotype-mixed sites. On the other hand, four sites that were previously considered to be cytotype-uniform (Šafářová 2004) were in fact a mixture of two cytotypes when repeatedly analyzed (Table 1). At all sites containing a mixture of two cytotypes (i.e. 4x+5x, 4x+6x and 5x+6x) no plants with a third ploidy level were detected.

Cytotype relative frequencies varied considerably between sites. At the sites consisting of tetra- and pentaploids and particularly of tetra- and hexaploids, tetraploids usually dominated but the reverse was also observed, although rarely. On the other hand, frequencies of both penta- and hexaploids varied at cytotype-mixed sites of penta- and hexaploids. At sites containing tetra-, penta- and hexaploids, one cytotype dominated over other cytotypes (Table 1).

Spatial and environmental distribution of cytotypes

At nearly half of the sites (47.6%) the significant Mantel statistics indicated that the cytotypes were not randomly distributed. When the spatial test based on the average distance between plants of the same cytotype was applied, the proportion of sites with spatially segregated cytotypes increased to 61.9% (Table 1). Spatial segregation of cytotypes was observed at all types of cytotype-mixed sites, but was slightly more frequent at sites with a mixture of tetra- and hexaploids and of penta- and hexaploids than at those containing a mixture of tetra- and pentaploids. Cytotypes were also spatially segregated at both cytotype-mixed sites with co-occurrence of three cytotypes.

Cytotypes were more frequently spatially structured at sites with a heterogeneous environment (based on the results of Mantel test: 66.7%; average distance test: 75.0%) than at those with a homogeneous environment (22.2% and 44.4%, respectively) but the difference between proportions was either significant (Mantel test, pooled data: $\chi^2 = 4.07$, $P = 0.044$) or insignificant (average distance test: $\chi^2 = 2.04$, $P = 0.153$). Cytotypes showed a significant association with different habitats at 5 out of 12 sites with a heterogeneous environment (Table 1). Except for cytotype-mixed sites with tetra- and hexaploids, there was a tendency for spatial segregation of cytotypes in heterogeneous environments when different cytotype compositions were treated separately. Because of the low frequencies of these, however, the data were not statistically assessed.

Fig. 2 shows examples of contrasting spatial structures of cytotypes at cytotype-mixed sites, while maps displaying the spatial distributions of cytotypes at all sites investigated are available in an Electronic Appendix 1. Site no. 18 (Fig. 2A) represents an example of a 'perfect' habitat and spatial separation between cytotypes, in which each cytotype forms a spatially segregated subpopulation inhabiting only one of two adjacent habitats. This situation is, however, rare, and cases of partial sympatry but different habitat preferences between cytotypes are more common (Table 1), as for example, at site no. 3 (Fig. 2B). On the other hand, spatial segregation of cytotypes was also observed at some sites with uniform environments, e.g. sites nos. 8 and 13 (Fig. 2C). There are also several examples of mutually random distribution of cytotypes at sites with either a uniform (e.g. site no. 15) or heterogeneous environment (e.g. site no. 14; Fig. 2D).

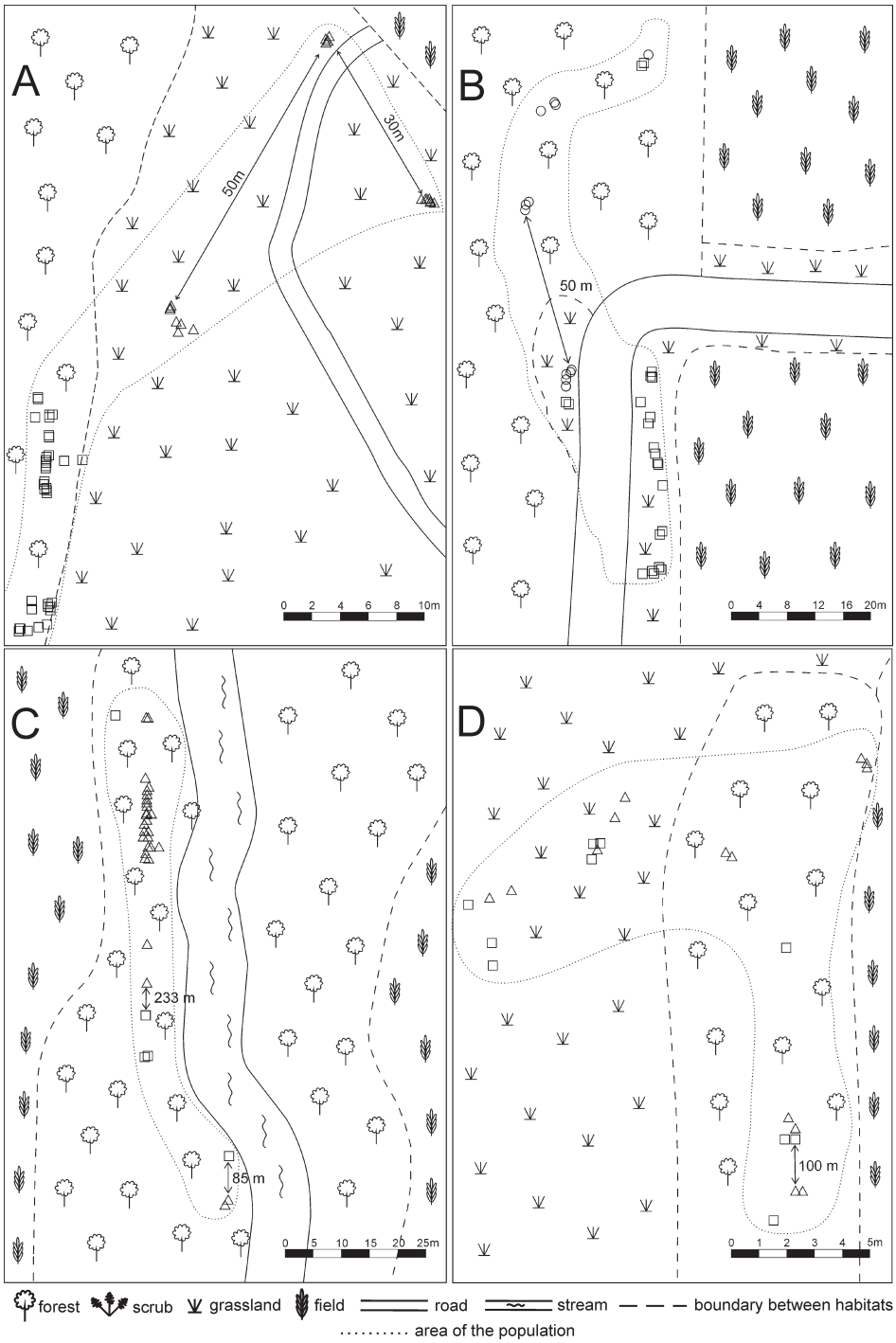

Fig. 2. – Examples of contrasting spatial structure of cytotypes at cytotype-mixed sites (A: site no. 18, B: site no. 3, C: site no. 13, D: site no. 14). Each plant sampled is represented by a symbol identifying its ploidy level (○= 4x, □ = 5x, △ = 6x). Population borders are demarcated by dotted lines and borders between habitats by dashed lines. Areas lacking *Allium* plants are not depicted in the real scale; lines ending in arrow-heads denote the distance between closest individuals. See Appendix 1 for site details.

Table 2. – Standardized Mantel statistics (r_M) of the distribution of cytotypes for different distance classes in some cytotype-mixed populations of *Allium oleraceum*. Statistically significant values of r_M after sequential Bonferroni correction (with an experiment-wide error rate of 0.05) are in bold.

Distance class	Distance (m)	Site/ploidy composition					
		3	9	11	13	18	19
		4x+5x	4x+6x	5x+6x	5x+6x	5x+6x	5x+6x
1	0–1.9	0.20	0.04	0.24	0.16	0.26	0.07
2	2–4.9	0.10	0.04	0.10	0.23	0.33	0.03
3	5–9.9	0.17	0.09	0.36	0.24	0.24	0.08
4	10–17.4	0.06	0.06	**–0.23**	0.20	0.32	0.04
5	17.5–24.9	0.09	–0.04	–0.02	0.01	0.00	–0.02
6	25–34.9	0.14	**–0.08**	0.10	0.03	–0.06	0.04
7	35–59.9	**–0.16**	0.05	**–0.23**	0.09	**–0.20**	0.01
8	60–99.9	**–0.30**	–0.14	**–0.29**	**–0.29**	**–0.58**	0.03
9	100–149.9	–0.07	0.06		**–0.21**		0.00
10	150–299.9		**–0.08**		**–0.73**		**–0.13**
11	> 300		0.10		–0.01		

Spatial distribution of the cytotypes at a microgeographic scale

Analysis of the spatial distribution of the cytotypes at a microgeographic scale at selected sites showed that at the majority of sites neighbouring individuals are likely to be of the same cytotype, as illustrated by positive Mantel statistics for distances smaller than 2 (–10) m. Except for one site, spatial autocorrelation between cytotypes largely disappeared at moderate distances. At large distances, negative Mantel statistics were detected at most sites, meaning that plants that are far apart have different ploidy levels (Table 2). However, after the application of Bonferroni adjustment, some significant, mostly positive, correlations disappeared. For all the populations analyzed the global and microspatial analyses gave similar results.

Discussion

Cytotype composition of populations and origin of cytotypes

The fine-scale sampling employed in this study revealed more complex patterns of cytotype structure in some populations previously considered to be cytotype-uniform (Šafářová 2004). These discrepancies are most probably caused by (i) differently delimited areas of the study populations and/or (ii) different sampling procedures used in the previous and the present study. The previous research employed a strictly hierarchical sampling design, which could lead to redundancy at the lowest (subsample) scale (Koenig 1999) if the cytotypes are associated with cytotype-homogeneous patches. Only a single cytotype was detected in 95% of the subsamples (Šafářová 2004, Duchoslav et al. 2010), and visual inspection of the microdistribution of cytotypes within mixed populations (Fig. 2 and Electronic Appendix 1) and the results of the microspatial analysis (Table 2) also support cytotype homogeneity over short distances. Generally, these observations suggest that the frequency of cytotype mixtures in *A. oleraceum* at a landscape scale reported in previous publications is underestimated (12%, Karpavičienė 2007; 23%, Šafářová 2004, Duchoslav et al. 2010). In fact, the higher percentage of mixed-cytotype sites in

A. oleraceum proposed here is more comparable with that recorded for some other well-investigated plants, such as *Andropogon gerardii* (Keeler 1992, 2004), *Galax urceolata* (Burton & Husband 1999), *Senecio carniolicus* (Suda et al. 2007) and *Vaccinium oxycoccos* (Suda 2002).

Although not expected, the results of the fine screening confirmed the frequent existence of cytotype mixtures consisting of two cytotypes (i.e. 4x+5x, 4x+6x, 5x+6x) and only the rare co-occurrence of tetra-, penta- and hexaploids in *A. oleraceum*. Assuming the origin of novel polyploids was via commonly accepted pathways, i.e. the fusion of reduced and unreduced gametes (Bretagnolle & Thompson 1995, Ramsey & Schemske 1998), then only 4x+6x and 4x+5x+6x mixed populations would be present. The supposed (recent) origin of hexaploid plants from tetraploids is also supported by: (i) the apparently contrasting frequencies of cytotypes within the populations consisting of tetra- and hexaploids, where tetraploids usually dominated over hexaploids (Table 1), and (ii) identical multilocus allozyme phenotypes of minority hexaploid plants and dominant tetraploids at two of the 4x+6x sites that were studied (sites nos. 7 and 9; cf. Staňková 2005). A similar pattern is described e.g. for 2x+4x and 4x+6x mixed populations of *Artemisia* subgen. *Tridentatae* (McArthur & Sanderson 1999) and *Dianthus* sect. *Plumaria* (Weiss et al. 2002). Subsequently, hybridization between tetra- and hexaploids could lead to pentaploid offspring and the establishment of 4x+5x+6x mixed populations. Since recent studies have shown that both the fusion of reduced and non-reduced gametes and cytotype hybridization are repetitive processes in plants (Ramsey & Schemske 1998, Soltis & Soltis 1999, Krahulcová et al. 2000, Peckert & Chrtek 2006, Mráz et al. 2008), polytopic origins for both penta- and hexaploids can be assumed. The cytotype data available for *A. oleraceum* at both regional and European scales (Karpavičienė 2007, Duchoslav et al. 2010) only partially support this mode of establishment of cytotype-mixtures because: (i) the overall frequency of 4x+6x populations is low in nature, despite the commonness of tetraploids, suggesting either a low probability of unreduced gamete production in tetraploids and/or a low probability of hexaploid establishment, and (ii) the gene flow is probably limited between tetra- and hexaploids as the latter very rarely produce flowers (Ohryzek 2007), which would hamper pentaploid formation.

The existence of mixed 4x+5x and 5x+6x populations is also difficult to explain by in situ de novo origin of pentaploids due to: (i) the absence of either hexa- or tetraploid parents and (ii) considerable cytotype variation both within and between populations, suggesting that 'minority cytotype exclusion' (Levin 1975) has little effect within these populations. Alternatively, we cannot exclude the possibility that tetra- or hexaploids can occasionally be produced by pentaploids via the fusion of partly reduced or unreduced gametes, respectively. This mode of mixed-population establishment in *A. oleraceum* is rather speculative but the data collected for some species, e.g. *Hieracium* subgen. *Pilosella*, show that pentaploids usually produce both euploid and aneuploid pollen grains ranging from 2x to 3x (Krahulcová & Krahulec 2000, Krahulcová et al. 2000). Because *A. oleraceum* pentaploids occasionally set well-developed seeds (Åström & Hæggström 2004, Ohryzek 2007) both euploids and aneuploids may be included within seed-sets (Fialová 1996). However, no adult aneuploid plants have been found in nature (Karpavičienė 2007, Duchoslav et al. 2010) suggesting they have a reduced fitness. Moreover, mixed 4x+5x and 5x+6x populations do not show a distinct geographical pattern but are sympatric with single-cytotype populations of participating cytotypes (Duchoslav et al. 2010).

In summary, the data presented show that the local co-occurrence of *A. oleraceum* cytotypes is more probably due to secondary contacts but also provides indirect support for polytopic and repeated polyploid origin, at least in the case of hexaploids. Currently an analysis of the cytotype composition of seeds, seedlings and adult plants within cytotype-uniform and mixed populations is being undertaken to gain a deeper insight into the evolutionary processes in this polyploid complex.

Microdistribution of cytotypes

Our results demonstrate a tendency for the *A. oleraceum* cytotypes in many populations to be spatially segregated, at least at the spatial scales addressed by our sampling. When the habitat composition of the sampled sites was taken into account, a clearer picture emerged: in a homogeneous environment, there was only a weak tendency for the cytotypes to be spatially separated, while in a heterogeneous environment they were spatially segregated and there was a more or less clear association of the cytotypes with different habitats. How can these discrepancies be explained?

Theoretical studies suggest that mixed-cytotype populations should be evolutionarily unstable except when cytotypes have similar fitnesses and reproduce predominantly via parthenogenesis (Yamauchi et al. 2004), or have strong pre-zygotic isolation (van Dijk & Bijlsma 1994, Husband & Schemske 2000, Husband et al. 2002), different microhabitat preferences (Levin 1975, Fowler & Levin 1984, Rodriguez 1996) and/or local pollen and seed dispersal (Li et al. 2004, Baack 2005). The latter factors are also responsible for the fine spatial segregation of cytotypes in a spatially heterogeneous environment, which results in a mosaic spatial pattern with different cytotypes occupying various local habitats but failing to colonize globally (Li et al. 2004).

Out of 15 studies, where within-population spatial cytotype structure was analyzed in detail, six record no spatial structuring. Meirmans et al. (1999) found no spatial correlation between 2x and 3x cytotypes in an analysis of four transects through a single population of *Taraxacum* sect. *Ruderalia* inhabiting ecologically homogeneous grassland, despite the significant differences in ecological niches between cytotypes recorded at a landscape scale. However, they did not consider other mechanisms that might have enabled the cytotypes to coexist at that site. Hardy et al. (2000) found no obvious spatial segregation of diploid and tetraploid *Centaurea jacea* within two mixed populations. Halverson et al. (2008) found no tendency towards the spatial segregation of diploid, tetraploid and hexaploid cytotypes at eight cytotype-mixed sites of *Solidago altissima* and no strong niche separation among cytotypes. These results are of particular interest because they indicate that the existence of cytotype mixtures may simply be the result of non-equilibrium processes and metapopulation dynamics (Levin 1975) and that these factors play an important role, especially in disturbance-tolerant plants.

Another three studies indicate that environmentally independent processes may explain the absence of spatial segregation of cytotypes. Sympatry and coexistence of diploid and tetraploid *Plantago media* in one mixed-cytotype population is thought to be a consequence of a pre-zygotic reproductive barrier between cytotypes, which greatly reduces the disadvantage of the minority cytotype (Van Dijk et al. 1992). McArthur & Sanderson (1999) record many 2x+4x mixed-cytotype populations in the subgenus *Tridentatae* of *Artemisia* with sympatric or closely parapatric distribution of cytotypes and attribute these

patterns to the recent origin of tetraploids in diploid populations, but in one case mention the close parapatry of cytotypes over a fine-scale environmental gradient. Suda (2002) records sympatry of cytotypes in many *Vaccinium oxycoccos* populations with intermingling of cytotypes even at a very fine spatial scale of 20 × 20 cm. In this case the existence of mixed populations is explained by the recurrent formation of cytotypes and their longevity and mainly vegetative reproduction, which may counteract minority cytotype exclusion.

In nine studies that show spatial segregation of cytotypes the segregation in five of them is indicated by ecological differentiation. Lumaret et al. (1987) explain the spatial segregation of diploid and tetraploid *Dactylis glomerata* in mixed-cytotype populations as a result of different habitat preferences of the cytotypes, i.e. their different responses to local light conditions. Similarly, Suda et al. (2004) found mixed populations of three *Empetrum* cytotypes in the Krkonoše Mts. (Czech Republic) and explain their existence in terms of small-scale patchy distribution of ecologically contrasting habitats for which the cytotypes show different ecological preferences. Husband & Schemske (2000) found different patches of plants with different ratios of diploids and tetraploids in a population of *Chamerion angustifolium*, but provide no explanation of the causes of this patchy distribution. In a previous study (Husband & Schemske 1998), these authors, however, speculated that the patchy distribution of cytotypes may be the result of slight differences in the ecological amplitudes of the cytotypes. Keeler (1992) did not detect any spatial segregation of cytotypes in different populations of the grass *Andropogon gerardii*, despite the contrasting ecological conditions at the study sites. A re-analysis of this data revealed significant autocorrelation patterns for two of the four populations and that the lack of a spatial structure was probably the result of a lack of statistical power and suggested that there is some ecological differentiation between the two cytotypes of *A. gerardii* (Meirmans et al. 2003). Meirmans et al. (2003) also investigated a diploid–triploid mixed population of *Taraxacum* sec. *Ruderalia* in detail and explain the patchy distribution of cytotypes they recorded in terms of the influence of elevation. However, these authors argue that elevation alone explains only a small part of the spatial autocorrelation in the distribution of cytotypes and that the heterogeneity in the distribution of cytotypes may be predominantly caused by ecological variables that were not measured or by demographic factors. Schönswetter et al. (2007) found a significant segregation of diploid and hexaploid cytotypes of *Senecio carniolicus* along an altitudinal transect in the Eastern Alps, with diploids exclusively at the higher and both cytotypes co-occurring at the lower altitudes. It was hypothesized that this is a result of ecological niche differentiation, but the design of the study prevents the separation of altitudinal from other ecological effects. At another site where there was little variation in altitude in the zone where cytotypes of *S. carniolicus* came into contact, the fine-scale segregation of cytotypes is linked to an environmental gradient, which is also reflected in the cytotype-associated plant assemblages (Hülber et al. 2009).

Recently, Kolář et al. (2009) record a non-random distribution of cytotypes in some mixed-ploidy populations of *Knautia arvensis* agg. and consider a founder effect and limited dispersal capacity of *Knautia* seeds as plausible, though non-exclusive explanations, of the spatial segregation of cytotypes, rather than only microhabitat differentiation (Kolář et al. 2009). Only Baack (2004) records a distinct spatial segregation of cytotypes of *Ranunculus adoneus* in a mixed population, with a transition zone between diploids and tetraploids occurring over 3 m, which is explained non-adaptively through reproductive exclusion of the minority cytotype.

Non-random distribution of *A. oleraceum* cytotypes in a heterogeneous environment is most probably explainable in terms of differences in the ecological niches of the cytotypes observed in a previous study on *A. oleraceum* (Duchoslav et al. 2010). A broader realized ecological niche for tetra- and pentaploid cytotypes than for the hexaploid cytotype and partial niche overlap among cytotypes could also explain cytotype intermingling under specific environmental conditions, e.g. in mesic and dry grasslands (e.g. site no. 9). Alternatively, at some sites (e.g. sites nos. 3, 18, 21) the pattern may be caused by demographic factors – different parts of the area may have different colonization histories and the current closely parapatric pattern represents secondary contacts between cytotype-different but uniform populations at an ecotone between habitats (e.g. between forest and field). It is also not possible to eliminate the possibility that the spatial segregation of cytotypes at some sites with a 'homogeneous' environment is due to the response of cytotypes to fine-scale variation in the environment. Common garden and reciprocal transplant experiments are now in progress to clarify the role of ecological differentiation in the microdistribution of cytotypes in *A. oleraceum*.

The lack of spatial structure in some populations (e.g. sites nos. 1, 2, 4) was probably the result of a lack of statistical power due to a strongly unbalanced representation of cytotypes and/or small sample sizes. The spatial aggregation of cytotypes is likely to be detected at finer scales (centimetres – decimetres) simply because *A. oleraceum* rarely produces daughter bulbs (Duchoslav 2000) but does produce high numbers of asexual bulbils within inflorescences (Åström & Hæggström 2004), which are locally dispersed around mother plants (Ronsheim 1994, Duchoslav 2001b). On the other hand, seed production varies both within and among cytotypes; hexaploids are almost sterile whereas tetra- and pentaploids produce a variable seed set ranging from zero to 20 seeds per plant (Åström & Hæggström 2004, Ohryzek 2007). Seed recruitment may, however, be inhibited by competition with clonal bulbils for safe sites (Abrahamson 1980, Eriksson 1997, Kliber & Eckert 2005). Fialová (2005) observed in a common garden experiment that the development of clonal progeny from bulbils is faster than that of sexual progeny. As a consequence, small-scale patches commonly occurring in populations of *A. oleraceum* (Duchoslav 2001b) are usually cytotype-homogeneous (Duchoslav et al. 2010). The predominance of vegetative reproduction (aerial bulbils), local dispersal and the longevity of *A. oleraceum* can result in the local co-occurrence of cytotypes, as is indicated by theoretical models (Li et al. 2004, Yamauchi et al. 2004, Baack 2005).

Considerable variation in cytotype composition and absence of spatial structure at some sites may also indicate that some populations have not reached a state of equilibrium in which all cytotypes but one are locally excluded. This is suggested for some cytotype-mixed populations of several species (van Dijk et al. 1992, Hardy et al. 2000, Keeler 2004, Halverson et al. 2008) and may also be applicable to *A. oleraceum*, a disturbance-tolerant hemerophilous species. Some populations of *A. oleraceum* are either relatively young or occur at sites at which the environmental conditions have recently changed, the most typical being abandoned arableland grassland or pasture that has subsequently become overgrown with shrubs, trees or afforested with plantations of *Robinia pseudacacia*. Records of land use at the sites using digitized old maps (2nd military mapping; 1836–1852) and contemporary aerial maps show that five of the fifteen presently fully or partially forested sites (sites nos. 4, 9, 11, 14, 18) were grasslands, pastures or arable fields in the past. A closed forest canopy restricts or even inhibits the completion of the normal life cycle of

A. oleraceum (Duchoslav 2009), which may induce remnant population dynamics, leading gradually to cytotype-uniform or even monoclonal populations (Eriksson 1989, Honnay & Bossuyt 2005) that can become extinct when unfavourable environmental conditions persist. Under such conditions, the effects of reproductive interactions or competitive exclusion influencing the co-occurrence of cytotypes may be obscured (Halverson et al. 2008).

In summary, our results indicate that the local co-occurrence of *A. oleraceum* cytotypes is not a rare phenomenon. When cytotypes co-occur in a heterogeneous environment, they are usually spatially segregated with a tendency towards habitat segregation. This suggests the presence of ecological differentiation among cytotypes, which is recorded at a landscape scale. The frequent co-occurrence of cytotypes, with or without significant spatial segregation, observed at many sites with either a heterogeneous or homogeneous environment, however, suggests that niche differentiation alone is insufficient to explain the existence of mixtures of cytotypes. It is likely that their mainly vegetative reproduction and local dispersal, abundance (Duchoslav 2001a) and the non-equilibrium processes influencing the establishment and extinction of *A. oleraceum* populations can result in the local co-occurrence of cytotypes. Additional research on the relative fitness of cytotypes and the role of pre- and/or postzygotic reproductive barriers between cytotypes is needed for a better understanding of their role in the dynamics of polyploid populations of *Allium oleraceum*.

See http://www.preslia.cz for Electronic Appendix 1.

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Souhrn

Studie se zabývá četností cytotypů a jejich prostorovým uspořádáním na jemné prostorové škále v cytotypově smíšených populacích evropského geofyta *Allium oleraceum*. Pro podrobné studium bylo vybráno 17 lokalit, na kterých byly předchozím výzkumem zaznamenány následující kombinace cytotypů: 4x+5x, 4x+6x, 5x+6x, 4x+5x+6x, a dále 4 populace, u kterých se údaje o cytotypovém složení rozcházely mezi předchozími studiemi. Opakovaný průzkum prokázal, že všechny studované populace jsou cytotypově smíšené, přičemž u zmiňovaných 17 lokalit potvrdil předpokládané složení. Byly tak potvrzeny neobvyklé kombinace cytotypů, mj. 4x+5x a 5x+6x. Ačkoliv byly relativní četnosti cytotypů ve smíšených populacích poměrně heterogenní, ve smíšených populacích tetra- a pentaploidů a především tetra- a hexaploidů převažoval vždy jeden cytotyp nad druhým. Výrazná disproporce v zastoupení tetra- a hexaploidů ve smíšených 4x+6x populacích může ukazovat na relativně recentní vznik hexaploidů v původně uniformních tetraploidních populacích. V závislosti na použitém statistickém testu bylo zjištěno, že na 47,6 % (Mantelův test) respektive 61.9% (test průměrné vzdálenosti) lokalit vykazovaly cytotypy vzájemně nenáhodné prostorové uspořádání. Pokud se v analýze zohlednily stanovištní charakteristiky jednotlivých lokalit, byly cytotypy prostorově strukturovány častěji v heterogenním než v homogenním prostředí. To může ukazovat na přítomnost ekologické diferenciace mezi cytotypy, která byla pozorována v předchozí studii. Byly však zaznamenány i smíšené populace cytotypů, ve kterých byly cytotypy vzájemně jak náhodně, tak i nenáhodně prostorově uspořádány, a to jak na lokalitách stanovištně homogenních, tak i heterogenních. Samotná diferenciace nik mezi cytotypy je tedy nedostatečným důvodem vysvětlujícím existenci cytotypově smíšených populací. Na jejich existenci se patrně podílejí i další faktory, mj. převažující vegetativní rozmnožování prostřednictvím pacibulek a dceřiných cibulí spojené s jejich prostorově lokálním šířením, dlouhá životnost jedinců, vysoká populační hustota druhu v krajině, a nerovnovážné podmínky na části lokalit (disturbance, sekundární sukcese aj.).

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Appendix 1. – Geographical location (WGS 84), habitat description (incl. habitat codes following Chytrý et al. 2001 in parentheses) and DNA ploidy levels for populations of *Allium oleraceum* at 21 sites.

Site no.	Ploidy level			Geographical coordinates		Locality	Habitat type	Altitude
	4x	5x	6x	Latitude (N)	Longitude (E)			(m a. s. l.)
1	x	x		50°07'35"	15°17'27"	Žehuň, Kozí hůra hill, oak-hornbeam forest (L3.1)	forest	230
2	x	x		50°04'03"	15°10'12"	Veltruby, Nature reserve Veltrubský luh, wet hardwood forest (L2.3)	forest	180
3	x	x		49°58'39"	15°53'56"	Dvakačovice, on the SE margin of the village, oak-hornbeam forest (L3.1) and adjoining mesic meadow (T1.1), in a ditch	grassland & forest	250
4	x	x		50°14'56"	15°59'37"	Libníkovice, degraded oak-hornbeam forest (L3.1) near collective farm buildings	forest	270
5	x	x		49°29'05"	17°44'05"	Opatovice, 2 km S of the village, semidry *Bromus erectus* grassland (T3.4), partly overgrown by *Prunus spinosa* shrubs (K3)	scrub & grassland	350
6	x		x	48°53'17"	17°34'32"	Suchov, 0.5 km S of the Trnovský Mlýn settlement, ash-alder forest growing on alluvium from the brook (L2.2)	forest	390
7	x		x	49°01'46"	17°16'06"	Syrovín, 1 km N of the village, mesic *Arrhenatherum elatius* meadow (T1.1) and adjoining cultivated *Robinia pseudacacia* forest (X9B)	grassland & *Robinia* forest	290
8	x		x	49°37'03"	14°00'03"	Lazsko, 1 km S of the road to the village of Ostrov, field margins around small hills partly overgrown by *Pinus sylvestris, Robinia pseudacacia* and eutrophic mesic scrub (X12)	field margin	530

Site no.	Ploidy level			Geographical coordinates		Locality	Habitat type	Altitude
	4x	5x	6x	Latitude (N)	Longitude (E)			(m a. s. l.)
9	x		x	49°33'38"	17°05'19"	Slatinice, 1 km W of the church in the village, secondary *Robinia pseudacacia* forest (X9B) in the valley of the brook and adjoining meadow dominated by *Arrhenatherum elatius* and *Bromus erectus* (T1.1)	*Robinia* forest & grassland	270
10		x	x	50°00'49"	16°53'42"	Komňátka, 0.5 km N of the village, small mesic *Arrhenatherum elatius* meadow (T1.1) by the road	grassland	380
11		x	x	49°34'49"	17°02'41"	Ludéřov, N edge of the village, secondary *Robinia pseudacacia* plantation (X9B) on former mesic grassland and adjoining field margin (X7)	*Robinia* forest & field margin	340
12		x	x	49°38'01"	16°44'13"	Jevíčko, mesic *Arrhenatherum elatius* meadow overgrown by mesic scrub dominated by *Prunus spinosa* (K3) and adjoining field margin near the railway-station (X7)	scrub & field margin	400
13		x	x	49°29'36"	17°04'21"	Kostelec na Hané, 2 km SE of the railway-station in the village, alluvial forest (L2.2) along the Romže brook	forest	240
14		x	x	49°36'08"	16°38'30"	Malá Roudka, SW margin of the village, mesic grassland with *Festuca rubra* by the road (T1.1) and adjoining oak-hornbeam forest (T3.1)	forest & grassland	440
15		x	x	50°05'04"	12°49'49"	Bečov n. Teplou, ravine forest (L4) near the railway-station	forest	510
16		x	x	49°55'57"	14°07'31"	Srbsko, S margin of the village, the Koda valley, wet alluvial forest (L2.2)	forest	330
17		x	x	49°19'08"	15°13'12"	Veselá, 0.5 km WNW of the village, stony ridges with acid semidry grassland (T2.3) and adjoining field margins (X7)	grassland & field margin	640
18		x	x	49°33'14"	17°32'59"	Dolní Újezd, 1 km NE of the village, oak-hornbeam forest (L3.1) and adjoining abandoned orchard invaded by shrubs (X13)	forest & orchard	340
19		x	x	49°10'29"	17°12'43"	Lísky u Kroměříže, Nature reserve Oulehla, broad-leaved dry grassland (T3.4) and adjoining field margins (X7)	steppe & field margin	290
20	x	x	x	49°42'19"	16°59'26"	Bílá Lhota, 1 km S of the village Měník, remnants of semidry grassland invaded by shrubs (T3.4), eutrophic scrub (X8) and oak-hornbeam forest (T3.1)	forest & grassland & ruderal scrub	300
21	x	x	x	49°33'37"	17°36'10"	Loučka u Lipníku n. Bečvou, 0.9 km SE of the village, wet floodplain forest (L2.2) in the alluvium of the brook, adjoining orchard with *Arrhenatherum elatius* (T1.1) and field margin (X7)	forest & grassland & field margin	300